DEVELOPMENT AND ASSESSMENT OF AN OXYTOCIN PARENTERAL DOSAGE FORM PREPARED USING PLURONIC® F127

By

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A Thesis Submitted to Rhodes University
in Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE (PHARMACY)

December 2006

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Grahamstown
South Africa

ABSTRACT

Post partum haemorrhage is one of the leading causes of maternal mortality in both the developed and developing world [1,2]. Post partum haemorrhage is caused by the loss of blood from the uterus following labour because of decreased uterine tone, retained placenta or placental fragments as well as lower genital tract trauma [3]. Routine management of post partum haemorrhage involves the use of parenteral oxytocin (OT) that is administered via the intramuscular or intravenous route to increase uterine tone and reduce bleeding. However, OT is rapidly metabolised in the liver and cleared from the body via the kidney [4]. The use of a long acting parenteral preparation of OT to maintain uterine tone is therefore proposed as a means of reducing maternal mortality by preventing post partum haemorrhage.

A variety of alternatives were investigated for the development of an appropriate dosage form for OT delivery. The use of Pluronic® F127 (PF-127) as a thermo-sensitive gel that exists as a viscous flowing liquid at low temperatures but forms a stiff gel on warming to body temperature is proposed. The properties of PF-127 allow for the administration of a cold liquid preparation via a syringe and needle into muscle tissue followed by the formation of a depot gel that has the potential for sustained delivery of OT *in vivo.*

Aqueous solutions of PF-127 were prepared using the cold method. PF-127 solutions were characterised with respect to critical micelle concentration and gelation temperature for different concentrations of gel. The temperature at which gelation occurs was found to be concentration dependent. The rheological properties of solutions of PF-127 were also investigated and a dramatic change in viscosity was found to occur simultaneously with the visual onset of gelation.

Due to the lack of compendial guidelines for *in vitro* release testing of controlled release parenteral preparations, different dissolution methods were evaluated for their potential to

discriminate between formulations of different compositions. Tests that were used to assess discriminatory behaviour were ANOVA analysis, the f_1 and f_2 difference and similarity factors, and Gohel's Similarity factor, S_d. The discriminatory behaviour was assessed by comparing the *in vitro* release of OT from 20%, 25%, and 30% w/w PF-127 containing preparations and it was observed that the USP Apparatus 3 showed the greatest potential to discriminate between all formulation compositions tested, compared to other test methods that were evaluated. The method was further optimised for OT per dose unit and to establish whether pH changes affected drug release from these systems.

The Korsmeyer-Peppas power law was used to assess the primary mechanism of drug release from the extemporaneously prepared dosage forms tested using different dissolution methods. The values of the release exponent, *n*, revealed that the mechanism of release of OT from PF-127 gels is generally a combination of diffusion and swelling controlled release or anomalous release. The extent to which diffusion or swelling impacts on the *in vitro* release of OT was dependent on the specific dissolution test that was used to evaluate the *in vitro* release of OT.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and thankfulness to the following people and organisations:

My supervisor, Prof. R.B. Walker, for his guidance and support and provision of laboratory facilities throughout the duration of my project.

The Dean and Head, Prof I. Kanfer, and the staff of the Faculty of Pharmacy, for the use of the facilities in the Pharmacy Faculty at Rhodes University.

Mr T. Samkange, for speedy technical support during my project and Mr. L. Purdon for assisting me in numerous ways.

The Andrew Mellon Foundation and Joint Research Committee of Rhodes University for financial support.

PolyPeptide Laboratories s.r.o. (Prague, Hostivar-Czech Republic) and BASF (Ludwigshafen, Germany) for their kind donations of oxytocin and Pluronic® F127, respectively.

Olarewaju Shaibu and Layla Cassim for assisting me with infrared and ultraviolet equipment, respectively.

My colleagues in the Biopharmaceutics Research Laboratory for their academic assistance, objectiveness, honesty, and pleasant company for the duration of my project.

My parents, Mr S. and Mrs J.M. Chaibva for their support of my dreams and hopes and always being objective and understanding as well as the financial support for my earlier studies.

My siblings, Tendayi, Patience, and Tatenda and my sister-in-law, Rhoda, for always being there for me and pushing me to do better each time.

Geoffrey Jjuko for listening to all my research woes and continuous support during this project.

STUDY OBJECTIVES

Post partum haemorrhage is one the primary causes of maternal morbidity and mortality [1,2]. The consequences of excessive haemorrhage following the delivery of a baby include hypo-volaemic shock and anaemia, which reduce the quality of life and care for both the mother and neonate. The incidence of post partum haemorrhage may be reduced by routine administration of oxytocin (OT) which maintains uterine tone and prevents bleeding. However, due to the short half-life of OT *in vivo*, the primary objective of the project was to develop and assess an alternate delivery system that would provide sustained and prolonged release of OT *in vitro*.

The objectives of this study were:

i. To develop and validate a suitable and sensitive High Performance Liquid Chromatographic (HPLC) method to accurately and precisely quantitate OT in pharmaceutical dosage forms and OT release from dosage forms during *in vitro* release testing,
ii. To review current trends of protein and peptide delivery and objectively evaluate and propose an alternative sustained release OT dosage form,
iii. To characterise a potential matrix for an OT dosage form for sustained delivery in order to understand factors that are likely to impact on the performance and integrity of the dosage form,
iv. To develop a discriminatory *in vitro* dissolution method for assessing the release of OT from sustained release dosage forms of different formulation compositions and,
v. To determine the mechanism/kinetics of OT release from the delivery matrix using model dependent approaches.

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CHAPTER 1

OXYTOCIN

1.1 INTRODUCTION

Oxytocin (OT) is synthesised in the cell bodies of the paraventricular and supraoptic nuclei in the hypothalamus from precursor proteins [5-7]. OT is attached to the carrier protein, neurohypophysin and is transported via axonal processes to the posterior lobe of the pituitary gland where it is stored and subsequently released when required [5,7].

OT possesses uterogenic and galactogenic activity in mammals resulting in uterine contractions and milk letdown following secretion, respectively [8-11], which can be considered the classical actions of OT. OT also possesses diverse peripheral and central effects due to the extensive expression of OT receptors (OTR) in several organs and tissues other than the uterus and mammary glands. Studies have shown that the activity of OT extends to stimulation of endometrial prostaglandin production, T-cell function, bone and muscle formation and secretion of prolactin in the pituitary gland, luteolysis and sperm transport [12,13].

OT is available commercially as parenteral and nasal solutions of the acetate salt [10]. Ampoules of OT containing 5 and 10 IU/ml are available on the South African market [14], whereas the nasal spray is not. The nasal spray is formulated to contain OT in a concentration of 40 IU/ml [15].

The commercial or brand names of OT products are Alpha-hypophamine®, Ocytocin®, Endopituitrina®, Pitocin®, Syntocinon®, Nobitocin S®, Orasthin®, Oxystin®, Partocon®, Synpitan®, Piton-S®, Uteracon® [16].

1.2 PHYSICO-CHEMICAL PROPERTIES

1.2.1 Description

OT comprises of nine (9) amino acids. The amino acid sequence of OT is shown in Figure 1.1 [8,11].

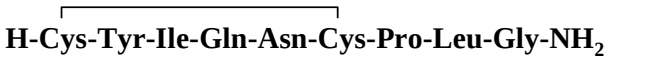


Figure 1.1 *The primary structure of OT [8,11]*

The amino acid moieties in the endogenous peptide exist in the L-configuration and a disulphide bridge links the two (2) cysteine residues resulting in a six (6)-membered ring linked to a tripeptide residue that is amidated at the carboxy terminal [8]. Figure 1.2 shows the complete chemical structure of OT [8].

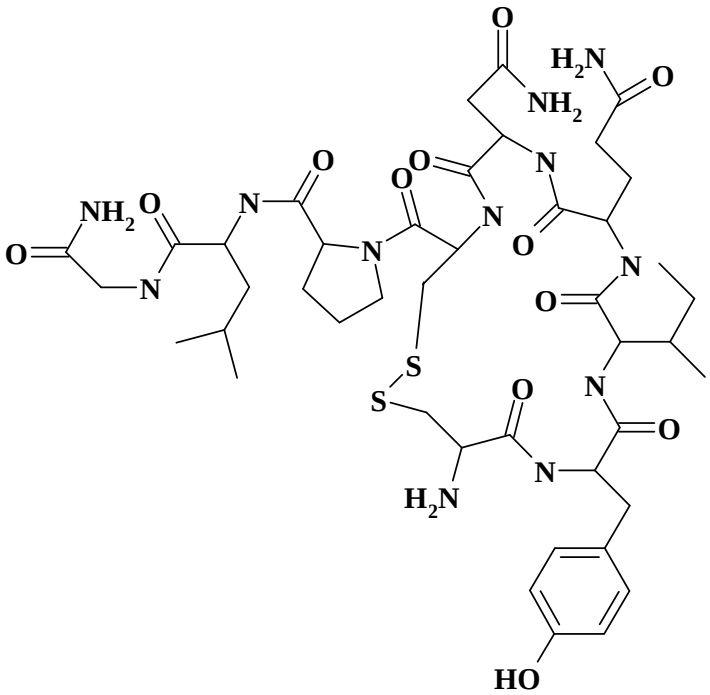


Figure 1.2 *The chemical structure of OT ($C_{43}H_{66}N_{12}O_{12}S_2$) MW = 1007.23 [8]*

The amino acid sequence of OT may define its chemical name. OT is L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide cyclic (1 → 6) disulphide or L-Hemi-cystinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-hemi-cystinyl-L-prolyl-L-leucyl-glycinamide [8].

The free base of OT has not yet been isolated in its crystalline form, but OT is available as the freeze-dried acetate salt. The acetate salt occurs as a white or almost white fluffy hygroscopic powder with a faint odour of acetic acid [8,11].

1.2.2 Biological Activity

OT can be manufactured using different synthetic pathways that may in turn, result in grades of OT with slightly different biological activity. The potency of synthetically produced OT samples may be standardised according to the contractility of the rat uterus, vasopressor activity in chickens and rats, as well as ejection of milk in lactating rats following administration [8]. The resultant activity is expressed in posterior pituitary units and a unit is equivalent to 2.0 – 2.2 μg of the pure hormone [15]. It has been reported that 12.5 IU of OT are contained in 21.4 μg of the synthetic peptide (584 IU/mg) [9] in one (1) ampoule of the Fourth International Standard (1978). The National Institute of Standards and Control (London, UK) indicates an activity of 595 IU/mg for the Fourth International Standard of OT [8]. The United States Pharmacopeia (USP) [10] recommends that the activity of OT must not be less than 400 IU/mg and the British Pharmacopoeia (BP) [11] by convention labels 1 mg of OT to be equivalent to 600 IU. The variability noted with regard to the standardisation of OT in the different compendia implies that the characterisation of OT samples may be mandatory. Characterisation may be achieved by determining the actual biological activity of OT using the biological assays described or by means of analytical assays and/or comparing a synthetic OT sample with an international reference standard [8].

1.2.3 Synthesis

In general, OT is produced on a commercial scale synthetically, but can be obtained from the pituitary glands of healthy domesticated animals [10]. However, the extraction of OT from pituitary glands is of no practical significance and therefore no longer used for the commercial preparation of OT [8].

OT and vasopressin (VP) were the first two (2) hormones for which the chemical structures were fully elucidated and chemical synthesis was successfully completed [17]. The first chemical synthesis of OT was reported by du Vigneaud *et al.* in 1953 [18,19]. In this early work, a highly purified form of OT was obtained, and isolated as a crystalline flavianate salt.

In the large-scale production of OT, protective species are necessary to hinder access to reactive amino and sulphydryl groups. Tosyl and carbobenzoxy groups are used for the protection of amino functionalities and a benzyl residue can be useful for the protection of the sulphydryl group in the cysteine residue [19]. The synthetic procedure used by du Vigneaud *et al*. [19] is shown in Figure 1.3.

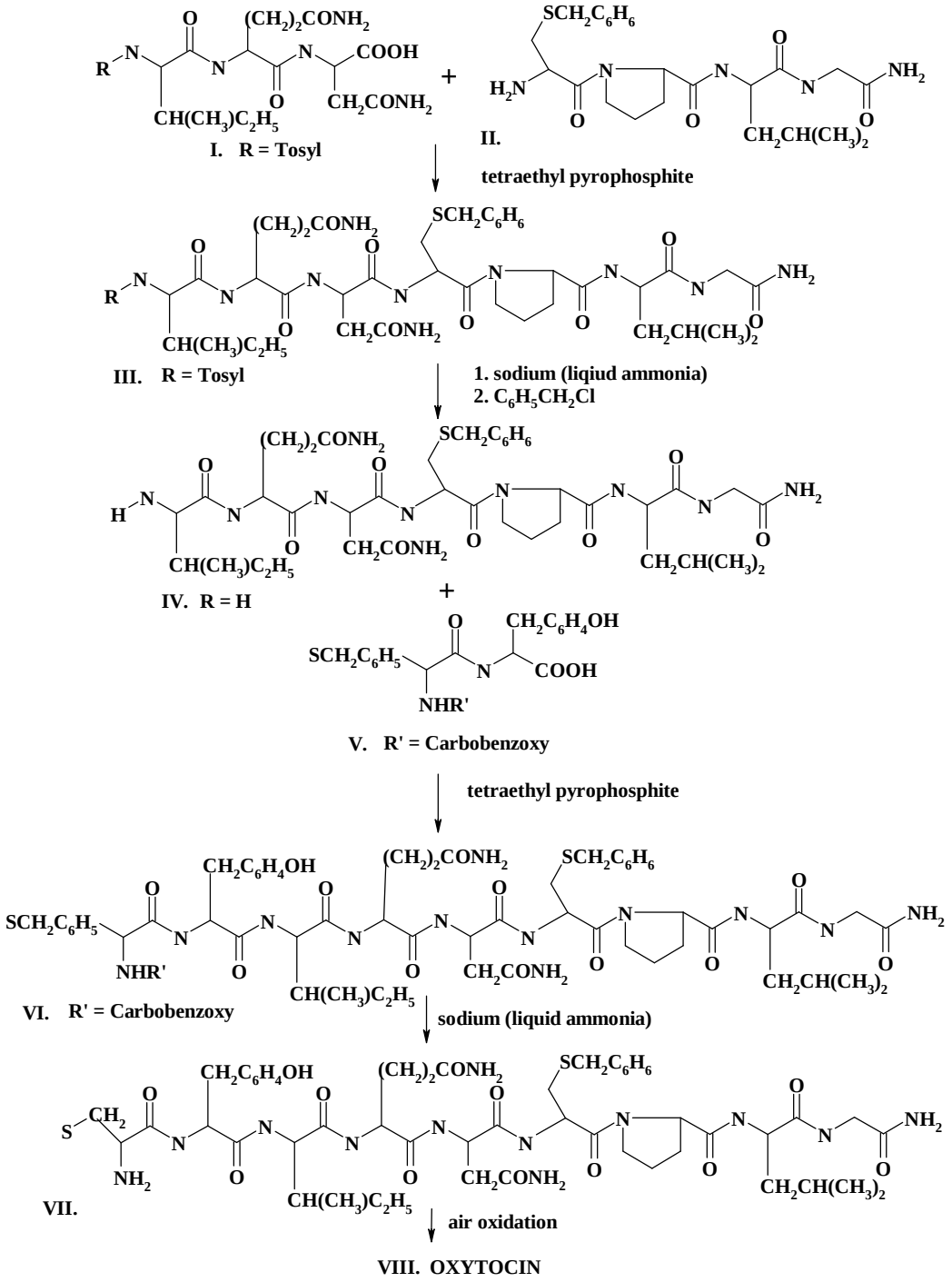


Figure 1.3 *The du Vigneaud synthetic pathway for OT (adapted from [19])*

The initial step in the synthesis of OT is the coupling of tosyl-L-isoleucyl-L-glutaminyl-L-asparagine (I) with S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (II) via a condensation reaction using tetraethyl pyrophosphite by a procedure known as the amide procedure. The heptapeptide (III), containing tosyl and benzyl protective groups, is formed and the protective

groups are removed in the presence of sodium in liquid ammonia. The reduced compound is re-benzylated with benzyl chloride to re-protect the sulphydryl group, forming L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV).

Condensation of this heptapeptide with a dipeptide, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine (V), is achieved in the presence of tetraethyl pyrophosphite to produce an open chain nonapeptide derivative, N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosinyl-L-isoleucyl-L-glutami-nyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide (VI). The resultant nonapeptide is further treated with sodium in liquid ammonia to remove both the protective benzyl and carbobenzoxy groups forming an open chain nonapeptide, L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide (VII). Air oxidation in a dilute aqueous solution at pH = 6.5 results in cyclisation of the nonapeptide by the formation of a disulphide bridge between the two cysteinyl residues to form OT (VIII) [19].

Subsequently OT has been produced by methods that have differed slightly in the chemistry of the synthetic process, use of different protective groups, methods used to make peptide linkages, and manufacturing plans used to synthesise the entire peptide [8].

Protective groups that have been used in the synthetic procedure include an S-acetamidomethyl group for protection of the cysteine residue [20]. p-methylbenzyl and p-methoxybenzyl, which were found to be better cysteine-protecting groups compared to benzyl [21], and S-benzamidomethyl [22] have also been used for this purpose. New protective groups for cysteine residue protection, 2,2,5,7,8-pentamethyl-3,4-dihydro-6-benzopyranylmethyl, 2,2,4,6,7-pentamethyl-2,3-dihydro-5-benzofuranylmethyl and 4,5,6-trimethoxy-2,3-dihydro-7-benzofuranylmethyl have been synthesised and tested in the OT synthetic procedure [23].

N-tert-butoxycarbamate (N-Boc) has been used successfully to protect amino groups in the synthesis of OT [24,25] and the use of an o-nitrophenylsulphenyl group has also been reported [24].

Mixed anhydrides, active esters, and azide methods have been used to make peptide linkages in OT [8]. In a stepwise addition, polychlorophenyl esters of three (3) equivalents of the butoxycarbonylamino acids were reacted in the presence of 1-hydroxybenzotriazole. This procedure was followed by aminolysis and deprotection of the reactive groups [26]. Furthermore, intermediates with pre-formed disulphide bridges have been used to synthesise OT [27].

Recently, biologically active OT has been synthesised using a ^{13}C and ^{15}N-labelled proline residue using Oppolzer's method. Sultam was used as the chiral auxiliary for the synthesis of the tripeptide fragment. N-Boc and 9-Fluorenylmethyloxy carbamate (N-Fmoc) were used for the protection of the amino groups in tocinoic acid, which is the twenty (20)-membered disulphide ring of OT. These were later removed using either trifluoroacetic acid or piperidine [28].

1.2.4 Solubility

The solubility of OT acetate in different solvents is summarised in Table 1.1.

Table 1.1 *The solubility of OT acetate in different solvents [8]*

Solvent	Concentration (IU/ml)	Concentration (mg/ml)
Water	37 800	63
Methanol	86 400	144
Dichloromethane	2.9	0.005

1.2.5 Isoelectric Point

OT has a free amino group on the cysteine[1] residue and a free acidic phenol group on the tyrosine[2] residue and is thus an amphoteric molecule. The isoelectric point is reported to occur at pH = 7.7 [8].

1.2.6 Ultraviolet Spectrum

The ultraviolet (UV) spectrum of OT in an aqueous solution of concentration 200 IU/ml over the range 200 – 600 nm at a scan speed of 600 nm/min and data interval of 0.800 nm is depicted in Figure 1.4. The UV spectrum was generated using a GBC UV/VIS 916 Spectrophotometer (GBC Scientific Equipment, Pty Ltd, Dandenong, Australia).

The UV spectrum depicted in Figure 1.4 is similar to that obtained by Nachtmann *et al*. [8]. The UV spectrum for OT shows two distinct wavelengths at which maximal absorbency occurs. OT has a λ_{max} at approximately 275 nm, with a shoulder at approximately 280 nm and shows another region of increased absorbency between 200 – 240 nm.

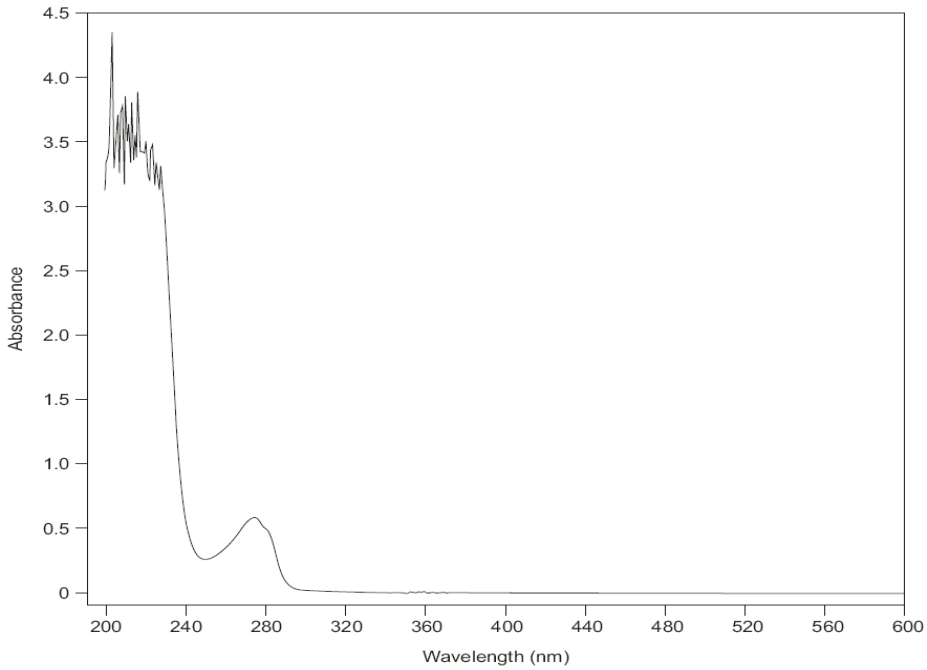


Figure 1.4 *UV absorption spectrum of OT acetate in aqueous solution*

1.2.7 Infrared Spectrum

The infrared (IR) spectrum of each compound is unique and serves as a useful tool to identify the chemical structure of an organic compound. Although the spectrum is characteristic of a compound, certain groups of atoms in organic molecules give characteristic absorption bands in the IR region, irrespective of the structure of the molecule. However, the precise interaction of the atoms in a molecule generates the unique spectrum for that molecule [29]. The IR spectrum of OT (470 IU/mg) is shown in Figure 1.5 and was generated from 4000 to 400 cm^{-1} using a Spectrum 2000 FTIR Spectrophotometer (Perkin Elmer Instruments LLC, Shelton, CT, USA) using a Mull technique with Nujol® or heavy liquid paraffin (UniLab, Redmont WA, USA).

The IR spectrum of OT shows characteristic absorption bands in the region of about 3500 – 3200 cm^{-1} that can be attributed to the presence of both primary amide bonds and the amine functionality, which show medium absorption bands in this region [29]. Alkanes give rise to medium intensity absorption bands around 3000 cm^{-1} and a sharp band can be observed in the IR spectrum at this region. In addition, bands in this region are also due to the presence of liquid paraffin, which has C-H bonds resulting in resonance at this frequency.

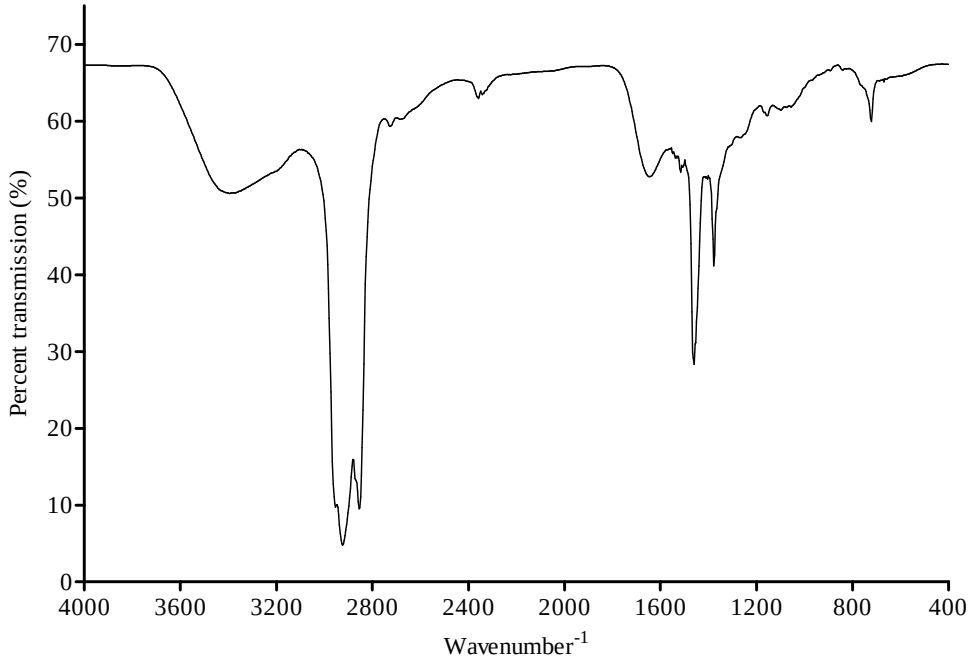


Figure 1.5 *Infrared spectrum of OT acetate*

The OT molecule has a disulphide linkage, and mercaptans result in weak absorption bands at about 2500 cm^{-1} and a weak absorption band is observed in this region in the spectrum of OT. Several functional groups absorb IR light in the 1700 – 600 cm^{-1} region. The tertiary amine in the proline residue produces a medium strength band at a wavenumber of approximately 1700 cm^{-1}. Primary amides in the peptide linkages give rise to an absorption band that is observed at about 1650 cm^{-1}.

The absorption observed for the rest of the region is primarily due to alkanes that are also present in liquid paraffin, primary amines, and amide groups which absorb IR light in this range, although only a weak absorption band is noted in the region between 1000 – 800 cm^{-1}. The IR spectrum obtained differs with one published in the literature [8] due to the use of a different method to obtain the IR spectrum. The IR spectrum reported in the literature [8] was generated using potassium bromide and this does not produce intense absorption bands due to the frequencies produced by C-H bonds present in the liquid paraffin that are observed in Figure 1.5.

1.3 STABILITY

1.3.1 Temperature

Freeze-dried OT acetate may be kept in a refrigerator for several years without significant loss of oxytocic activity. However, inactivation of the peptide stored under these conditions may occur as a result of disulphide interchange in which intramolecular disulphide linkages convert to form intermolecular bridges [8], and it may therefore be necessary to test the biological activity of samples following long-term storage.

OT sample concentrates that were stored in a refrigerator at 5 °C showed no significant loss of activity after twelve (12) [8] or eighteen (18) months [30]. Whereas samples that had been kept at 21 °C, showed only a slight loss of activity of 1.5% per annum, and samples maintained at 30 °C showed an increased loss of activity of approximately 10% per annum [8].

The compendial and pharmacopoeial guidelines for OT storage concur with the stability characteristics of commercial OT preparations described above. The USP [10] and BP [11] recommend that OT must be stored in airtight containers in a refrigerator set between 2 and 8 °C and must be discarded after 30 days if OT ampoules have been stored between 15 and 25 °C [31].

The thermal stability of OT in aqueous media has been reported previously [32]. Only 38% of the original OT that was added to the solution remained after evaporating an aqueous solution of OT to dryness by maintaining the solution at 35 °C in a water bath. Heating at 100 °C for 30 min resulted in a 39% loss of OT, whereas heating for 15 min at 120 °C resulted in no loss of activity [32]. After heating an aqueous solution of OT (10 IU/ml) at 50 °C for 10 min, as described in § 2.5.6.3, only 3.55 $\pm$ 1.07% of the OT degraded. This experiment was conducted in triplicate (n = 3) and was analysed using a validated HPLC method (Chapter 2, *vide infra*).

1.3.2 pH

The stability of OT is pH dependent and pH therefore affects the shelf life of OT in formulations. At low pH, OT undergoes hydrolysis of the peptide linkages [8]. However OT has been found to be stable between pH = 5 and pH = 8.5 and has a pH of maximal stability at pH = 3. As the pH increases towards a neutral value and proceeds to a more alkaline range, the formation of dimers and other polymeric forms of OT occurs as a result of the conversion of intramolecular disulphide

linkages to form intermolecular links between the cysteine residues in two (2) different OT chains [8].

Approximately 10.58, ± 0.51%, of OT degrades in 0.1 M solutions of hydrochloric acid and 70.33, ± 0.25%, of OT degrades in 0.1 M sodium hydroxide solutions in 2 hours as described in detail in § 2.5.6.1 and 2.5.6.2, respectively. The degradation studies were performed in triplicate (n = 3) and analysed using a validated HPLC method (Chapter 2, *vide infra*).

OT parenteral preparations are formulated with acetate buffer at a pH of approximately 3.9 using a sodium acetate buffer and acetic acid to adjust the pH to the required value, to ensure maximal stability of the peptide formulation [33].

1.3.3 Oxidation

OT is susceptible to oxidation and 21.67, ± 0.49%, of OT degrades in twenty (20) volume hydrogen peroxide solutions, as described in § 2.5.6.5, and analysed using HPLC, as described in detail in Chapter 2, *vide infra*.

1.4 CLINICAL PHARMACOLOGY

1.4.1 Physiological Role of OT in the Uterus

The primary role of OT is to cause uterine smooth muscle tissue contraction during parturition [5,12,15,34,35,36]. In the pregnant and puerperal uterus, OT causes a dose dependent increase in the frequency and amplitude of uterine contractions and at high doses causes an increase in the uterine resting tone [15].

Successful parturition depends on coordinated changes in the uterine contractility and sensitivity of the uterine smooth muscle tissue to OT because of increased OTR and changes in the connective tissue that allows cervical ripening and dilation [34]. In mammals, the changes in the uterus are a result of an increase in the levels of circulating oestrogen and a corresponding decline in circulating progesterone levels [5,34].

There is an almost thirty (30) fold increase in the number of OTR in the uterus in early labour compared to their expression in early pregnancy [5,37]. Consequently, there is an increased sensitivity and responsiveness of the uterus to OT. This sensitivity may increase up to eight (8)

fold in the last nine (9) weeks of pregnancy, with an associated increase in contractility of the uterine smooth muscle tissue [5,13]. This is associated with the up-regulation of OTR messenger, ribonucleic acid (mRNA), and the density of the myometrial OTR, which reaches a peak in early labour [13,38]. In addition, there is an increase in myometrial weight and actinomycin content, which also promote uterine muscle tissue contraction [15,34]. Furthermore, an increase in spontaneous motor activity of the uterus in the third trimester, as compared to the first and second trimesters of pregnancy can be considered to parallel the increase in the responsiveness of uterine tissues to OT stimulation [5]. The synergistic effects result in contraction of the uterus at full term pregnancy at the commencement of labour [5,8,9,15].

Progesterone has been shown to antagonise the effects of OT *in vitro* and therefore may have an effect on the responsiveness of the uterus to OT. The decline in the progesterone levels prior to birth may therefore also play a role in the activity of OT and initiation of labour [5,37].

1.4.2 Physiological Role of OT in the Mammary Glands

Milk ejection from the mammary glands is triggered by the suckling action of an infant on the nipple. Suckling stimulates the mammary tactile receptors that generate sensory impulses and directs them to the paraventricular and supraoptic nuclei via the spinal cord [5,8,9,13,15]. The oxytocinergic neurons in the hypothalamus display a high frequency-bursting activity with each burst resulting in a massive release of OT into the systemic circulation, which is carried to the breasts of lactating mothers. In the mammary gland, OT causes a contraction of the myoepithelial cells in the walls of lactiferous ducts, sinuses, and breast tissue alveoli. In humans, milk ejection occurs within 30 – 60 seconds following the commencement of suckling [13]. OT is considered essential for infant nursing and a deficiency of OT in mice has been shown to result in failure of test animals to effectively nurse their offspring [39].

1.4.3 Additional Physiological Roles of OT

OTR are expressed in several other issues, thereby revealing the diverse nature of the physiological roles of this nonapeptide in the human body [13].

In the peripheral tissues, OTR are expressed in the ovary [40] and corpus lutetium [41] and OTR expression has been observed in males [42] including in the testes [43] and prostate gland [44]. OT synthesis has also been recently observed in the breast [45] and lung tissues [46], where it may be involved in the proliferation of cancerous cells.

OTR are also expressed in the kidney [47], heart [48], thymus [49], and pancreas [50] among other peripheral organs, indicating a diversity of physiological roles for OT. In the brain, OT plays a role in sexual and maternal behaviour [51,52], stress related behaviour [53] and eating disorders [54], amongst other diverse functions and roles.

1.4.4 Mechanism of Action

OTR is a G-protein coupled receptor (GPCR) and is functionally coupled to $G_{q/11\alpha}$ class of guanidine triphosphate (GTP) binding proteins [13]. The GTP binding protein, coupled with $G\beta\gamma$ stimulates the activity of phospholipase C-β forms, which results in the hydrolysis of phosphoinositide, generating two entities, specifically, inositol 1,4,5-triphosphate and 1,2-diacyl glycerol. Inositol 1,4,5-triphosphate triggers the release of calcium ions from intracellular stores and 1,2-diacyl glycerol causes the stimulation of protein kinase C, which phosphorylates, as yet unidentified, target proteins [55-57]. The increase in intracellular calcium ions results in a variety of intracellular events including the formation of calcium-calmodulin complexes. In smooth muscle tissues of the uterus, the increase in calcium ions triggers the activation of myosin light-chain kinase activity, which results in contraction of the myometrial tissue resulting in uterine contraction [13,58,59].

1.4.5 Structure-Activity Relationships

The conformation of a drug at a receptor is important for eliciting a biological response *in vivo*. For OT a myriad of possible conformations exist and therefore understanding the elements of the molecule necessary to stimulate biological activity is imperative to comprehend the subsequent biological events. In addition, understanding the structure-activity relationships (SAR) of OT facilitates the design of stable agonists and antagonists that can be used in clinical practice [7,60,61].

Hormone-receptor interactions actually involve three different states for the hormone-receptor complex. At the outset, hormone-receptor binding interaction occurs and is considered to be the recognition step between the hormone and the receptor. The recognition step is necessary for binding but is not adequate to elicit a biological response. The transduction state follows and involves hormone-receptor interactions with a regulatory protein, and leads to activation of a second messenger compound such as cyclic adenosine monophosphate (cAMP) and/or calcium ions. The third step in the process is the dissociation of the hormone-receptor complex and return

of the cell to a basal state. The recognition and transduction states are known to involve structural and conformational properties of hormones and research has shown that the two steps for peptide hormone-receptor systems can actually involve two different conformations of the same hormone [62].

In order to understand the agonist activity of OT, the structure of OT must be separated into the 20-membered backbone and a tripeptide side chain. Early SAR studies of the activity of OT showed that the 20-membered ring was essential for the activity of OT. In support of this theory it was noted that a synthetic ring-opened form of OT was found to have significantly reduced potency as compared to the closed form. The ring-opened structure however had a slower onset of activity that was attributed to the slow oxidation of the linear form of the compound prior to transformation into a cyclic peptide form [63].

The tripeptide tail has no known activity but plays a critical key role in the potency of the molecule and binding affinity of the OT molecule to receptors. The replacement of the C-terminal amide group with a carboxylic acid group forming oxytocinoic acid, results in a molecule that has only $1/400^{th}$ the activity of OT [64].

It is generally believed that the tripeptide side chain and the disulphide ring work cooperatively in the interaction of OT with OTR or in transducing the receptor, thereby eliciting a biological response [7].

The SAR of OT at the uterus receptor have been summarised by Hruby *et al*. [62] as follows:

i. The 20 membered disulphide ring system is sufficient for activity at the uterine receptor,
ii. There is cooperativity that occurs between the disulphide ring and the tripeptide tail, which is important for the full binding interaction of OT with OTR,
iii. The tripeptide tail of OT is essential for binding of OT to the receptor but is not important in the transduction of the uterine OTR,
iv. Important residues for both binding and transduction are cysteine residues (1 and 6), the tyrosine^{2} and asparagine^{5} residues, which appear to be involved in a cooperative manner in the transduction process and,
v. The OTR in the uterus appears to have three (3) separate topological binding sites for OT. There is a lipophilic site associated with first and third residues in OT, a hydrophilic site with hydrogen bond accepting properties for the fourth and fifth residues and a more lipophilic site that encompasses the tripeptide tail of the hormone.

1.4.6 Clinical Use, Indications and Dosage

OT may be used for the induction of labour or augmentation of labour when medically indicated [5,9,36,65,66]. For this purpose OT may be given by a slow intravenous infusion in which 5 IU of OT are placed in 500 ml of a physiological compatible fluid such as normal saline, although higher doses may be used [5,9,15,67]. Initially, 0.5 – 2 mU/min may be administered intravenously and the dose may be increased every 15 – 60 min as is necessary [68]. There is general debate about the correct dosage for labour induction, although the consensus is that a 10 mU/ml of OT administered at an initial dose level of 1 mU/min [5] is sufficient to promote labour. Guidelines [69] recommend 10 or 20 IU in 1000 ml normal saline and that the infusion is initiated at a rate of 1 – 2 mU/min, gradually increasing every 30 min, until a maximum of 3 – 4 contractions per 10 minute interval is observed [9,31,69]. It has been recommended that increments be implemented at 40 minute intervals [65] or even 60 minute intervals in order to prevent excessive uterine contractions and foetal distress [70,71] based on the pharmacodynamic profile of OT [70].

An administration rate of 12 mU/min is the maximum that is usually required for labour induction, although a rate of 6 mU/min is sufficient to produce physiological levels of OT usually present at the commencement of natural labour [9]. Foetal heart rate and uterine contractions should be monitored in the process once labour has commenced and is progressing and at that stage, the OT infusion may be gradually withdrawn [9].

OT has also proved useful for the prevention of postpartum haemorrhage and management of postpartum or postabortal uterine atony in the third stage of labour [5,9,15,72,73]. In order to promote routine third stage prophylaxis, a recommended dose of 5 – 10 IU as an intravenous bolus [68,74] or intramuscular [69] dose of OT may be used. This may be followed by a slow intravenous infusion in severe cases, where 5 – 20 IU [9] or 10 – 40 IU of OT in 1000 ml of a compatible and non-hydrating diluent are administered following a normal vaginal delivery [69], or a high dose of OT, 2667 mU/min, administered over 30 min for caesarean delivery [69,75]. A dose of 10 IU is recommended at a rate between 20 – 40 mU/min [9]. Initially, the infusion is given at a rate of 10 ml/min for a few minutes until the uterus has contracted and then is reduced to 1 – 2 ml/min until the mother is ready to be transferred to the postpartum unit [5]. OT may also be administered as an intramuscular injection (10 IU) for the treatment of uterine atony [5]. Furthermore, as a prophylactic measure OT may be administered intramuscularly (5 IU) in combination with ergometrine maleate (500 μg) to non-hypertensive patients with or after the delivery of the shoulders of the baby, or if OT administration alone fails to produce adequate

uterine tone [5,9]. In the case of a missed abortion, 5 IU of OT is administered via slow intravenous injection or as an infusion at a rate of 20 – 40 mU/min or higher [3,5,9,15,76].

OT may also be used to stimulate lactation and may be recommended for nursing mothers following the premature delivery of infants [77]. A double-blind randomised controlled clinical study showed the effectiveness and safety of nasal OT for the promotion of lactation [78]. However, a recent double blind randomised clinical trial [79] comparing the effectiveness of nasal OT to a placebo preparation for the promotion of lactation in mothers of pre-term infants, showed that the administration of nasal OT resulted in faster initial milk production compared to the placebo formulation. However, there was no significant difference between the use of the OT nasal spray and the placebo, suggesting the presence of a significant placebo effect. A recommendation for promotion of lactation has been that the encouragement and support of mothers, with unrestricted feeding, may diminish the need for OT use to stimulate lactation [80]. In order to stimulate lactation, OT is administered as a nasal spray and one (1) puff is sprayed into one (1) or both nostrils 3 min before suckling is initiated [9,15]. There is a risk of dependence and therefore OT use for lactation stimulation is not always recommended [9].

1.4.7 Overdose

An overdose of OT results in uterine hyperactivity, hypertone or tetany, which are potentially detrimental to both mother and baby, causing severe adverse reactions as described in § 1.4.8. OT administration must be discontinued immediately and symptomatic and supportive treatment is advised in cases of overdosing [31].

1.4.8 Adverse Drug Reactions

OT is an endogenous hormone and has not been reported to have any adverse effects following administration on its own. However, administration of OT in high doses may result in uncontrolled and violent uterine contractions or uterine tetany that may result in uterine rupture and decreased utero-placental blood flow, resulting in foetal bradycardia, foetal arrhythmia, asphyxia and even maternal and foetal death [9,15]. Nausea, vomiting, and cardiac arrhythmias may also occur [81].

OT is structurally similar to VP and possesses weak anti-diuretic properties due to interaction with VP receptors [35,82]. Prolonged infusions over 120 min and high doses of OT may result in water retention with associated water intoxication and hypo-natraemia, resulting in pulmonary

oedema, convulsions, coma or even death, in serious cases [81]. The vasopressor effect of OT is more likely with OT of natural origin, but may occur when synthetic OT is administered [9,15,83].

Rapid administration of commercial OT may also result in hypotension and reflex tachycardia [9,15]. Some studies have shown that the impact of commercial OT preparations is not due to the OT itself, but the combination of OT and chlorbutol, the preservative in commercial formulations. The combination results in cardio-depressive and negative inotropic effects, which are similar in magnitude to those caused by chlorbutol when administered alone, resulting in vasodilation, precipitating hypotension, and consequent tachycardia in patients receiving such commercial preparations [82,84-87].

A rare case of water intoxication and hypo-natraemic encephalopathy has been reported [88] following excessive use of OT administered via the nasal route. Following administration of intravenous fluids, water intoxication resulted, probably due to the anti-diuretic properties of OT with a result of hypo-natraemic encephalopathy and convulsion. It is therefore recommended that the use of nasal OT for promotion of lactation be monitored to prevent such deleterious effects [88].

The use of OT to induce labour has been associated with an increase in the incidence of neonatal jaundice [89,90]. OT may cause neonatal jaundice by inhibiting hepatic glucoronyl transferase [91] and by increasing erythrocyte deformation and haemolysis at high doses [89].

Anaphylactic and hypersensitivity reactions to OT, although rare [81], include cardiac arrhythmia, pelvic haematomas in addition to nausea and vomiting [9,15]. Following administration of OT via the nasal route, insulin and glucagon secretion is increased [92], which may result in low plasma glucose levels.

1.4.9 Contraindications

OT should not be administered in cases where its action is likely to harm the mother or the foetus i.e., where there is a poor risk: benefit ratio for the patient [9,15]. OT use is contraindicated for obstetrical conditions such as cephalopelvic disproportion, foetal malpresentation, foetal distress, placenta praevia, and in cases of mechanical obstruction to delivery. Moreover, OT is contraindicated in cases where there is a predisposition to uterine rupture as in the case of

multiple pregnancy or when there has been previous entry into the uterine cavity, for example a previous caesarean section, myelectomy or any other genital tract related surgery [9,15,36,81].

A rare contraindication to the use of OT for induction of abortion has been reported, where sepsis as a result of ligamentary ectopic pregnancy occurred and abortion was indicated at over twenty four (24) weeks of pregnancy [93]. The administration of OT failed to induce labour resulting in persistent bleeding and OT is therefore contraindicated in such gynaecological complications [93].

OT should not be administered in cases of uterine hyperactivity and in patients in which there is an increase in the basal tone of the uterus, as this may result in uterine rupture [9,15,81].

OT is contraindicated in patients that have hypersensitivity to OT [15]. Hypersensitivity usually occurs as a result of administration of very high doses of OT and may be reduced by administration of OT with small increments in the infusion rate thereby preventing hyper-stimulation of the uterine smooth muscle and hypersensitivity [15]. OT must also be used with caution in patients with cardiac disorders [81].

1.4.10 Drug Interactions

Drug interactions with OT are not common; however, OT may increase the vasopressor effect of sympathomimetic drugs [9]. This is the consequence of administration of high doses of OT resulting in high circulatory levels of the hormone, and therefore OT acting on the VP receptors due to the similarity in their primary amino acid sequence and structure. Therefore, there is an associated drop in blood pressure following this interaction [35]. Prostaglandins must be used with caution if administered concomitantly with OT as this may result in uterine hyperactivity [81].

1.5 PHARMACOKINETICS

1.5.1 Absorption

OT is a labile peptide that is not susceptible to trypsin-mediated degradation in the gastro-intestinal tract (GIT). The degradation of OT in the GIT is due to metabolism by chymotrypsin, which digests the tyrosine^{2} – isoleucine^{3} and leucine^{8} – glycine^{9} bonds [94]. OT was found to

degrade rapidly within 60 min in intestinal fluid contents, but the degradation of OT in the gastric, colonic and rectal membranes and in the intestinal microvilli membranes is thought to relatively slow and could be a result of membrane-bound proteins such as endopeptidase 24,11 and amino peptidase, which digest proteins [94]. Due to extensive metabolism in the GIT, OT is not active orally. It is however rapidly absorbed across mucous membranes and OT has been formulated as a sublingual dosage form, although this has been discontinued due to erratic and unpredictable absorption of OT from the buccal mucosa when this dosage form was administered [95].

1.5.2 Distribution

OT distributes into the extracellular fluid space, occupying approximately one third of the total volume of the extracellular fluid [96] and has a volume of distribution of approximately 0.3 l/kg [97] indicating that OT has little or no apparent protein binding characteristics [96].

Administration of OT by different routes, *viz.*, nasal, intramuscular, intravenous, and buccal routes, results in different plasma concentrations. Basal levels of OT are reported to be < 10 pg/ml. Following nasal administration of between 65 – 100 μg OT, the plasma concentration of OT increased to between 36 – 85 pg/ml. Administration of an intramuscular dose of OT (25 μg) produced resultant plasma concentrations that were markedly higher that those attained following nasal administration, reaching between 360 – 480 pg/ml. However, the administration of buccal OT (70 μg) was ineffective as the plasma concentrations observed were lower than 10 pg/ml [98].

1.5.3 Metabolism

OT is rapidly cleared from the systemic circulation by metabolism in the liver and is excreted via the kidneys [4,8,99]. OT has a plasma half-life of only a few minutes, reported to be 3.2 min following a single intravenous bolus injection and 4.8 min following an intravenous infusion at 0.5 IU/min for 25 min [96,100].

OT is metabolised primarily by two (2) enzyme systems that are responsible for the removal of glycinamide from the C-terminal of the polypeptide and removal of the Leu-Gly-NH_2 sequence [8].

Glutathione plays an important role in the metabolic fate of OT in the kidney, increasing the rate of degradation observed in the renal microvilli by membrane-bound enzymes [4]. *In vivo*, glutathione may be involved in the metabolism of OT by cleavage of the disulphide link between

the cysteine moieties [101]. An alternate mechanism by which OT may degrade in the kidney in the absence of glutathione may be a result of the presence of a membrane bound enzyme, endopeptidase 24.11, which attacks lipophilic bonds i.e. proline^{7} – leucine^{8} [4].

Degradation of OT in the liver does not occur as a result of membrane-bound enzymes, but occurs in the hepatocytes [4] and OT has been found to be susceptible to enzymatic attack by peptidases in the liver *in vitro*. Peptidase enzymes are located mainly in the lysosomes and microsomes [102]. In addition, OT undergoes receptor-mediated endocytosis into the hepatocytes and undergoes intracellular metabolism in these regions, as has been observed for VP [103]. The rate and extent of degradation in the kidney in the presence of glutathione is higher than in the liver [4].

Furthermore, pregnant women produce plasma oxytocinase [104-107], which is an aminopeptidase enzyme that cleaves OT at the cysteine^{1} – tyrosine^{2} bond resulting in the formation of an inactive acyclic compound [8]. An enzyme present in the placenta identical to plasma oxytocinase also hydrolyses OT [107-109]. Figure 1.6 shows a summary of this degradation reaction.

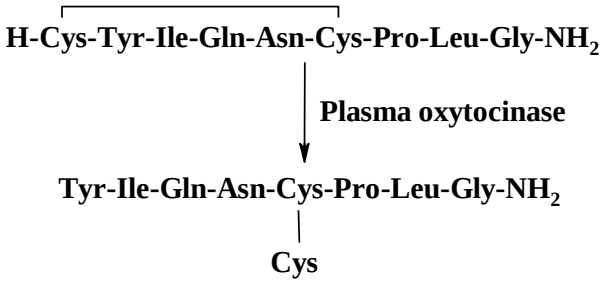


Figure 1.6 *Metabolic degradation of OT by plasma oxytocinase (Redrawn from [8])*

Reported half-life and mean metabolic clearance rates of OT vary according to the route of administration and the dose delivered. The plasma half-life of OT in males is reported to be between 3.2 – 10.3 min [97] and in non-pregnant females is between 3.3 – 4.5 min, whereas in pregnant females the range is 1.8 – 4.9 min [110]. The mean metabolic clearance of OT has been reported to be 21.5 $\pm$ 3.3 ml/kg/min [97] and 16.4 ml/kg/min [111]. Following the use of OT for labour induction, the metabolic clearance rate has been reported to be 7.97 ml/min [112]. There are no significant differences in the mean metabolic clearance rate of OT between men, non-pregnant and pregnant women. Reported values of mean metabolic clearance are 27 $\pm$ 1.8 ml/kg/min in men, 20.6 $\pm$ 2.8 ml/kg/min in non-pregnant women and 23.1 $\pm$ 2.6 ml/kg/min in

pregnant women, using the corrected weight pre-pregnancy weight for pregnant women to give a metabolic clearance rate of 25.4 $\pm$ 2.0 ml/kg/min [113].

The administration of an intravenous dose of OT results in a rapid onset of action that occurs within 1 minute, with a duration between 30 and 60 min and with a half-life less than 5 min [31,69]. The onset of action following intramuscular administration is within 3 – 5 min and lasts 2 – 3 hours [31].

1.5.4 Elimination

OT is eliminated primarily via the renal route [31,110]. Less than 1% of the administered dose is excreted unchanged in the urine of pregnant women as compared to 20% recovery in the urine of non-pregnant women [110].

1.6 CONCLUSION

OT is a powerful uterotonic agent, useful for the induction and augmentation of labour, prevention of postpartum haemorrhage and promotion of lactation. The instability of OT in the GIT is primarily due to the high acidity of the stomach, the alkaline pH of the small intestine and the presence of enzymes in the GIT. The compound is therefore unsuitable for formulation for delivery via the oral route.

The short half-life of OT makes it a suitable agent for formulation into a sustained release parenteral formulation. However, the use of a sustained release formulation is limited for labour induction, as careful monitoring of the patient for this indication is required to prevent complications to mother and/or baby and the use of an implanted sustained release parenteral preparation would be unsuitable. However, after the delivery of the shoulders of a baby, in the third stage of labour, the routine use of a sustained release OT preparation could be advocated to prevent postpartum haemorrhage and incidences of hypo-volaemic shock [114], which is one of the leading causes of maternal morbidity and mortality. A sustained release OT product would assist in maintaining uterine tone and prevent excessive bleeding.

CHAPTER 2

HPLC METHOD DEVELOPMENT AND VALIDATION

2.1 INTRODUCTION

2.1.1 Overview

The analysis of OT has traditionally been based on biological assays that measure uterine contractions in the rat or a drop in the blood pressure of chickens [8]. The BP 1993 [115] and USP 1990 [116] recommended the use of these assays to quantitate OT activity. However, biological assays are time consuming and the applicability of high performance liquid chromatography (HPLC) in comparison to biological assay methods has ensured that this analytical tool has become a method of choice for the quantitation of OT in biological samples [117,118], pharmaceutical formulations [119-124] and for quality control purposes [125,126]. Furthermore, there is a good correlation that exists between the activity of OT measured using biological assays and the use of HPLC as a quantitative analytical tool [8,121,124]. In addition, current compendial methods recommend the use of HPLC for the assay of OT containing formulations [10,11].

The objective of these studies was to develop and optimise an HPLC method for the quantitation of OT in pharmaceutical dosage forms. Furthermore, the development of a stability-indicating analytical method for the assessment of OT was desired. The method for OT analysis was further optimised by increasing the sensitivity in order to allow for the use of the method in the assessment of OT release from extemporaneously prepared OT gel formulations for parenteral delivery of the peptide.

2.1.2 Principles of HPLC

HPLC has become one of the most versatile tools in a wide variety of fields, finding application in the pharmaceutical sciences [127-129], chemical [130-132], cosmetic [133-135], biotechnological [136-138], biomedical and clinical fields [139-141].

The term HPLC encompasses numerous forms of the technique including liquid-liquid, liquid-solid, ion exchange, and size-exclusion chromatography [142-144]. Modification of liquid-liquid chromatography gave rise to bonded phase chromatography and the original form of bonded

phase chromatography is referred to as normal phase HPLC. Normal phase chromatography uses a polar stationary phase and a non-polar solvent or mobile phase is used to ensure elution of the analyte of interest. However, a more commonly used form of HPLC is reversed-phase HPLC (RP-HPLC) where the stationary phase is non-polar and the mobile phase is comprised of polar materials. RP-HPLC is a more useful technique for the analysis of pharmaceuticals as the majority of compounds of interest are polar in nature, being either weak acids or bases and are ionisable, necessitating the use of polar solvents to effect rapid elution from hydrophobic stationary phases [143-145].

The stationary phase is usually retained in a column made of stainless steel and is primarily made up of a silica backbone, chemically bonded to different functional groups, such as for example, alkyl chains. The mobile phase is pumped through the stationary phase in the column and carries the analyte(s) of interest to the detection system for analysis. Usually the detector system is coupled to a computer system, integrator, or chart recorder for data capture and integration of the detector response. The components of a mixture undergoing analysis interact differently with the stationary and mobile phases, resulting in differential migration or varying rates of movement and consequently, different retention times of the compounds of interest thus effecting a separation [143-145].

The separation of compounds in HPLC is a poorly understood complex process, despite numerous efforts to elucidate the precise nature of the interaction between an analyte and the stationary and mobile phases. Several mechanisms for the retention of analytes in RP-HPLC have been proposed, each having distinct advantages and/or shortcomings. The failure to reach consensus for the mechanisms of retention is probably due to the complexity of interactions that occur in RP-HPLC systems, which change with the nature of the mobile phase composition, type of stationary phase and the analyte in question [146-148]. The understanding of mechanisms of retention in RP-HPLC is useful for the prediction of performance of a specific separation in chromatographic systems and the logical optimisation of that separation, especially in complex systems that contain a wide range of solutes or analytes to be separated [147,149,150].

The retention of an analyte in RP-HPLC is a function of its distribution between a liquid polar mobile phase and a non-polar stationary phase. The equilibrium existing in a system is not strictly liquid-solid equilibrium, as the chemically modified hydrophobic layer is made up of mobile chains and therefore there is no sharp interface between the stationary and mobile phases. Furthermore, the equilibrium cannot be considered as a liquid-liquid equilibrium as the stationary phase, although it contains mobile chains, is chemically bonded to a stationary silica backbone

[146]. When the layer of bonded alkyl chains is exposed to the contents of a mobile phase, it adsorbs some of the contents of the mobile phase and swells to a degree, forming a complex, thick solid-liquid interface [146].

Analyte retention was initially proposed to be due to interactions between a solute and the mobile phase [151,152]. Karger *et al.* [151] proposed that hydrophobic molecules tend to cluster in aqueous media and result in the formation of a cavity, of which the surface area is directly proportional to the molecular connectivity providing a topological index. The retention of solutes increases with an increase in molecular connectivity of a molecule and therefore, more hydrophobic entities, which occupy larger surface areas, have longer retention times. The solvophobic theory [152] also describes the importance of the aqueous medium in reducing the surface area of non-polar solutes forming a cavity in the media to accommodate the solute. The solvophobic tendency results in the interaction of solutes with the stationary phase and hydrophobic molecules therefore tend to interact more with the stationary phase compared to more hydrophilic compounds. The size of the molecule and the surface tension of the mobile phase are therefore important factors in determining the mechanism of retention [152].

Later studies have provided evidence of the importance of the stationary phase in the retention process [153-156] and models that include both the characteristics of the stationary and mobile phases have been developed. Experimental data reveal that the density and length of the alkyl chains in a stationary phase are important components in determining the retention mechanism for small non-polar solutes [157]. Tan and Carr [155] investigated the role of cavity formation, dispersion interaction, polarity, hydrogen bond acidity and basicity on the retention behaviour of analytes. The stationary phase was found to be an important determinant in the retention mechanism.

The mechanism of retention can be described by essentially three mechanisms [158]:

i. Partitioning of an analyte between a stationary and mobile phase [149,154],
ii. Adsorption of an analyte onto the surface of a non-polar adsorbent [159] and,
iii. The combined effects of adsorption and partitioning [160].

Partitioning implies that a solute becomes fully embedded in a stationary phase, whereas in adsorption, a solute interacts with a stationary phase only on the surface of that phase [158].

In the partitioning model [158], the stationary phase can be considered an amorphous bulk fluid medium and the transfer of solutes between the stationary medium is driven by the relative

chemical affinity of a solute for the mobile and stationary phases. The solvophobic theory [152] is an example of the early partitioning models, although it fails to provide a complete picture for the partitioning process as it neglects to describe the impact of a stationary phase on solute retention [149]. Equation 2.1 depicts the analyte distribution/partition coefficient used to describe the partitioning theory [143,148].

$$K = \frac{C_s}{C_m} \qquad \textit{Equation 2.1}$$

Where,

C_s = Concentration of analyte in the stationary phase

C_m = Concentration of analyte in the mobile phase

In this model, the migration of a solute may be assumed to occur only when the molecules are in the mobile phase and a greater distribution of the solute in the stationary phase results in a higher partition coefficient. As a result, the rate of migration of a solute is inversely proportional to the partition coefficient and in cases where there is a larger amount of analyte in the stationary phase, longer retention times result [143]. A correlation exists between the partition coefficient of a solute in water and *n*-octane and the retention factors for RP-HPLC [146]. Relationships to determine and predict the migration of analytes within RP-HPLC columns can be derived from the partition coefficient [148]. The partitioning of solutes in RP-HPLC can be considered to occur in three steps *viz*., the creation of a solute-sized cavity in the stationary phase, which is followed by the transfer of the solute from the mobile phase leaving a cavity and is finally closed by the mobile phase in the subsequent step [158].

The contribution of adsorption to the retention process was initially underestimated, but adsorption is now recognised to play a vital role in the retention of analytes in RP-HPLC [154,158,159]. Adsorption describes an accumulation of one component in close proximity to an adsorbent surface, under the influence of surface forces. When a liquid binary solution is used, the accumulation of an analyte onto a stationary phase surface occurs with the simultaneous displacement of solvent from the surface region near the adsorbent, into the bulk solution. At equilibrium, a certain amount of analyte will remain accumulated on the adsorbent surface at a higher concentration than that present in the bulk solution [148]. This model has been considered more logical in explaining retention mechanisms of solutes as compared to the partitioning model. This is because column-packing material is porous, with a large surface area that is impermeable to analytes, allowing the adsorption process to occur primarily in the liquid phase [148]. The retention mechanism for proteins can be considered as adsorption of the analyte at the

hydrophobic stationary surface described above, although the mechanism of retention for proteins may be altered as a result of changes in protein conformation during RP-HPLC analysis [161].

The partitioning and the adsorption models are extremes in a spectrum of all possible mechanisms of retention and the overall retention of analytes is due to a complicated process of different interactions occurring simultaneously in RP-HPLC [146,148].

The organic modifier used in a mobile phase can also be adsorbed at the stationary phase interface thereby forming a thick layer suggesting that a modified model may be necessary to explain the retention process. In the first stage of the process, an analyte partitions between an adsorbed organic layer and the bulk mobile phase and subsequently, the analyte is adsorbed onto the surface of the stationary phase [148]. Jaroniec [156] proposed a partition-displacement model in which the formation of the solvent-surface stationary phase occurs via a displacement mechanism and the distribution of the solute between the mobile and stationary phases follows partitioning of an analyte between the stationary and mobile phases of a system.

Differential migration of analyte(s) is influenced by variables that affect the interaction(s) between the analyte of interest and either the stationary and/or mobile phase. The retention process is a complex system that is affected by several factors, including mobile phase composition and the nature of the organic modifier in the mobile phase [146,162,163], flow rate and temperature [163,164], pressure [146], presence of salts, buffer molarity and pH [146,165].

Several methods and relationships have been used to add clarity on the mechanism of retention in RP-HPLC systems. A semi-logarithmic relationship shown in Equation 2.2 has been applied to describe analyte retention in binary RP-HPLC systems where the relationship shows a linear dependence of the logarithm of the retention factor, *k'*, (log *k'*) and the composition of the mobile phase, Φ_b [166,167].

$$\log k' = \log k_w - S\Phi_b \qquad \textbf{\textit{Equation 2.2}}$$

Where,

k' = isocratic capacity factor

Φ_b = volume fraction of organic modifier

k_w = extrapolated value of k' when $\Phi_b = 0$

The limitation of this method is that the relationship that exists often deviates from linearity as the aqueous component exceeds 90% (Φ_b < 0.1) and is only linear when the volume fraction of the organic modifier is between 0.2 – 0.8. However, this limitation is overcome by use of a quadratic function that is shown in Equation 2.3, of the mobile phase composition to represent log *k* [168,169].

$$\log k' = A\Phi_b^2 + B\Phi_b + E\sqrt{\Phi_b} + \log k'_w \qquad \textit{Equation 2.3}$$

Where,

k' = isocratic capacity factor

Φ_b^2 = volume fraction of organic modifier at high volume fractions (>0.8)

Φ_b = volume fraction of organic modifier (between 0.2 – 0.8)

$\sqrt{\Phi_b}$ = volume fraction of organic modifier at low volume fractions (<0.2)

k_w = extrapolated value of k' when $\Phi_b = 0$

Linear free energy relationships have also been used [170-173] to describe retention mechanisms, although the major limitation of this method of description is that a large amount of retention data is necessary for the calibration of a chromatographic system and some solutes deviate from the model [174].

Quantitative structure-retention relationships (QSSR) have played an important role in predicting the retention characteristics of solutes in RP-HPLC and explaining the retention mechanism in these systems [150,175,176]. This has improved the understanding of retention mechanisms and the selectivity in RP-HPLC. QSSR are useful in predicting the retention characteristics of a new solute, identifying the most informative descriptors and gaining insight into the mechanism operating in RP-HPLC, evaluation of physicochemical properties of analytes, and the prediction of relative biological activities within a set of test drugs [150].

2.2 LITERATURE REVIEW

Prior to the development of a specific HPLC method for the analysis of OT, a literature review was conducted and the published conditions used for the analysis of OT are summarised in Table 2.1. The initial conditions used for the development of the HPLC method for use in these studies were based on the data presented in Table 2.1.

Table 2.1 *HPLC methods for OT analysis*

Column	Mobile phase	Flow rate	Detector	Retention time	Reference
Nucleosil® C_{18}, 5 µm, 100 X 4.6 mm	35% v/v ACN in 0.83 mM phosphate buffer, pH = 5 containing 0.05 % sodium tetradecyl sulphate.	2.5 ml/min	UV	8 min	[124]
Partisil® SCX, 10 µm, 250 X 4.6 mm	10% v/v MeOH in 20 mM in phosphate buffer, pH = 5	0.5 ml/min	UV 209 nm	18 min	[177]
Column RP 18, 10 µm, 250 X 4.6 mm	18% v/v ACN in 67 mM phosphate buffer, pH = 7	2.0 ml/min	UV 210 nm	8 min	[8]
Nucleosil® C_8, 5 µm, 150 X 4 mm	20% v/v ACN in phosphate buffer, pH = 7	2.0 ml/min	UV 210 nm	5 min	[178]
Spherisorb® S5 ODS, 5 µm, 75 X 3 mm	17.5% v/v ACN in borate buffer, pH = 10	1.0 ml/min	UV 220 nm	2.5 min	[178]
Nucleosil® C_{18}, 10 µm, 150 X 4 mm	20% v/v ACN in 67 mM phosphate buffer, pH = 7	4.0 ml/min	UV 210 nm	4 min	[122]
Zorbax® TMS, 5 µm, 250 X 4.6 mm	18% v/v ACN in 50 mM phosphate buffer, pH = 5, internal standard ethyl hydroxyl benzoate	1.0 ml/min	UV 210 nm	15 min	[123]
LiChrosopher® 60 RP-select, 5 µm	18% v/v ACN in phosphate buffer pH = 2.1	1.0 ml/min	UV 220 nm	10 min	[121]
Partisphere® C_{18}, 5 µm, 125 X 4.6 mm	21% v/v ACN in 0.1 % phosphoric acid	1.3 ml/min	Fluorescence ex 250 em	12 min	[179]
RP 8® (Merck) 10 µm, 250 X 3 mm	20% v/v ACN in phosphate buffer pH = 7	1.47 ml/min	Fluorescence ex 390 em	6 min	[180]
RP-C_{18} (e.g. Shandon Hypersil®), 5 µm, 125 X 4.6 mm	A: 50% v/v ACN B: 100 mM sodium dihydrogen phosphate Gradient: 30% A to 60% A in 30 min	1.0 ml/min	UV 220 nm	8 min	[125]
Alltech Hypersil® ODS, 5 µm, 120 X 4.6 mm Beckman Ultrasphere® ODS, 5 µm, 150 X 4.6 mm	A: 100mM sodium phosphate monobasic with pH varied from pH = 3.1 to pH = 4.5 B: 50% v/v ACN Gradient elution	1.5 ml/min	UV 220 nm	10 min	[120]

The analysis of OT in both biological samples and pharmaceutical dosage forms has been performed on silica support columns, with C_8 and C_{18} columns being used most frequently, although ion exchange columns such as Partisil® SCX columns [177] have also been used.

The most commonly used organic modifier that has been used is acetonitrile (ACN) [119-123,125,178], although methanol (MeOH) has also been used [177]. The content of organic modifier content is usually relatively low and ranged from between 10 – 35% v/v ACN [124].

The use of ion-pairing reagents in RP-HPLC for analysis of peptide containing samples has been recommended as they result in improved peak shapes [181]. The use of an ion-pairing reagent, sodium tetradecyl sulphate, has been reported for the analysis of OT in dosage forms [124], but the use of ion-pairing reagents has a drawback of producing long equilibration times and the need for removal of salts from a column, thereby also reducing the column life span [182]. The majority of published methods summarised in Table 2.1 did not support the need for use of an ion-pairing reagent in analysis of OT samples.

OT is an amphoteric molecule (§ 1.2.5) and buffered systems are used for the successful analysis of OT using RP-HPLC systems. Phosphate buffer at pH of between pH = 5 and pH = 7 have most commonly been reported [122-124,177,178,180] due to the stability of OT in this pH range (§ 1.3.2), although a borate buffer at pH = 10 has also been successfully used [178].

HPLC with UV detection is the preferred method of analysis for many assays as its use is well documented in the scientific literature [119-122,178] and official compendia [10,11]. In addition, the use of fluorescence [179,180] and coulometric detection [117] has also been reported.

2.3 EXPERIMENTAL

2.3.1 Reagents and Materials

OT was obtained from PolyPeptide Laboratories s.r.o. (Prague, Hostivar-Czech Republic) and Inter-Chemical Hongkong Ltd (Shenzhen, China). The potencies of OT used in these studies were 541 IU/mg and 470 IU/mg, respectively. Methyl hydroxybenzoate (MHB) and propyl hydroxybenzoate (PHB) were obtained from Sigma Aldrich (St Louis, MO, USA). Acetonitrile (ACN) (HPLC grade, far UV) was purchased from Romil Ltd, (Cambridge, United Kingdom).

Sodium hydroxide pellets and ortho-phosphoric acid (85% w/w) were obtained from Merck Chemicals Ltd, (Modderfontein, South Africa). HPLC grade water was purified using a Milli-Ro® -15 Water purification system (Millipore, Bedford, MA, USA), which is made up of a Super-C® carbon cartridge, two Ion-X® ion-exchange cartridges and an Organex-Q® cartridge. The water was filtered through a 0.22 μm Millipak® stack filter prior to use (Millipore, Bedford, MA, USA). Syntocinon® (Batch Number S0042, Novartis, Johannesburg, South Africa), a locally available dosage form of OT at a concentration of 10 IU/ml was purchased from a local pharmacy. All reagents were used without further preparation and were at least of analytical reagent grade.

2.3.2 Preparation of Stock Solutions

Approximately 10 mg of OT (541 IU/mg) was accurately weighed, transferred into a 100 ml A-grade volumetric flask, and made up to volume with HPLC grade water to produce a final concentration, in solution of 54.1 IU/ml. The standard stock solution was used to prepare calibration standards in the concentration range 0 – 12 IU/ml by serial dilution using A-grade glassware. Standards were prepared by serial dilution of the standard stock solution to produce solutions of 1, 2, 4, 6, 8, 10, and 12 IU/ml for use when assessing OT content in pharmaceutical dosage forms. Standards for the optimised method were prepared from a 1 IU/ml stock solution of OT to produce solutions of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 IU/ml.

A stock solution of internal standard was prepared as follows; approximately 5 mg of MHB was accurately weighed and dissolved in 200 ml of HPLC grade water using a 200 ml A-grade volumetric flask, to produce a concentration of 25 μg/ml. Then, 6 ml was pipetted using an A-grade pipette, into a 20 ml A-grade volumetric flask and made up to volume with water to produce a solution of a concentration, approximately 7.25 μg/ml.

2.3.3 Preparation of Buffer Solutions

80 mM buffer solutions were prepared by pipetting 5.460 ml of 85 % w/w ortho-phosphoric acid into a 1000 ml A-grade volumetric flask. Approximately 500 ml of HPLC grade water was added and the pH was titrated to pH = 5 with sodium hydroxide pellets using a Crison Model GLP 21 pH meter (Crison Instruments, SA, Barcelona, Spain) to monitor the pH. The volume was made up to 1000 ml using HPLC grade water.

2.3.4 Preparation of Mobile Phase

Appropriate volumes of buffer and ACN were measured separately using A-grade measuring cylinders. The individual components were then mixed in a 1000 ml Schott® Duran bottle (Schott Duran GmbH, Wertheim, Germany). The mobile phase was filtered and degassed under vacuum, after mixing, using an Eyela Aspirator A-2S vacuum pump (Rikakikai Co., Ltd, Tokyo, Japan) and a Millipore® HVLP 0.45µm filter (Millipore, Bedford, MA, USA).

2.3.5 HPLC System A

The method for OT analysis in pharmaceutical dosage forms was developed and validated on HPLC System A. The modular isocratic HPLC system consisted of a Spectra-Physics Isochrom LC pump (Spectra-Physics Inc., San Jose, CA, USA), Waters WISP 712 autosampler (Millipore, Waters Chromatography Division, Milford, MA, USA), Linear UV/VIS-500 Model 6200-9060 detector (Linear Instrument Co., CA, USA) and a Perkin Elmer 561 strip chart recorder (Perkin Elmer Instruments LLC, Shelton, CT, USA). A Phenomenex Hypersil® column, 5 µm, 4.6 x 150 mm (Phenomenex, Torrance, CA, USA) was used at ambient temperature (22 °C). The separation was conducted under isocratic conditions with UV detection at 220 nm. The volume of injection was 20 µl and a flow rate of 1.5 ml/min was used.

2.3.6 HPLC System B

HPLC system B was used for the optimisation of the analytical method for use in the subsequent development of an *in vitro* release test method for OT and the *in vitro* assessment of OT dosage forms. HPLC System B differed from System A. It consisted of, a Model P100 dual piston solvent delivery module (Thermo Separation Products, San Jose, CA, USA), a Model AS100 autosampler (Thermo Separation Products, San Jose, CA, USA) fitted with a Rheodyne® Model 7010 injector (Rheodyne, Reno, Nevada, USA) and a fixed volume 20 µl loop and a GASTIGHT® 250 µl Model 1725 syringe (Hamilton Co., Reno, NV, USA). Data was collected using a Spectra Physics SP 4600 integrator (Thermo Separation Products, San Jose, CA, USA). The detector and column used for the analysis was the same as that used in HPLC System A (§ 2.3.5).

2.4 METHOD DEVELOPMENT AND OPTIMISATION

2.4.1 Introduction

The initial step in the development of an analytical method is to consider the nature of the sample that is to be analysed, including the chemical structure and acidic/basic nature of the analyte, the presence of impurities, degradation products and any compounds that may interfere with the analytical procedure [183-186]. In addition, the intended purpose or use of the HPLC method has also been considered in the development stages [185,187-189] as this is directly related to the desired sensitivity and range of the analytical method that must be developed.

Traditional means of method development and optimisation have focused on selecting the initial conditions based on the literature or by assessing the nature of the molecule to be analysed and predicting the most appropriate initial conditions for an analysis. The method is then optimised by determining the effect of operating variables such as organic modifier content, type of organic modifier, column type, and temperature on the resultant separation [183].

Current trends in the development of analytical methods depend on the application of automated column selection systems that can be used to compare a series of HPLC columns varying in hydrophobicity and silanol activity, in combination with computer software such as DryLab® software [190-192] and ChromSword® optimisation software [193]. The basic strategy involves automated screening of various columns and mobile phase combinations initially, and then selection of the best starting conditions for the separation and finally, the optimisation of that separation [193]. Another example of current trends in method development is a three-step method development/optimisation strategy for pharmaceuticals including a multiple-column/mobile phase screening test, which uses a column-switching device and allows further optimisation of the separation by using multiple organic modifiers in a mobile phase. Multiple-factor method optimisation, where several aspects are changed simultaneously, is also possible with automated systems [194]. Intelligent software such as LabExpert®, which operates in real-time for analytical method development has also been described [195].

Analytical procedures for use in quality control and formulation development studies should have relatively short run times in order to allow multiple analyses to be conducted in as short a time as possible. A run time for each sample should generally be less than 20 min although run times between 5 – 10 min are preferable for these purposes to expedite analyses [183].

2.4.2 Challenges in RP- HPLC Analysis of Proteins and Peptides

RP-HPLC analysis of biomolecules such as proteins and peptides can be a challenge as there are often problems associated with analytical systems such as excessive band broadening, peak tailing or mis-shaped bands, low recovery, ghost peaks and the appearance of one protein in two or more distinct bands [196]. Understanding the impact of process variables in RP-HPLC can help minimise or eliminate these undesirable effects.

The analysis of biochemical entities such as peptides, proteins, and oligonucleotides by RP-HPLC pose different challenges as compared to the analysis of small chemical molecules since they have larger hydrodynamic radii and different functionalities in the molecules that may result in different interactions with RP-HPLC stationary and mobile phases. These factors must be considered in the development of an analytical method for proteins and peptides [183,197]

2.4.3 Column Selection

A description of the relevant physicochemical properties of OT was reported in Chapter 1 and reveals that the molecule is a peptide, with a molecular weight of 1007.23. OT has free amino and acidic phenol groups, making it an amphoteric molecule with an isoelectric point at pH = 7.7. This fact is important since the separation of OT is dependent on the pH of the mobile phase and elution in RP-HPLC can be modulated by the presence of buffers.

2.4.3.1 Column Packing and Stationary Phases

Stationary phases used in RP-HPLC are primarily made of a silica backbone that is chemically bonded to various aliphatic functional groups such as methyl, butyl, octyl and octadecyl, although other functional groups such as –phenyl, cyano, amino, diol and hydroxyl have also been used [142,143,145]. The stationary phases are typically prepared by covalently bonding an organosilane with the relevant functional group onto a support surface producing siloxane linkages (Si – O – Si – R) between the silica support and organosilane [183].

Silica based bonded phases dominate the separation of proteins and peptides [198-202]. Peptide separations are frequently achieved on C_{18} columns, but protein separations are carried out on C_3 and C_4 columns [203]. Different types of stationary phases that may be used in RP-HPLC for protein and peptide separations include micropellicular stationary phases and gigaporous packings, which increase the efficiency of biomolecular separations [197].

There are several advantages of a silica backbone for stationary phases that have made it the most widely accepted and used stationary phase support [183]. Advantages include the fact that the support consists of porous silica particles that can be prepared in a wide range of pore and particle sizes, thereby increasing the variety of columns that can be produced. This allows for the separation of a wide variety of molecules with diverse molecular weights, increasing the utility of RP-HPLC columns. In addition, the silica backbone can be attached to a wide variety of functionalities, thereby further enhancing the applicability of RP-HPLC in a wide range of analytical applications [183].

One of the primary advantages of silica as a support in RP-HPLC is its mechanical strength that enables tight packing of stationary phase beds, thereby increasing the surface area for interaction of analytes and subsequent retention. In addition, tightly packed beds allow the column to withstand the high-pressure conditions that are usually encountered in RP-HPLC [204]. Furthermore, silica does not swell or shrink when exposed to organic solvents making it inert under these conditions. Importantly, the production and bonding chemistry for silica supports is also reproducible, well understood and can be performed with different morphologies to give a wide variety of stationary phases, increasing the applicability and the diversity of molecules that can be separated using RP-HPLC [183,204].

Column failure in RP-HPLC may occur because of the vulnerability of silica-based stationary phases to deterioration effects. One of the major disadvantages of silica support materials is the instability of silica and chemically modified silica as a function of pH. The typical operating range of such stationary phases is between pH = 2 and pH = 8, where only slight dissolution of the stationary phase occurs [205]. The organic ligands of most of the currently manufactured stationary phases are covalently bonded to the silica surface by mono- or polyfunctional siloxane bond(s) (Si – O – Si) [183,206]. In acidic media, siloxane bonds in silica support stationary phases undergo hydrolysis and release bound organo-silane from the stationary phase surface, resulting in elution of this organic moiety. Kirkland *et al*. [207] showed that the main mechanism resulting in the degradation of silica support stationary phases at low pH is due to the hydrolysis of the covalently bonded Si – O – Si organic ligands. This loss of bound silane in the stationary phase increases the population of surface silanols and results in a decrease of the retention of neutral and/or low polarity solutes, which would otherwise be retained on the hydrophobic stationary phase. With the increase in surface silanols, organic cations usually elute with increased peak widths and greater peak asymmetry [131]. The mechanism of deterioration changes above a pH of approximately 7, where the dissolution of silica is substantially

accelerated causing an impairment of the silica backbone. The resultant effect is a reduction of theoretical plate numbers and column efficiency and finally column clogging [207].

Another disadvantage of silica support columns for use in RP-HPLC is the strong affinity of such phases for strongly basic compounds, which often results in unsatisfactory separations, with poor resolution, peak asymmetry, and substantial peak tailing [205].

Despite the major disadvantages of silica, a silica-based column was found to be appropriate for the development of an analytical method for OT. This decision followed considering the properties of this type of packing and reviewing published analytical methods of OT, which have used silica columns for the analysis of OT. In addition, the pH range within which the separation of OT was to be undertaken would not cause the degradation of the column and therefore the life span of the column is unlikely to be negatively affected.

2.4.3.2 Internal Diameter and Length

The internal diameter (i.d.) of a column plays an important role in the performance of that column. Separations for proteins and peptides have been mostly achieved on columns of i.d. of between 3 – 5 mm, although narrow bore columns with diameters of between 0.5 – 2 mm are gaining significance and even microbore columns with diameters less than 0.5 mm are considered useful [197]. Microbore columns of 1.0 mm i.d. have been used [208,209] and found to have greater efficiency allowing for the detection of low concentrations of analyte. However, the primary disadvantage to smaller i.d. columns is that for the same column length, a microbore column will require a larger inlet pressure than that required by a wider column [143] and this has detrimental effects on both the column and the solvent delivery module reducing their life span [145].

Peptide and protein separation is normally carried out under gradient elution conditions using short columns with high velocity flow rates. However, protein separations have been achieved using columns as short as 3 – 5 cm in length and peptide separations can be achieved on columns between 10 – 25 cm in length [197].

An appropriate column diameter that allows for adequate separation efficiency and a reduced column backpressure, with reduced deleterious effects to the column and the HPLC system that is regularly used is usually of 4.6 mm i.d. and 15.0 cm in length. A column with these dimensions was selected for the analysis of OT as a compromise on the factors discussed above.

2.4.3.3 Particle Size

The particle size of a stationary phase affects the efficiency of a separation for protein and peptide samples. Smaller particle sizes have greater separation efficiency as compared to larger particles due to the associated increased surface area for adsorption and interaction. Moreover, the separation of bio-molecules results in smaller plate numbers and smaller particles are usually required to ensure a good separation is achieved [197]. A decrease in the particle size of the packing material results in an increase in the efficiency of separation and a decrease in analysis time, with a consequent increase in column backpressure [210]. Particle sizes of 5 µm are routinely used for biochemical sample analysis [183] and therefore a column with these dimensions was selected for use in these studies.

2.4.3.4 Pore Size

In RP-HPLC, the interaction between an analyte and the stationary and mobile phases governs the retention mechanism of that solute as discussed in § 2.1.2. The pore size of the stationary phase material dictates the extent to which analyte molecules penetrate/interact with the interior of that phase. Large molecular weight molecules require larger pore sizes to prevent restricted diffusion, which is likely to occur if the column porosity is of the wrong dimensions [183]. For peptides that contain more than 50 amino acid residues, column packings with pore sizes of between 250-350 Å are essential. For a molecule with a molecular weight of less than 2000 Da, a pore size of 100 Å is acceptable [183]. OT has a molecular weight of 1007.23 Da, with only nine (9) amino acids and therefore a column with a pore size of 100 Å is appropriate for the analysis of this nonapeptide.

2.4.3.5 Column Selection

The column selected for use in the development of an analytical method for the analysis of OT was a bonded phase silica column. The column that was selected was a Phenomenex® Hypersil 150 X 4.6 mm i.d. (Phenomenex, Torrance, CA, USA) with a 5 µm particle size stationary phase and 100 Å pore size.

2.4.4 Method of Detection

Following the separation of analytes from a column, specific and sensitive detector systems are required for the quantitation of an analyte of interest. An electronic signal generated as a result of changes in a detector response due to the presence of analytes is produced and this is recorded as a peak on a chromatogram [211].

Detector systems that have found application in RP-HPLC include UV/VIS, spectrofluorometric, diode array, electrochemical, and refractometer detectors and more recently mass spectrometers have been used [183,212]. Hyphenated systems such as HPLC-MS and HPLC-NMR, which couple other analytical techniques to HPLC are also gaining recognition and increase the sensitivity and utility of RP-HPLC analyses allowing for complete peak identification and characterisation [212,213].

The UV/VIS detector is the most versatile detector used in HPLC for analysis of proteins and peptides at wavelengths of 210, 220, and/or 280 where absorption maxima are observed [183]. In addition, a large number of compounds are capable of detection by UV absorption in the wavelength range of 200 – 400 nm further extending the applicability of this type of detector in HPLC analysis [183]. UV/VIS detection is not always ideal as it lacks specificity but the method is extensively used since it produces a linear response, allows for accurate calibration and the use of a single standard to calibrate the detection system following validation [212]. Another challenge in the use of UV detection for protein and peptide analysis is that UV spectroscopy can only be applied to a small range of protein concentrations if no other absorbing components that may interfere with detection are present in the solution being analysed. The use of diode array detection in the UV range overcomes the lack of selectivity of this method, but is less sensitive in comparison to conventional UV/VIS detection [214].

The detection of OT has mainly been achieved using UV/VIS detectors set at a wavelength of 220 nm as shown in Table 2.1. The selection of the wavelength for the detection of OT in this system was 220 nm since the UV absorption spectrum shown in Figure 1.4 shows a region of increased absorbance at this wavelength, which is greater than the λ_{max} of approximately 280 nm that was observed in the spectrum.

2.4.5 Temperature

Temperature is also an important variable for protein and peptide sample separations [215-217] ultimately affecting the selectivity, column efficiency and sample run times in RP-HPLC. Temperature ranges for effective separations of proteins and peptides can vary from 20 °C to 60 °C [216,217] or even 90 °C [196], where higher temperatures were found to increase the recovery of an analyte. In addition, increasing the temperature also decreases bandwidths and lowers column backpressures. However, elevated temperatures result in a decrease in the life span of alkyl silica-based columns such as C_8 and C_{18}. The instability of silica columns to high

temperatures that may be used in RP-HPLC separations has been overcome by the availability of modified columns, resistant to the detrimental effects of high operational temperatures [196].

Temperature has been found to impact on the conformation of proteins and affecting the quality of the chromatogram produced. Cohen *et al*. [218] demonstrated these effects for ribonuclease, which shows asymmetric peaks when analysed at low temperatures and shows a significant improvement in peak shape with increased temperatures. This was due to a change in the conformation of the protein during HPLC separation and the denaturation at higher temperatures. At lower temperatures, a dynamic equilibrium between the different conformations of the molecule results in poorly shaped peaks.

The use of elevated temperatures may offer advantages for the separation of proteins and peptides due to an increase in the kinetic and transport properties of the analytical procedures for biomolecules [197]. There is also a decrease in the viscosity of the mobile phase at higher temperatures, which increases the diffusivity of large macromolecules such as proteins and increases the efficiency of the separation [197].

The analytical methods for OT, reported in Table 2.1, do not indicate the use of elevated temperatures. OT is a small peptide and is unlikely to suffer from a change in conformation as described for the ribonuclease assay [218]. Small peptides respond in a similar manner to individual amino acids and with an increasing number of amino acid residues, the secondary, tertiary, and quaternary structures become increasingly important and affect the retention behaviour of the compounds in RP-HPLC analysis [219]. OT has only nine (9) amino acids, therefore conformational changes are unlikely to impact on the retention mechanism and characteristics of the molecule, and therefore there is no need for use of elevated temperatures to improve separations during OT analysis.

The use of a constant temperature increases the robustness of an analytical method and it is therefore important that a column oven be used in order to ensure that the separation is achieved under constant temperature. However, the use of an air-conditioned laboratory with a constant temperature is acceptable and ensures the reproducibility of retention times and quality of separation [220]. The method for OT analysis was developed under ambient conditions of 22 ^{o}C.

2.4.6 Quantitative Measurement

Calibration curves of detector responses to standards are used to quantitate samples in analytical procedures, where the concentration or amount of unknown analyte can be extrapolated from an appropriately constructed calibration curve [183].

Either peak area and/or peak height responses can be monitored in quantitative analysis by RP-HPLC. The peak area is determined by the sum of all individual responses of sample molecules over a given period from the start to the end of the peak, but the peak height is determined by the maximal concentration of a sample that passes through the flow cell [183]. The maximum concentration that can be monitored is dependant on the amount of band spreading that may occur on a column during chromatography while the peak area is independent of the amount of band spreading, as it measures all the components present in a system. However, peak area is influenced by the time that it takes the molecules to pass through the detector flow cell and varies if there are changes in the flow rate as the flow cell transit time changes [145,183]. Fluctuations in mobile phase flow rates result in the components of a sample moving along a column and into the detector flow cell at different speeds and this is reflected on a recorder that traces a chromatogram reflecting sample concentration versus time. The peak remains approximately the same height, but becomes broader as there is an increase in the number of particles passing through the flow cell as a function of time [183].

The extinction coefficient can be used to quantitate analytes in RP-HPLC with UV detection. Once the extinction coefficient of an analyte is determined with an appropriate standard, future measurements can be related to the extinction coefficient, negating the need for the preparation of calibration standards [214]. The use of a response factor calculated from different parameters including the extinction coefficient, detector response in millivolts (mV), and the absorbance for protein and peptide analysis has been proposed by Erberlein [214]. This method was found to be at least as accurate as using a peak area calibration curve for sample quantitation.

Due to the possible variability of flow rate and the overall constant response of peak height as opposed to peak area, peak height was selected as the preferred parameter for all quantitative analyses of OT in these studies.

2.4.7 Mobile Phase Selection and Flow Rate

The efficiency of a RP-HPLC separation depends on several factors, including the use of an appropriate mobile phase, since the interactions between an analyte and a stationary phase also depend on the interaction of the analyte with the mobile phase as described § 2.1.2. Intermolecular interactions between these three interacting variables in RP-HPLC are of paramount importance in order to achieve a successful and possibly rapid separation in an analytical method. Polarity is important in the interaction, for example, if the sample components and the mobile phase have polarities that are similar, there is likely to be poor retention and the components will be eluted rapidly. In RP-HPLC, the most polar component(s) will be eluted first as there is little or no interaction with the stationary phase and an increase in the polarity of the solvent will result in an increase in the elution time for non-polar analytes [221].

Mobile phases for protein and peptide samples usually contain ACN in gradient elution systems, with the use of low concentrations of trifluoroacetic acid to maintain mobile phase pH at < 2 [196,203,216,217].

Ion-pairing reagents such as the alkylsulphonates, trifluoroacetic acid and triethylammonium phosphate (pH = 4) may be used as mobile phase additives for protein and peptide separation [222]. These additives increase the hydrophobicity of peptides and proteins by forming uncharged entities through ionic interactions, thereby increasing the interaction of otherwise polar molecules with a stationary phase resulting in sharper and more symmetrical peaks [181,196]. The use of ion-pairing reagents for OT analysis is not widely advocated as reported in Table 2.1 and discussed in § 2.2 and therefore their use was not considered since successful separations have been achieved without the use of these agents.

2.4.7.1 Choice of Organic Modifier and Appropriate Buffer

The mobile phase selection process for RP-HPLC usually involves an assessment of various solutions of MeOH and/or ACN that may or may not be buffered. It is important that the mobile phase of choice does not absorb UV radiation at the wavelengths used in analytical procedures, and that the minimum wavelength that can be used or the cut-off wavelength of the solvent of choice is known. This is because the use of wavelengths below the cut-off wavelength will result in the mobile phase absorbing UV light thereby preventing accurate detection of the

analytes of interest. The UV cut-off wavelength of ACN and MeOH are 190 nm and 205 nm, respectively [183].

ACN is a less viscous solvent than MeOH and therefore results in comparatively lower column backpressures that arise due to the difficulty in pumping viscous liquids through the analytical column [183,220]. The differences in retention behaviour of molecules when using ACN or MeOH is a function of their different properties in aqueous media, more specifically, their ability to form hydrogen bonds with water. MeOH is able to form hydrogen bonds by accepting or donating protons but ACN is unable to do so and the difference in the ability to form hydrogen bonds with water affects the efficiency of a separation and the retention mechanisms of analytes of interest [155]. Differences in the interaction of ACN and MeOH with water result in the production of sharper peaks with ACN containing mobile phases and lower percentages of ACN are required in RP-HPLC separations due to its comparatively higher elution strength [220]. Other benefits when using ACN include lower solvent consumption in comparison to the use of MeOH and better resistance of silica stationary phase gels to dissolution in an ACN-based mobile phase as compared to MeOH-based mobile phases [220]. ACN appears to be the organic modifier of choice for OT separations (Table 2.1) and the benefits of using ACN as an organic modifier resulted in its selection as the organic solvent of choice for the mobile phase for OT chromatographic analysis.

In some cases, it is important to use a buffer as a means of controlling the pH when the separation of acidic and basic compounds is undertaken. In general, buffer concentrations in the region of 25 mM are considered as suitable for RP-HPLC analyses. The UV cut-off wavelength of phosphate buffers is < 200 nm, which makes it an ideal buffer system as it does not absorb light at the longer wavelengths that are normally used for the analysis of drug molecules [183].

Column stability is also of importance for the analysis of peptides since mobile phases of low pH are commonly used to effect the separation of peptides. The use of a low pH may result in the instability of the silica based stationary phase [145,183] (§ 2.4.3.1). The stability of the stationary phase was not of major concern for the analysis of OT since the pH range of the mobile phases that have been reported for the analysis of OT were between pH = 5 and pH = 7 (Table 2.1, § 2.2).

2.4.7.2 Mobile Phase Composition

The initial mobile phase composition that was selected for use was based on literature data [122,178] and was comprised of 20% v/v ACN in 67 mM Phosphate buffer, pH = 7. The influence of changing ACN content on the retention time and the peak shape of OT was then monitored. In addition, the impact of flow rates of 1 ml/min and 1.5 ml/min on these parameters was also assessed. Figure 2.1 shows the impact of the changes in mobile phase composition on retention time.

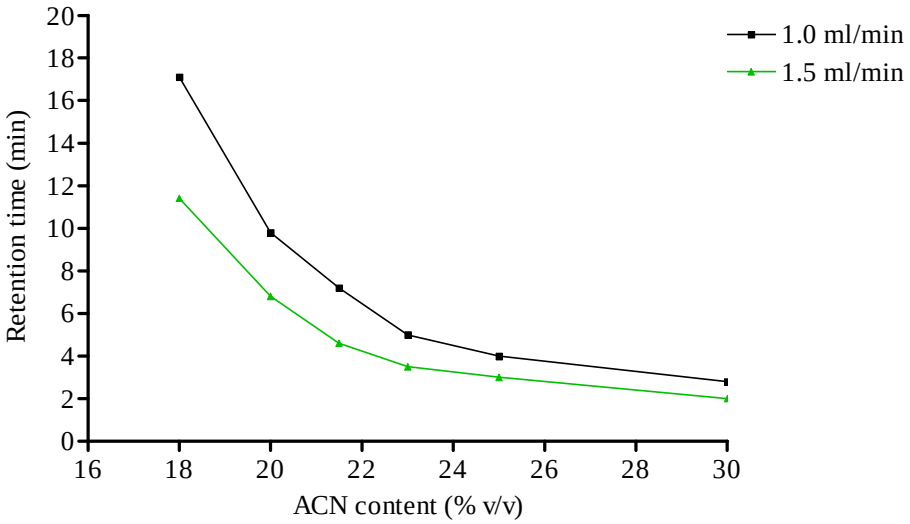


Figure 2.1 *The effect of changing ACN content and flow rate on the retention time of OT*

It is clear from the data shown in Figure 2.1 that as the percentage v/v of ACN increases there is a decrease in the retention time of OT. The decrease in polarity of the mobile phase as ACN content increases results in the preferential partitioning/adsorption of the drug into the mobile phase as compared to compositions with lower amounts of organic modifier, favouring elution of the molecule. This is despite the polar nature of the drug, since OT has a carbon backbone that provides non-polar characteristics to the molecule. The retardation of elution of proteins and peptides in RP-HPLC has been explained based on the solvophobic theory [152]. The interaction of the non-polar portion of the molecule with the hydrophobic stationary phase is promoted by the forces of attraction between the stationary phase and the molecule compared to those between the solvent and the molecule [161]. The increase in the content of organic modifier decreases the attractive forces between the peptide and the stationary phase and promotes the elution of OT in RP-HPLC [161]. Figure 2.2 shows the effect of changing the flow rate on the peak shape and height of OT using mobile phases containing 18% w/v ACN and 23%w/v ACN as examples.

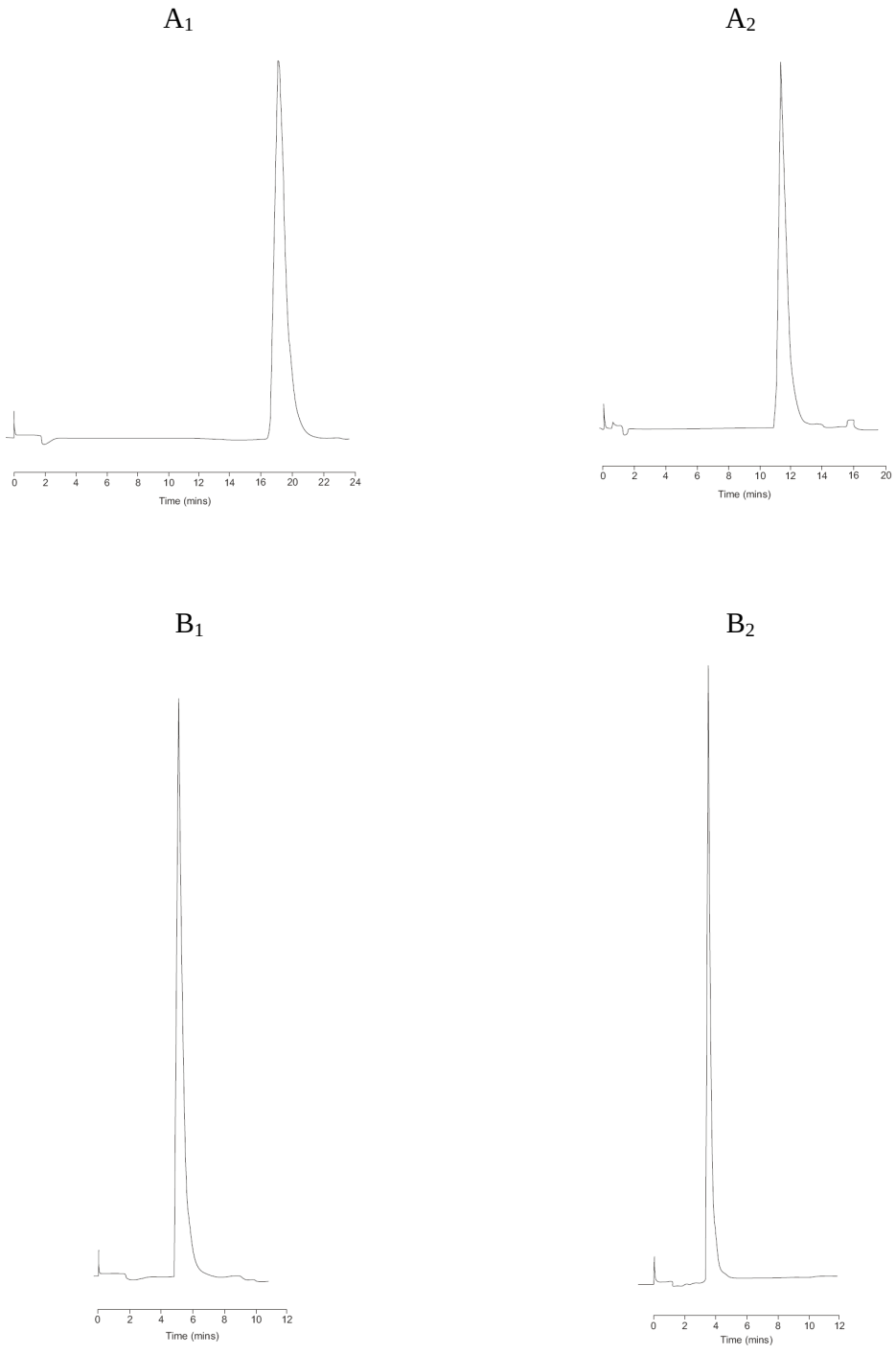


Figure 2.2 *The impact of changing flow rate on the peak shape and height of OT using 18 % v/v ACN (A) from 1 ml/min (A_1) to 1.5 ml/min (A_2) and using 23 % v/v ACN (B) from 1 ml/min (B_1) to 1.5 ml/min (B_2)*

It can be seen that the faster the flow rate of the mobile phase, the faster the elution of OT from the chromatographic system. The peaks produced from the slower flow rate were broader than those from 1.5 ml/min, as shown in Figure 2.2, in which the peak shapes obtained for mobile phase compositions containing 18% v/v ACN and 23% v/v ACN for the different flow rates, respectively, can be compared.

Peak symmetry for the different mobile phase compositions and different flow rates was compared using the peak asymmetry factor A_s, measured at 10% of full peak height and the peak-tailing factor (PTF) measured at 5% of full peak height. Excellent columns give an A_s value of between 0.95 and 1.1 although values of < 1.5 are acceptable. PTF values of between 1.0 and 1.4 are also acceptable [183]. The relationships of PTF and A_s are schematically illustrated in Figure 2.3.

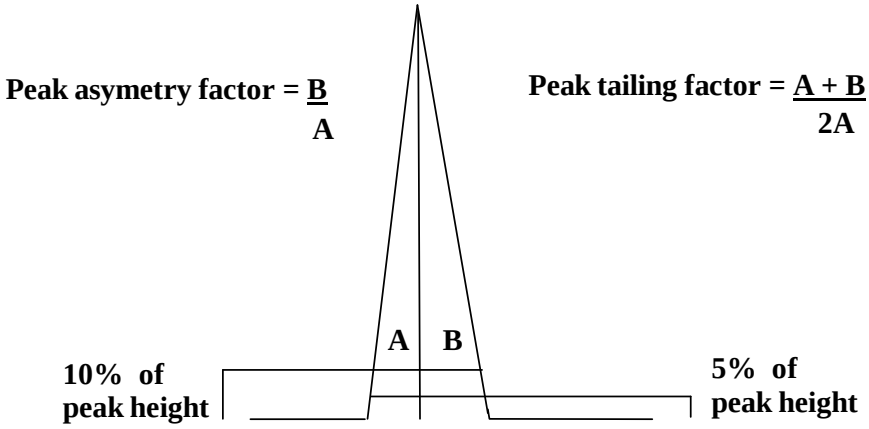


Figure 2.3 *Peak asymmetry and peak tailing factors (adapted from [183])*

The A_s and PTF values for the mobile phase compositions and the flow rates that were used to assess the peak symmetry are reported in Table 2.2 and therefore allowed for the selection of a mobile phase composition for further optimisation.

Table 2.2 *Peak asymmetry and tailing factors for OT for different mobile phase compositions*

ACN content (% v/v)	**1 ml/min**		**1.5 ml/min**	
	***A*$_s$**	**PTF**	***A*$_s$**	**PTF**
18.0	1.17	1.28	1.11	1.17
20.0	0.97	1.17	1.05	1.10
21.5	0.93	1.21	0.97	1.08
23.0	1.10	1.38	0.96	1.15
25.0	1.18	1.29	0.91	1.27
30.0	1.27	1.33	0.89	1.44

The mobile phase flow rate affected peak shape and the retention time of OT. As the flow rate is reduced, the analyte of interest takes longer to pass through the detector cell and the peaks that were observed are broader than peaks observed when using faster flow rates (Figure 2.2) [145]. OT peaks that were observed at the higher flow rate also indicated improved symmetry, compared to the lower flow rate. However, the use of higher flow rates resulted in higher column backpressures, which may be deleterious to the solvent delivery module and column and thereby may reduce the working lifetime of these system components. The column backpressure resulting when flow rates of 1 ml/min and 1.5 ml/min were used, was monitored, and compared over a period of 3 days. A flow rate of 1.0 ml/min resulted in a column backpressure of 958 $\pm$ 42 psi and at the higher flow rate, a pressure of 1246 $\pm$ 56 psi was observed. Although this pressure was higher, the backpressure was not considered to be excessive and would more than likely not result in excessive damage to the column and/or pump and therefore a flow rate of 1.5 ml/min was deemed acceptable for use.

For the separation of complex peptide and protein mixtures, lower flow rates are often desirable as this results in better resolution of the peaks of interest [183]. However since the separation of OT in these studies is not complex, low mobile phase flow rates are not necessarily desirable or required. The decision for the selection of the most favourable mobile phase was based on an appropriate retention time and the peak shape produced following introduction of the sample onto the HPLC system. Shorter retention times and peaks of better shape were produced using a flow rate of 1.5 ml/min as shown in Figures 2.1 and 2.2, respectively and summarised in Table 2.2. The most favourable retention times were obtained for mobile-phase compositions of 20% v/v and 21.5% v/v ACN at flow rates of 1.5 ml/min, (6.8 min and 4.6 min respectively) and for 21.5% v/v ACN at a flow rate of 1 ml/min (7.2 min). The mobile phase selected for further studies was therefore 20% v/v ACN in 67 mM phosphate buffer at pH = 7 as this composition was easy to produce with suitable reproducibility.

2.4.7.3 pH

Acidic mobile phases are preferred for the separation of proteins and peptides, as acidified media provide for the optimal interaction between peptide residues and stationary phases used for analysis. Acidic conditions result in the suppression of silanophilic interactions between the analyte of interest and the stationary phase. In addition, adjusting the eluent pH allows for optimisation of the chromatographic selectivity of a specific method [131]. The pH of the buffer used to produce a mobile phase in addition to the ultimate pH of the mobile phase mixture has an effect on retention times and peak shape in RP- HPLC. pH is particularly relevant for the

elution of compounds that have ionisable functional groups, such as weak acids and bases and amphoteric compounds that have both acidic and basic functional groups. Changing the pH of a mobile phase changes the ionisation state of the molecules of a mixture, thereby altering their polarity and hence their retention times. The effect of pH on the retention time and the peak shape of OT were investigated using a mobile phase composition of 20% v/v ACN in 67 mM phosphate buffer. The results of pH effects on retention time are depicted in Figure 2.4.

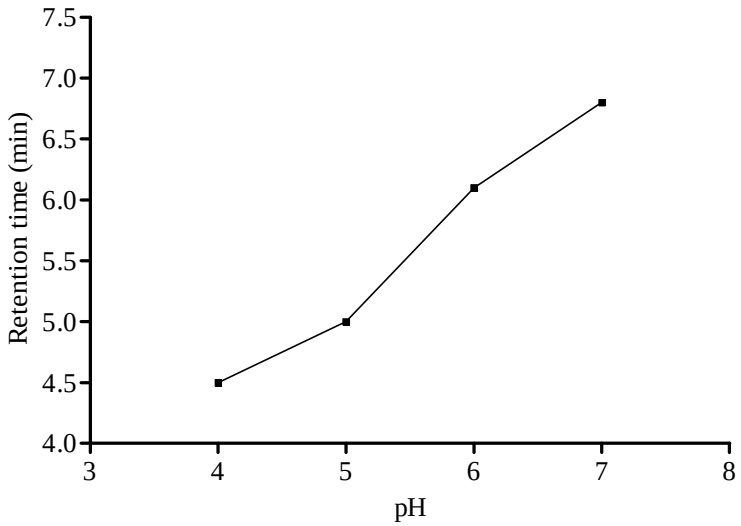


Figure 2.4 *The effect of changing pH on the retention time of OT*

An increase in buffer pH resulted in an increase in the retention time of OT, which is more than likely due to a decrease in the polarity of the OT molecule. The ionisable functional groups of OT are ionised at low pH values and as the pH approaches 7.7, the isoelectric point at which OT is neutral is reached and the polarity of the molecule is reduced and is therefore better retained on a non-polar stationary phase. The pH chosen for further optimisation was pH = 5 as at this pH, OT peaks were sharp and representative chromatograms of OT under these conditions is shown in Figure 2.5 with a retention time of approximately 5 min, which also shows OT peaks at pH values, 6 and 7.

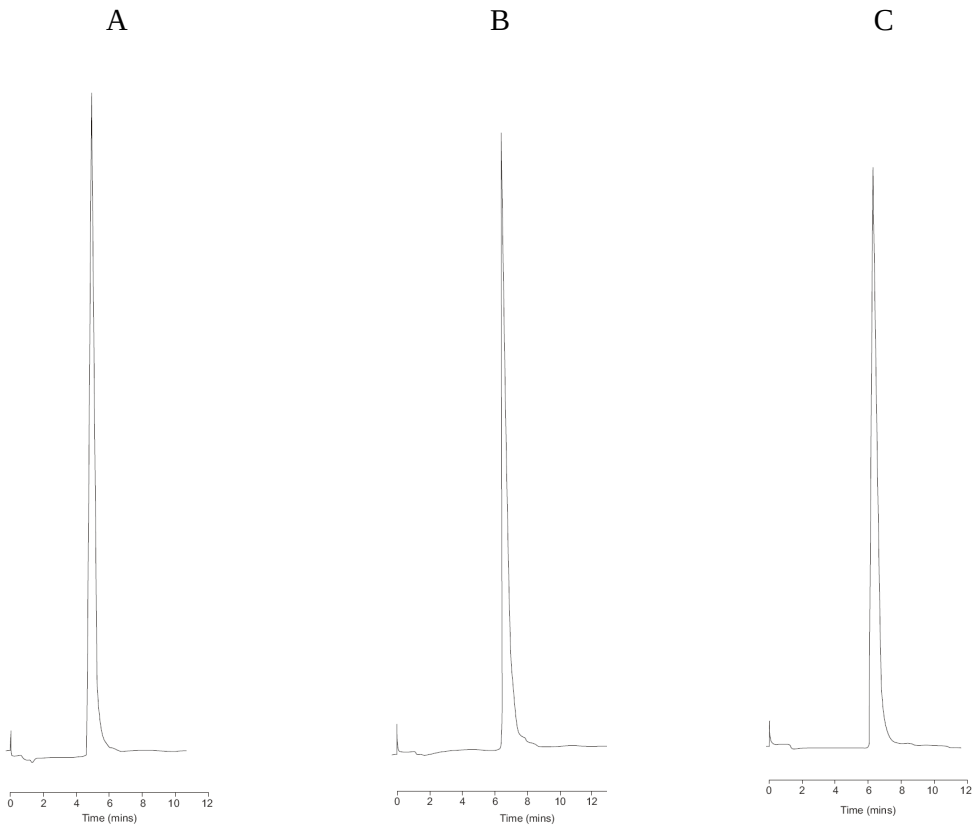


Figure 2.5 *The effect of pH on the peak shape for pH = 5 (A), pH = 6 (B) and pH = 7 (C), using a mobile phase comprised of 20% v/v ACN in 67 mM phosphate buffer*

2.4.7.4 Buffer Molarity

The molarity of a buffer in a mobile phase also has an effect on peak shape and retention time and buffers of 50 mM, 67 mM, 80 mM and 100 mM strength were compared to determine the optimal buffer concentration to be used for HPLC analysis. The effect of buffer molarity on retention time is depicted in Figure 2.6.

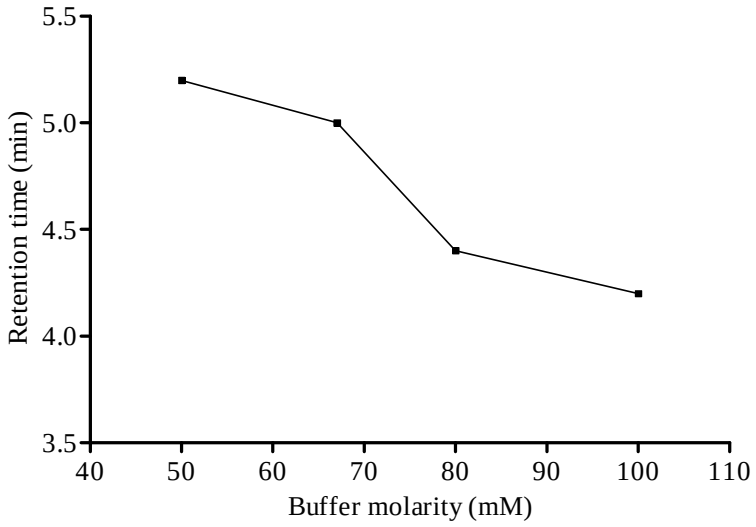


Figure 2.6 *The effect of buffer molarity on the retention time of OT*

As the buffer molarity was increased, there was a corresponding decrease in the retention time of OT. This is in agreement with previously reported results obtained following the analysis of proteins, OT, and other nonapeptides [178,183] where this is attributed to a corresponding increase in the solubility of OT and other peptides as the concentration of buffer salts in the mobile phase increases. The increase occurs up to a maximum, after which the solubility of OT and other peptides in the mobile phase will decrease. Therefore, the increase in the solubility of OT in the mobile phase with increasing buffer concentration resulted in a corresponding decrease in the retention time of OT as the peptide fraction/distribution in the mobile phase increased. Buffer strength affects the number of ions in a mobile phase that are available to interact with the eluent and as the buffer ionic strength is increased, there is increased number of ions in the mobile phase and therefore an increased interaction of the mobile phase with OT and a resultant decrease in the retention time.

The selection of appropriate buffer strength was based on the number of theoretical plates calculated from the chromatograms following the injection of samples onto the chromatographic system and eluted with mobile phases containing buffers of different molarities. The number of theoretical plates may be useful in evaluating the performance of an analytical column and describes the ability of a column to produce sharp narrow peaks with adequate resolution between those peaks [183]. The theoretical plate number of a column is affected by the diffusivity of a molecule in that column, which increases with an increased plate number [197]. A column with a length of 150 mm and a particle size diameter of 5 μm, should have a

theoretical plate number between 10000 – 12000 for small molecules [183], although the Center for Drug Evaluation and Research recommends that a theoretical plate number of at least 2000 is acceptable [223]. The theoretical plate number for OT under the specified conditions was calculated using Equation 2.4 [183].

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right) \qquad \textit{Equation 2.4}$$

Where,

N = the number of theoretical plates

t_R = the retention time of the drug

$W_{½}$ = the width of the peak at half the peak height [183]

The results of calculation of the theoretical plate count for the column used in these studies and obtained following the analysis of OT are shown in Table 2.3.

Table 2.3 *The effect of buffer strength on the theoretical plate number using a Hypersil® column for OT*

Buffer strength (mM)	Plate number
50	3745
67	4522
80	4766
100	2443

It can be seen from the data summarised in Table 2.2 that as the buffer strength increases, there is an increase in the theoretical plate number of the column, which was greatest when a buffer of an ionic strength of 80 mM was used. Thereafter there is a decrease in the plate number. The buffer strength that was chosen for further development and optimisation was 80 mM at which OT had a retention time of approximately 4.4 min.

The theoretical plate numbers obtained for the column used for the analysis of OT in these studies indicated that the column is operating well below the recommended efficiency level. It should however be noted that the separation of bio-molecules on such columns results in smaller theoretical plate numbers. This is because of the low diffusivity of the larger molecules in the column, which affects the velocity at which the molecules move along the column resulting in a smaller plate number count [183,197]. The column was however, found to be useful in achieving the intended purpose since the column theoretical plate number affects the resolution of compounds and the resolution obtained for this separation when an internal standard was used (§ 2.4.8) was found to be acceptable.

2.4.8 Selection of an Internal Standard

Calibration in chromatographic test methods can be achieved by use of an external or internal standard. In external standard methods, a standard is analysed on a separate chromatogram from a sample and quantitation is based on comparison of the peak height/area of a sample to that of a reference standard. This method may be more appropriate in analytical procedures with single/narrow concentration ranges and simple sample preparation procedures [183,223].

Alternatively, an internal standard may be used in a calibration method and in such cases, a different compound with similar structural and physicochemical properties to the analyte is added in known concentrations to the sample to be analysed. The compound must be well resolved from the peak(s) of interest and the ratio of response i.e. either the peak height or peak area, of the analyte of interest to that of the internal standard is used for the calibration plot and all subsequent quantitative measurements [183,223].

The use of an internal standard is particularly valuable in quantitative analyses for pharmaceutical [224-226] and pharmacokinetic studies [227-229] and especially for the analysis of samples that require significant pre-treatment that may undergo significant loss during the preparation procedure, such as the case when analysing biological samples. Furthermore, where the expected concentration ranges are wide and/or low concentrations of the analyte of interest are likely to occur, the use of an internal standard is recommended. Where variability of instrument responses is prevalent, the use of an internal standard is also recommended to enhance the precision of such methods. Instrumental variation in HPLC analyses can be a consequence of flow rate variability that in turn results in variability in the detector response to analytes of the same concentration. The use of an internal standard compensates for the variability in a method, thereby increasing the accuracy and precision of an analytical method [230]. The relative responses of two compounds, *viz.*, a drug, and internal standard will remain constant despite differences in instrumental operation and use of an internal standard ensures that the response that is measured is constant irrespective of equipment operation [183,223].

In addition, to achieve adequate resolution between an analyte of interest and an internal standard, the standard must have similar retention characteristics and times to the compound of interest. An internal standard should also be absent from the original sample matrix, available as a high purity sample commercially, chemically stable and no reactivity with the analyte of interest, the mobile and/or stationary phases used for the separation [183]. Furthermore, an

internal standard must also exhibit similar ultraviolet absorbance patterns to the compound of interest but need not necessarily be chemically similar to the analyte [183].

The selection of an internal standard was based on the data published in the available literature. Although most reported analyses of OT did not include the use of an internal standard, Ohta *et al*. [123] used ethyl parahydroxybenzoate as an internal standard for the quantitative analysis of OT in pharmaceutical preparations. Methyl hydroxybenzoate (MHB) and propyl hydroxybenzoate (PHB) were tested as potential internal standards using analytical conditions of 20% v/v ACN in 80 mM phosphate buffer, pH = 5 and the retention times of the MHB and PHB were approximately 7 min and more than 20 min, respectively. The internal standard that was therefore deemed appropriate and selected for use for the analysis of OT was MHB. The MHB peak was well resolved from that of OT, which eluted at 4.4 min under the specified conditions and its use resulted in a total analytical run time of < 10 min for each sample.

The response of the internal standard should be approximately half that of the maximum expected concentration of the analyte of interest. Therefore, the concentration MHB that was used was approximately 7.25 µg/ml and was quantitatively added to each of the samples to be analysed prior to the analysis.

2.4.9 Chromatographic Conditions Selected

The optimal chromatographic conditions that were established for the quantitative determination of OT are summarised in Table 2.4 and a typical chromatogram of the optimised conditions is shown in Figure 2.7.

Table 2.4 *Optmised chromatographic conditions for quantitation of OT in pharmaceutical dosage forms*

Column	**Phenomenex® Hypersil C_{18}, 5 µm, 150 X 4.6 mm**
Mobile phase	**20% v/v ACN in 80 mM phosphate buffer at pH = 5**
Detection wavelength	**220 nm**
Detection sensitivity	**0.005 AUFS**
Injection volume	**20 µl**
Chart speed	**5 mm/min**
Temperature	**22 °C**
OT retention time	**4.4 min**
MHB retention time	**7 min**

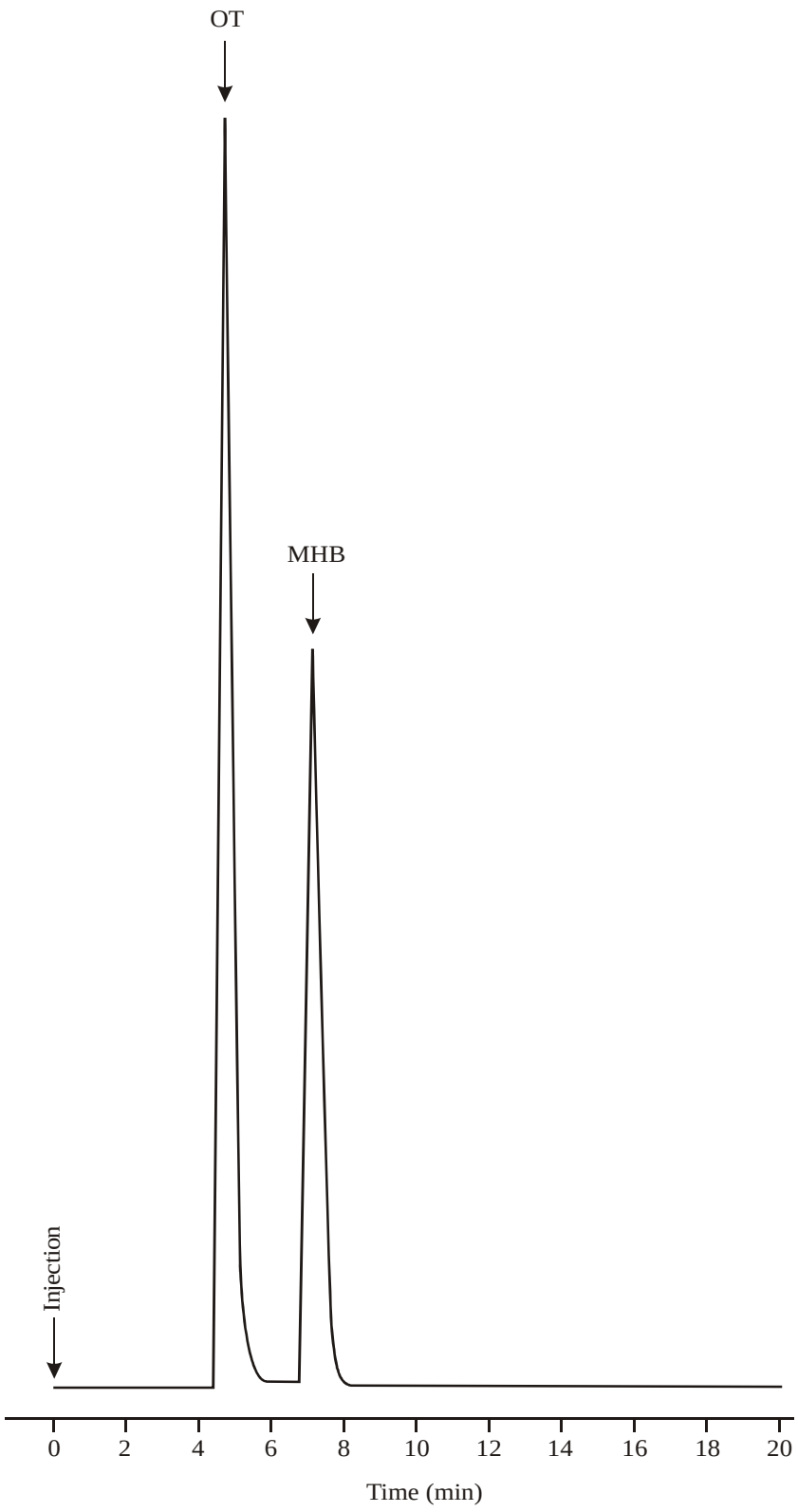


Figure 2.7 *Typical chromatogram of the separation of OT (4.4 min) and MHB (7.0 min)*

2.5 METHOD VALIDATION

2.5.1 Introduction

The USP [10] states that the validation of an analytical method is a process that establishes the performance characteristics of a developed analytical method and ensures that it meets its intended purpose and analytical application. Method validation includes an assessment of the adequacy of the analytical procedure by means of statistical testing, including linear regression analysis, and relative standard deviation determination in order to demonstrate the validity of the method [231]. During validation, an analytical method is tested for reliability, accuracy, and preciseness of the intended purpose of that method [223]. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [232] recognises accuracy, precision, repeatability, intermediate precision, specificity, limits of quantitation and detection, linearity, and the range of a method as important validation parameters. The guidance for the methodology and statistical tests and associated limits for the validation procedure is provided in the ICH Q2B document [233].

2.5.2 Linearity

The ICH [232] defines the linearity of an analytical procedure as its ability, within a given range to obtain test results that are directly proportional to the concentration or amount of an analyte in a sample. The linearity of response to the concentration is in agreement with the Beer-Lambert Law [234] where it was established that the absorbance of a solute in dilute solutions is directly proportional to the concentration of that solute or analyte in that solution [183,223].

To demonstrate linearity across a given concentration range, dilution of a standard stock solution and/or separate weighings of synthetic mixtures of a drug product and its components can be used. A minimum of five concentrations is required to demonstrate/establish linearity [233]. Linearity was assessed by analysis of seven (7) concentrations in the range of 0 – 12 IU/ml (n = 5) of OT. Calibration solutions of the required concentrations were prepared, including a solution that contained 0 IU/ml of OT with the internal standard as previously described in § 2.3.2.

To evaluate linearity, the ICH [233] recommends that a visual inspection of a plot of response signal as a function of analyte concentration or content initially, and if there is a linear

relationship then statistical testing should be conducted. Testing can be achieved by means of calculation of a regression line by a least squares linear regression method to determine the degree/extent of linearity of the analytical method. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares and a plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity and the precision of a method. Figure 2.8 depicts a typical calibration curve for the analytical procedure developed and validated for analysis of OT.

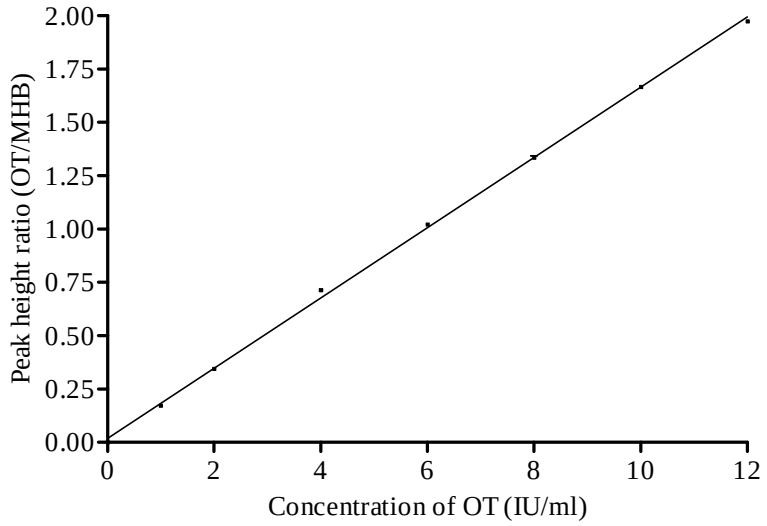


Figure 2.8 *Typical calibration curve for OT in the concentration range 0 – 12 IU/ml*
$y = 0.1667x + 0.0008$, $r^2 = 0.9995$

The equation for the best-fit linear regression line was $y = 0.1667x + 0.0008$ with a coefficient of determination of 0.9995 indicating that the calibration curve is linear over the calibration range studied. Under most circumstances a regression coefficient of 0.999 [183,223,235] is considered sufficient to deem the relationship between two variables linear. Furthermore, the y-intercept should be < 2% of the response or near zero [235] and the intercept obtained (0.0008) satisfies this criterion.

The use of the correlation coefficient as a means of determining the degree of linearity of a curve may have shortcomings, in particular if the responses are measured over one order of magnitude or deviate from linearity at the extremes of the calibration range [235]. In this case, the parameters of a linear regression equation may be affected by skewed data at the low and/or high concentration samples. A plot of the response factor versus concentration in order to ascertain the impact these data may have on the linearity of a method can be plotted and

evaluated. The response factor is determined by the detector response or peak height ratio divided by the concentration of the standard producing that response. If the response obtained at each concentration is equal or is similar then the points should form a straight line with a slope of zero [183,235]. The response factor graph obtained for these studies is depicted in Figure 2.9.

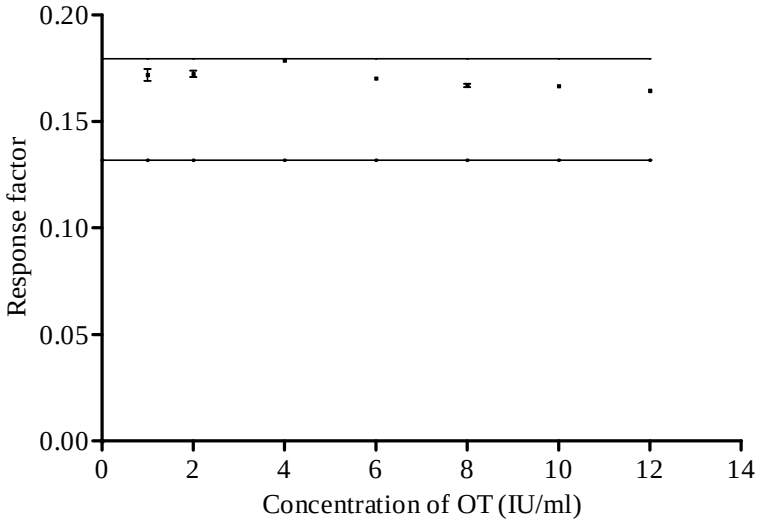


Figure 2.9 *The response factor curve for OT in the concentration range 0 – 12 IU/ml, showing the upper and lower limits of the standard deviation*

The average response factor was determined to be 0.1557 $\pm$ 0.0238 and the response obtained for most concentrations are all within one (1) standard deviation. The equation of the line is $y = 0.0029x + 0.1429$. The slope of the response factor slope has a near zero slope and shows that the relationship can indeed, be considered linear.

2.5.3 Precision

The precision of an analytical method is a measure of the degree of scatter or agreement among individual data values, when the method is applied to multiple samples. In addition, precision is a means of evaluation of how close the data values are to each other for a number of measurements taken using the same analytical conditions [10,223,232]. The ICH guidelines define precision on three levels, *viz.*, repeatability, intermediate precision, and reproducibility. The ICH [233] recommends that the standard deviation, relative standard deviation or coefficient of variation and a confidence interval be reported for each type of precision that is investigated.

2.5.3.1 Intra-assay Precision (Repeatability)

Repeatability refers to the precision of an analytical method used under the same operating conditions by the same analyst and measured over a short period of time [10,145,223,232,235]. The percent relative standard deviation (% RSD) of a group of samples is used to assess the precision of a method and the permitted % RSD depends on the intended use of the method and the matrix from which the active pharmaceutical ingredient (API) of interest is isolated. For an assay method, the limit of the % RSD would be 2%, whereas that for determining impurities at trace levels is 10% [235]. Repeatability of the method was determined by interpolation of data from the calibration curve at three levels covering the specified range *viz.*, 3 IU/ml, 7 IU/ml and 11 IU/ml. The samples were analysed with five samples (n = 5) at each concentration and the results are summarised in Table 2.5. In addition the precision of the calibration curve data (n = 5) shown in Figure 2.8 are summarised in Table 2.6.

Table 2.5 *Intra-assay precision for OT in the concentration range 0 – 12 IU/ml*

Concentration (IU/ml)	Peak height ratio (OT/MHB), (n = 5)	Standard deviation	% RSD
3.0	0.492	0.00210	0.43
7.0	1.165	0.00305	0.26
11.0	1.788	0.00376	0.21

Table 2.6 *Intra-assay precision for the calibration curve of OT (0 – 12 IU/ml) shown in Figure 2.8*

Concentration (IU/ml)	Peak height ratio (OT/MHB), (n = 5)	Standard deviation	% RSD
0.0	0.000	0.00000	0.00
1.0	0.172	0.00280	1.63
2.0	0.345	0.00300	0.87
4.0	0.715	0.00170	0.24
6.0	1.021	0.00256	0.25
8.0	1.335	0.00686	0.51
10.0	1.667	0.00503	0.30
12.0	1.974	0.00404	0.20

The results shown for repeatability of the assay reveal % RSD values of less than 2% for all concentrations of OT studied thereby indicating that the method is repeatable.

2.5.3.2 Inter-day Precision (Intermediate Precision)

The intermediate precision of an analytical method evaluates the reliability of the method in a different environment other than that used during development of the method to ensure that the method will provide the same results once method development has been completed [223]. The ICH [232] defines intermediate precision as the long-term variability of a measurement process, expressing within laboratory variation, analysis on different days, different analysts, and/or equipment. The extent to which inter-day precision is determined is dependant on the

circumstances in which the analytical method is to be used and therefore the expected variation [233]. Intermediate precision can be determined by comparing the results of an analytical procedure run over a period of days to weeks. Intermediate precision was determined for three (3) concentrations, injected in triplicate (n = 3) on three (3) consecutive days. The results for intermediate precision studies are summarised in Table 2.7 and all % RSD values were less than 2% indicating that the method has day-to-day precision.

Table 2.7 *Inter-day precision for OT in the concentration range 0 – 12 IU/ml*

Concentration (IU/ml)	**Day 1**		**Day 2**		**Day 3**	
	Peak height ratio	**% RSD (n = 3)**	**Peak height ratio**	**% RSD (n = 3)**	**Peak height ratio**	**% RSD (n = 3)**
3.0	0.49	0.39	0.50	0.26	0.49	1.45
7.0	1.16	0.25	1.18	0.24	1.16	0.24
11.0	1.78	0.44	1.83	0.40	1.79	0.10

2.5.3.3 Reproducibility

The reproducibility of an analytical method is a measurement of the precision of a method that is used in more than one laboratory, i.e. precision between laboratories. The assessment of reproducibility serves to show that a method that has been developed can be transferred between laboratories. Inter-laboratory trials must be considered in the standardisation of a procedure such as those intended for inclusion in an official compendial publication [233]. Reproducibility may be determined by analysing samples from homogenous lots in different laboratories using different analysts. Reproducibility of this method was not established as it was to be used in one laboratory by the same analyst for the duration of these studies.

2.5.4 Accuracy

The accuracy of an analytical method is defined as the closeness of a measured value to the true value for that sample [10,183,223,232,235]. There are several ways of determining the accuracy of an analytical method including comparison to a reference standard, recovery of spiked analyte, and the standard addition of an analyte to a sample [233]. For drug products, it is recommended that accuracy be performed at 80, 100, and 120% of the label claim [223]. Accuracy should be assessed using a minimum of nine (9) determinations over a minimum of three (3) concentration levels covering the specified range, for example, three (3) concentrations analysed in triplicate (n = 3) [233].

The accuracy of a method may be determined by recovery which is expressed as the amount/weight of a compound of interest reported as a percentage of the theoretical amount present in a medium and where full recovery of the analytes is desirable [223,233]. Percent bias (% Bias) can also be used to determine the extent of deviation of a result for a sample from the true value for that sample. The closer the recovery is to 100% and the lower the % Bias, the more accurate an analytical method can be considered and in general, a % Bias of less than 5% is desirable.

Accuracy was determined at three (3) concentrations and each solution was analysed in replicates of five (n = 5) and the % Bias was calculated by interpolation of the data from a calibration curve. The resultant concentration was compared to the theoretical concentration for each of the samples and it was observed that the resultant % Bias for this method was low, and the results are summarised in Table 2.8.

Table 2.8 *Accuracy for OT in concentration range 0 – 12 IU/ml*

Concentration of OT (IU/ml)	Determined concentration (IU/ml)	% RSD	% Bias
3.0	2.89	0.43	3.83
7.0	6.96	0.26	0.54
11.0	10.80	0.21	1.81

2.5.5 Specificity

Specificity is defined as the ability of an analytical procedure to accurately and quantitatively measure the concentration of an analyte in the presence of all sample materials, including extraneous components from which it must be well resolved [10,183,223,232]. The determination of the specificity of an analytical method is considered one of the most important steps in the development and validation of that method.

The chromatographic procedure that is developed must resolve the peak of the compound(s) of interest from any possible excipients or contaminants that may be present in a dosage form, during analysis of that dosage form. Commercially available OT preparations include chlorbutol (CB) as a preservative at a concentration of 5 mg/ml [31]. A solution containing OT (10 IU/ml) and the preservative was prepared and on analysis no interference was observed between the OT and CB peaks, which was eluted at 4.4 min and 15.2 min respectively was observed. A typical chromatogram showing the separation of OT and CB is shown in Figure 2.10.

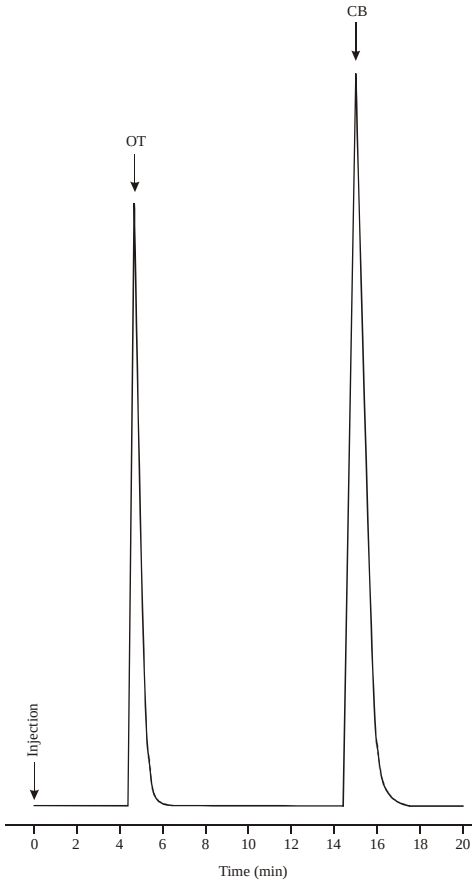


Figure 2.10 *Typical chromatogram showing OT (4.4 min) and CB (15.2 min)*

2.5.6 Stress studies

A stability-indicating assay can be defined as a validated quantitative analytical procedure that is able to detect changes in the pertinent properties of the drug substance and/or drug product [231]. A stability-indicating assay must also be capable of accurately measuring the specific analyte without interference from degradation products, process impurities, excipients, or other potential impurities. It is recommended that assay procedures used in stability studies should be stability-indicating, unless otherwise scientifically justified [231].

To demonstrate specificity in stability-indicating assays the quantitation of an analyte is attempted in the presence of degradation information obtained from stress studies in which the analyte(s) of interested are exposed to acid and base, heat, light and oxidation conditions to assess the impact of these conditions on the hydrolytic, thermal, photolytic and oxidative properties of the molecule. It is important that impurities and degradation products from the

analyte do not interfere with the quantitation of the active ingredient in an analytical method [231]. Specificity is vitally important in the development of stability-indicating assays and stress studies may be used to demonstrate of specificity of a developed method. Stress studies entailed exposing OT to acid, alkali, hydrogen peroxide, light, and heat conditions [10,183,223,233]. OT solutions (10 IU/ml) were prepared as described in § 2.3.2 and by Chaibva and Walker [119] and exposed to acidic, alkali, oxidative, light and heat conditions.

2.5.6.1 Acidic Conditions

Two millilitres (2 ml) of 0.1 M hydrochloric acid was added to eight millilitres (8 ml) of a 10 IU/ml solution of OT to produce a solution of OT that was 8 IU/ml. The solution was allowed to stand for one hour at 22 ºC [119]. The resultant chromatogram generated following injection of a sample that was allowed to stand in acidic conditions for one hour is shown in Figure 2.11.

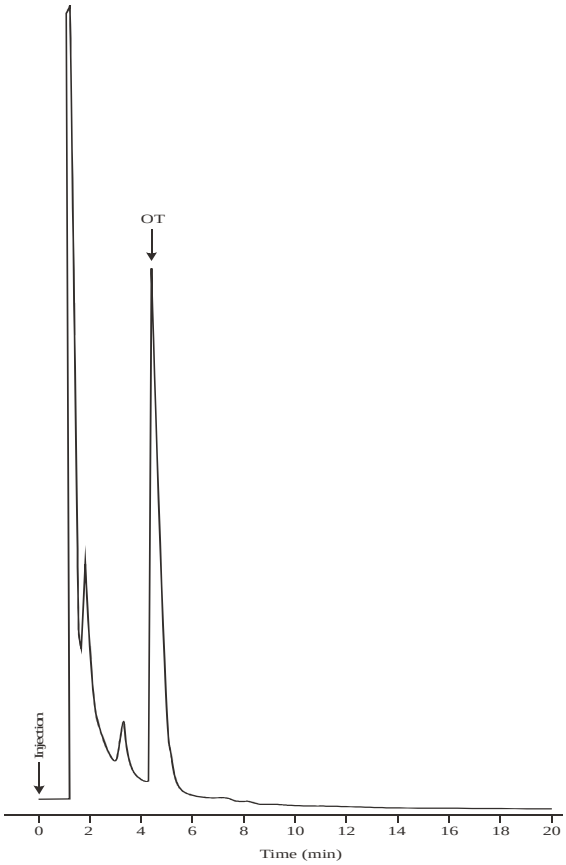


Figure 2.11 *Typical chromatogram showing degradation of OT after exposure to 0.1 M hydrochloric acid for 1 hour*

OT underwent degradation, under acidic conditions, and the resultant degradation product peaks were well resolved from that of the OT peak. An assay of the sample using the validated HPLC method, to determine the extent of OT degradation revealed that 10.58 $\pm$ 0.51% of OT had degraded under these conditions.

2.5.6.2 Alkali Conditions

Two millilitres (2 ml) of a 0.1 M sodium hydroxide solution was added to eight millilitres (8 ml) of a 10 IU/ml solution of OT to produce a solution of 8 IU/ml. The solution was allowed to stand for one (1) hour at 22 ºC [119]. The resultant chromatogram generated following injection of a sample that was allowed to stand for 1 hour is shown in Figure 2.12. It is clearly evident that OT is highly unstable in alkali conditions [8] and that after exposure as described, almost all of the OT in solution had degraded and only a small peak corresponding to OT was observed on the chromatogram. The extent of degradation of OT under these conditions was determined and showed that 70.33 $\pm$ 0.25% of OT had degraded.

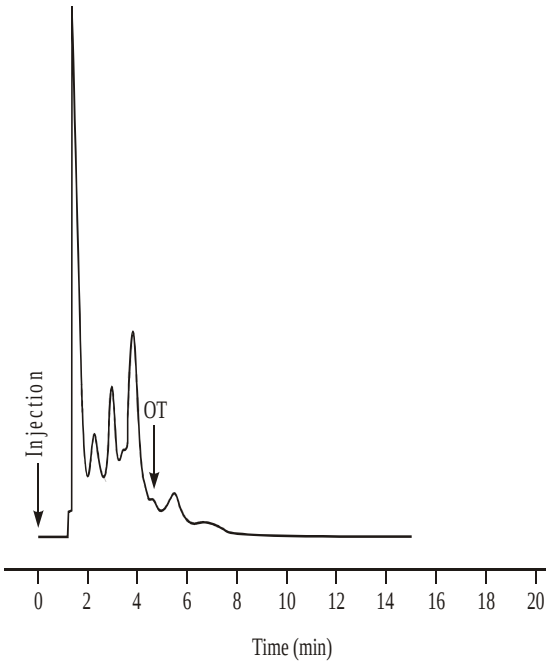


Figure 2.12 *Typical chromatogram showing degradation of OT after exposure to 0.1 M sodium hydroxide for 1 hour*

2.5.6.3 Heat

Twenty millilitres (20 ml) of a 10 IU/ml solution of OT was heated and maintained at 50 °C for 10 min [119]. The solution was allowed to cool to room temperature prior to injection onto the RP-HPLC system. The resultant chromatogram generated following injection of a sample

heated at 50 °C for 10 min is shown in Figure 2.13. The extent of degradation under these conditions was determined and it was observed that 3.55 ± 1.07% of OT had undergone degradation.

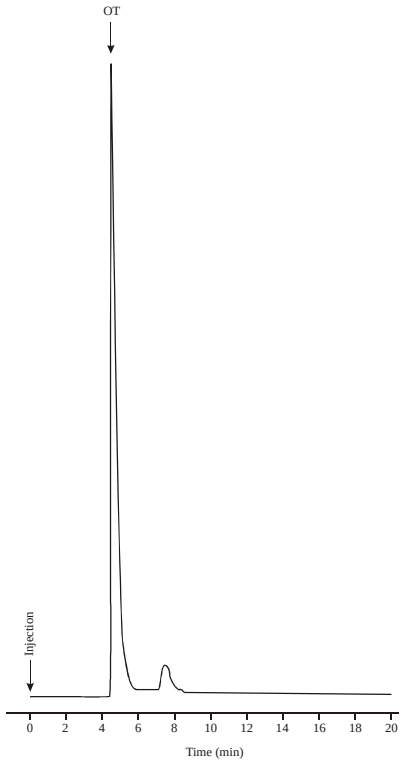


Figure 2.13 *Typical chromatogram showing degradation of OT after exposure to 50 ℃ for 10 min*

2.5.6.4 Light

A 10 ml OT solution at a concentration of 10 IU/ml in a clear volumetric flask was exposed to natural sunlight for 8 hours, by placing the solution on a windowsill in the laboratory for the duration of the experiment [119]. No degradation was observed as a single OT peak was obtained on the chromatogram as shown in Figure 2.14, indicating that OT is stable in light after exposure for a period of 8 hours.

2.5.6.5 Oxidation

Two millilitres (2 ml) of a 3% hydrogen peroxide solution was added to eight millilitres (8 ml) of a 10 IU/ml solution of OT to produce a solution of 8 IU/ml. The solution was allowed to stand for 2 hours at 22 ºC in a dark cupboard [119]. The resultant chromatogram generated following injection of a sample that was allowed to stand for two hours under the specified conditions is shown in Figure 2.15. An assay of the resultant solution showed that 21.67 ± 0.49% of OT had degraded under these conditions.

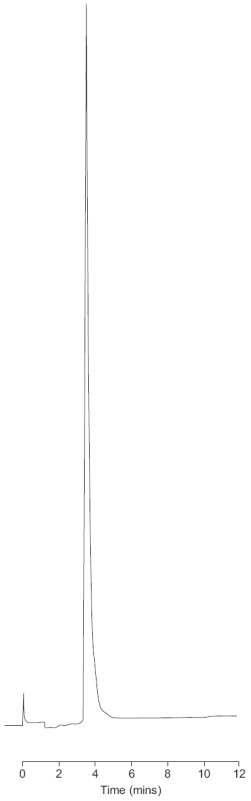


Figure 2.14 *Typical chromatogram showing degradation of OT after exposure light for 8 hours*

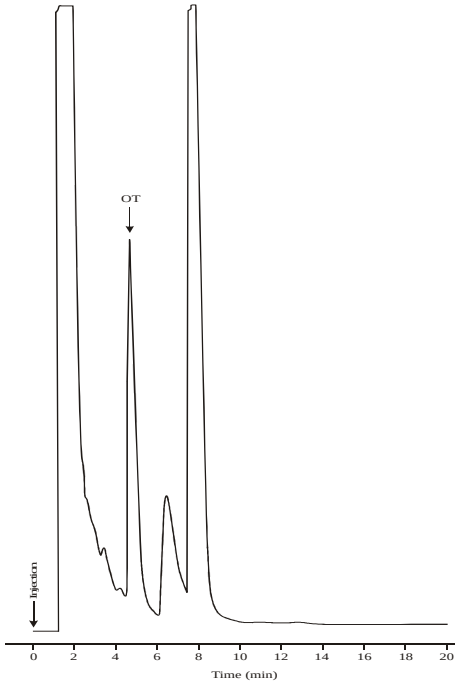


Figure 2.15 *Typical chromatogram showing degradation of OT after exposure to 3% hydrogen peroxide for 2 hours*

2.5.6.6 Conclusion

The specificity of the assay was clearly demonstrated by the ability of the analytical test to discriminate between OT peaks and those of degradation products that resulted after exposure of OT to stress conditions. Stability-indicating assays are essential for quality control purposes and give an indication of process or storage conditions that are likely to impact on the amount of drug that is present in a dosage form.

The method developed for the analysis of OT in dosage forms is able to detect changes in the quantity of drug present in solution. The limitation of the stability-indicating method is however that the presence of degradation products of different retention times, limits the use of the internal standard (MHB) in the quantitation of the residual amount of OT. This is because the MHB peak would not be not resolved from thermal and oxidative degradation products peaks that are shown in Figures 2.13 and 2.15, respectively and that elute at approximately 7 min. Despite this limitation, the ability of the method to resolve OT and degradation products makes it invaluable for quality control purposes in pharmaceutical dosage forms.

2.5.7 Limits of Quantitation (LOQ) and Detection (LOD)

2.5.7.1 Introduction

The limit of quantitation (LOQ) of an individual analytical procedure is defined as the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy [223,232]. The limit of detection (LOD) is defined as the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value, under the defined experimental conditions [223,232]. UV detectors may result in diminished precision at low concentrations of an analyte because of a potential and/or gradual loss of sensitivity of detector lamps as they age or due to noise level variation of detector because of different manufacturers or even model number of that detector [223].

The ICH [233] recommends three different techniques that can be used to determine the LOQ and LOD of a given method. The LOQ and LOD can be determined based on visual evaluation of analytical data. The quantitation and detection limits can determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can reliably be quantified with acceptable accuracy and precision or detected, respectively.

The signal-to-noise ratio can be used to determine the LOQ and LOD of a method [10,233]. However, this approach can only be applied to analytical procedures that exhibit substantial baseline noise. To determine the signal-to-noise ratio, measured signals from samples with known low concentrations of analyte and of blank samples are compared establishing the minimum concentration that is required to give a signal-to-noise ratio of 10:1 for the LOQ and between 3 and 2:1 for the LOD. Even though this is recommended by the USP [10], this approach is not considered very practical, as the noise level on the detector may be different when the samples are assayed on different detectors [223]. The Center for Drug Evaluation and Research (CDER) [223] therefore recommends the use of an additional standard in the test method at the LOQ and a repeatability assessment of samples at the LOQ level.

Another method that can also be used for determination of the LOQ and LOD is based on the standard deviation of the response and the slope of the line [233]. These relationships are shown in Equation 2.5 and Equation 2.6 respectively.

$$LOQ = \frac{10\sigma}{S} \qquad \textbf{\textit{Equation 2.5}}$$

$$LOD = \frac{3.3\sigma}{S} \qquad \textbf{\textit{Equation 2.6}}$$

Where,

σ = standard deviation of the response

S = slope of the calibration curve

The calibration curve may be used to estimate the requisite slope and the standard deviation may be determined by analysing the standard deviation of several blank samples or by the construction of a calibration curve in the range of the LOQ or LOD, as appropriate. The residual standard deviation of the y-intercept may be used as the standard deviation in these equations [233].

The ICH [233] recommends that if the LOQ or LOD are determined from visual evaluation of chromatograms or are based on the signal-to-noise ratio method then the presentation of the relevant chromatograms is required if the LOQ and LOD are obtained by calculation or extrapolation. This estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

2.5.7.2 LOQ

The analytical method did not have any baseline noise and therefore the use of methods incorporating the signal to noise ratio were not deemed appropriate. The LOQ was determined using Equation 2.5 and a calibration curve in this region gave an equation, $y = 0.1184x + 0.0008$, $R^2 = 0.9942$. The LOQ was determined to be approximately 0.3 IU/ml with an average peak height ratio of 0.04 and a % RSD = 5.71 (n = 6).

2.5.7.3 LOD

As noted in § 2.5.7.2, the absence of baseline noise hindered the use of the signal-noise-ratio. LOD was determined using Equation 2.6 and the linearity curve described in § 2.5.7.2 and this was found to be approximately 0.1 IU/ml.

2.5.8 Range

The range of an analytical procedure is defined as the interval between the upper and lower concentration/amount of an analyte in the samples being analysed and include the upper and lower concentrations for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity [232]. The range of a method depends on the purpose of the analytical procedure that has been developed. For assay procedures, the range would fall between 80 – 120% of the target concentration, but for combination analytical methods for assay and determination of impurities the range would be the limit of quantitation to 20% above the target concentration [223,233]. The range is derived from linearity and the LOQ studies and the range of this analytical method was found to be 0.3 – 12 IU/ml.

2.5.9 Stability of the Analyte in the Mobile Phase

During validation, additional data deemed necessary include the stability of all analytical sample preparations that will be used for the duration of the analysis [231]. The test method must be used to analyse sample stability, especially where samples are analysed overnight or following long storage periods after collection or preparation. This is especially a concern for drugs that undergo hydrolysis, photolysis, or that are adsorbed onto glassware [223]. Data to support sample stability in solution under normal operating laboratory conditions for the test procedure are therefore required.

The stability of the analyte in the mobile phase was investigated. A solution of concentration of 10 IU/ml of OT was prepared as described in § 2.3.2 and the stability in the mobile phase was tested over a 3-day period, after storage of the samples in a refrigerator at 5 °C. No additional degradation products were observed during chromatographic analysis and the OT content over three consecutive days was determined to be 10.012 $\pm$ 0.016 IU/ml with 0.16 % RSD. It was therefore established that OT was stable in the mobile phase, and the method developed for the quantitative analysis was suitable for analytical procedures that run over at least three days.

2.6 ASSAY OF SYNTOCINON®

An assay of the commercially available form of OT in South Africa was undertaken using the analytical method that had been developed and Syntocinon® 10 IU/ml was purchased from a local pharmacy. The analysis was performed in triplicate using three (3) individual ampoules and a 1.0 ml aliquot of the parenteral formulation was diluted to 5.0 ml with HPLC grade water in an A-grade volumetric flask and then subjected to analysis using the validated procedure described in this Chapter. A typical chromatogram obtained during analysis of the commercially available product is shown in Figure 2.16 and the results of these analyses are summarised in Table 2.9.

Table 2.9 *Result of the analysis of an assay of commercially available Syntocinon®*

Sample number	**Peak height ratio (OT/MHB)**	**% RSD (n=3)**	**Determined concentration (IU/ml)**
1	0.35	1.75	10.08
2	0.35	0.76	10.05
3	0.33	0.62	9.55

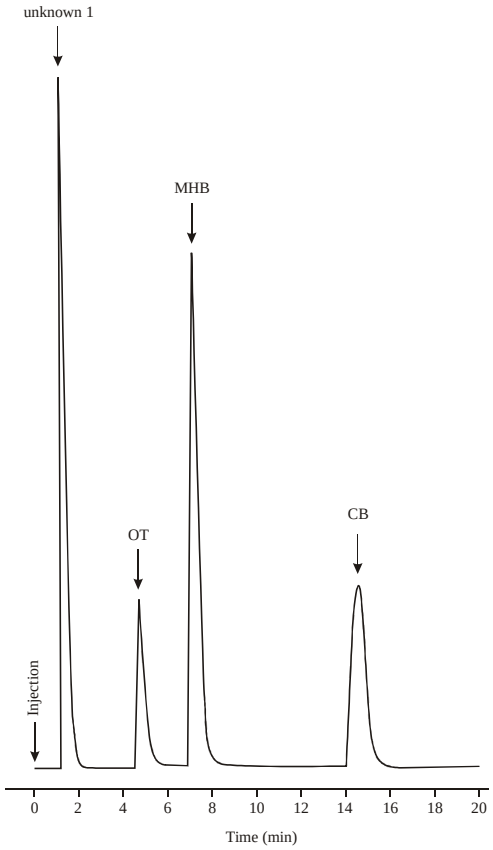


Figure 2.16 *Typical chromatogram obtained following the assay of Syntocinon®*
showing an unknown peak (2.2 min), OT (4.4 min), MHB (7.0 min), and CB (15.2 min)

2.7 CONCLUSION

The method that was developed is linear, accurate, precise, sensitive, and selective and is appropriate for the analysis of the current pharmaceutical dosage form of OT that is available on the market.

2.8 OPTIMISATION OF THE ANALYTICAL METHOD FOR *IN VITRO* USE

The analytical method that was developed and validated was further optimised for use in the analysis of *in vitro* release of OT from hydrogel systems that were developed in subsequent studies (See Chapter 5, *vide infra*). The sensitivity of the method was increased in order to ensure that the lower concentrations expected during assessment of *in vitro* release could be quantitated. HPLC system B (§ 2.3.6) was used for the optimisation of the previously validated method. The method was optimised in terms of wavelength of detection and volume of injection by monitoring the change of response of OT. The method was thereafter revalidated to ensure

that the method would meet the necessary criteria to ensure that reliable and valid data were obtained.

2.8.1 Wavelength

A sample solution of OT of concentration of 0.3 IU/ml was injected under the same conditions that had been established using HPLC system A (§ 2.4.9) except that the wavelength was varied within the range of 200 – 230 nm. Samples were injected in triplicate (n = 3) and a plot of the effects of wavelength change on response are depicted in Figure 2.17.

As the wavelength was increased, there was a corresponding decrease in the peak height response of OT. The greatest response was observed at a wavelength of 200 nm. However, since there is a decrease in the selectivity and excessive baseline noise when using this wavelength, 210 nm was selected as the optimal wavelength for the detection of OT. This is because using a wavelength 210 nm provides an adequate analyte response and limited baseline noise, making it a suitable wavelength for detection of the peptide.

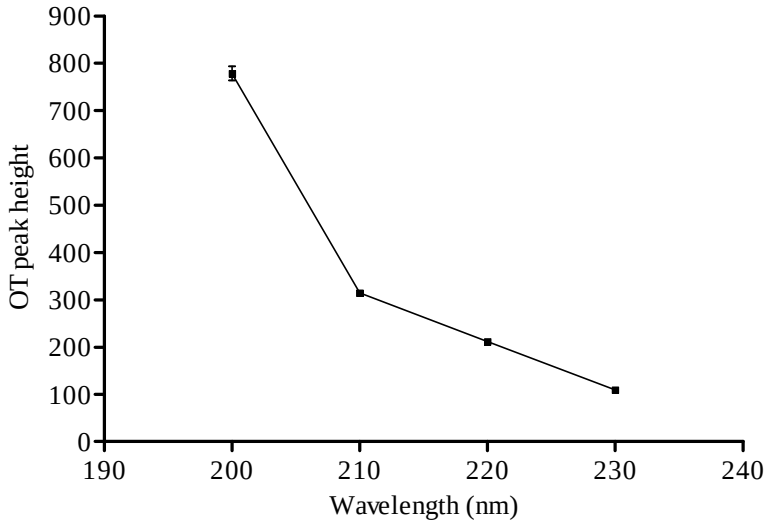


Figure 2.17 *The effect of changing wavelength on the peak height of OT*

2.8.2 Volume of Injection

Two volumes of injection were compared, *viz.*, 20 µL and 50 µL and testing was performed in triplicate (n = 3). The results of these studies are depicted in Figure 2.18.

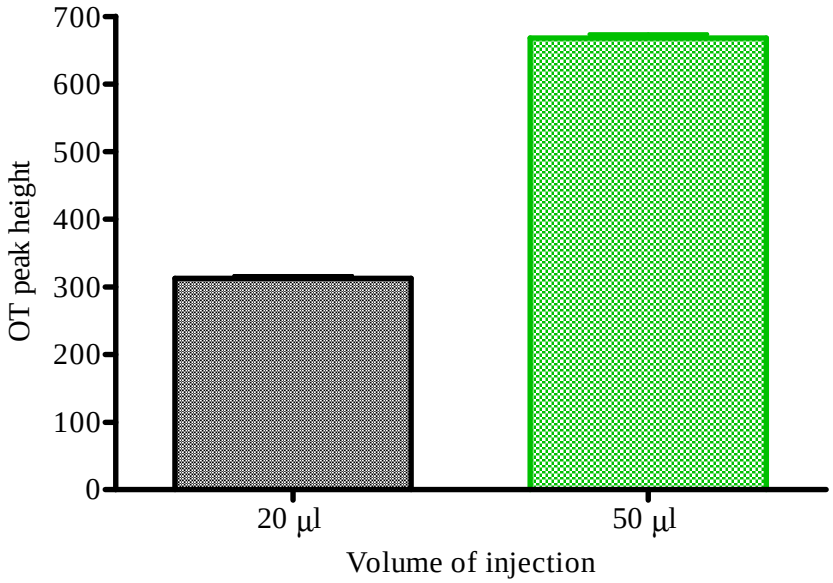


Figure 2.18 *The effect of changing injection volume on the peak height of OT*

As the volume of injection is increased, there is a corresponding increase in the amount of analyte that passes through the column to the detector and an increase in detector response [122]. This is important as it permits very small concentrations of analyte to be detected because of the increase in response and higher peaks that are observed. However, when the volume of injection is increased excessively column overload may occur and this phenomenon must be evaluated prior to selecting a suitable volume for injection. Column overloading was not a major concern for this particular application as the maximum possible concentration that would be expected to be observed during *in vitro* testing was calculated to be no more than 0.50 IU/ml and therefore the maximum possible column load following an injection of 50 µl would be 0.025 IU.

No internal standard was used for the optimised analytical method for use in the assessment of OT release from Pluronic® gels (See Chapter 5, *vide infra*) because of the potential for the hydroxybenzoate preservatives to be solubilised by micelles [236]. The formation of micelles by Pluronic® F127 (PF-127) in aqueous solution (§ 4.5.4) may cause an interaction with the internal standard and result in interference with *in vitro* release studies of OT from PF-127 dosage forms.

2.8.3 Optimised HPLC Conditions

The optimised conditions for the separation of OT for *in vitro* release testing are summarised in Table 2.10 and Figure 2.19 summarise the optimised conditions for OT analysis and show a typical chromatogram of OT that was obtained using the optimised method, respectively.

Table 2.10 *Optimised conditions for in vitro analysis of OT release from hydrogel dosage forms*

Column	**Phenomenex® Hypersil C_{18}, 5 µm, 150 X 4.6 mm**
Mobile phase	**20% v/v ACN in 80 mM phosphate buffer at pH = 5**
Detection wavelength	**210 nm**
Detection sensitivity	**0.005 AUFS**
Injection volume	**50 µl**
Chart speed	**2.5 mm/min**
Temperature	**22 °C**
OT retention time	**4.38 min**

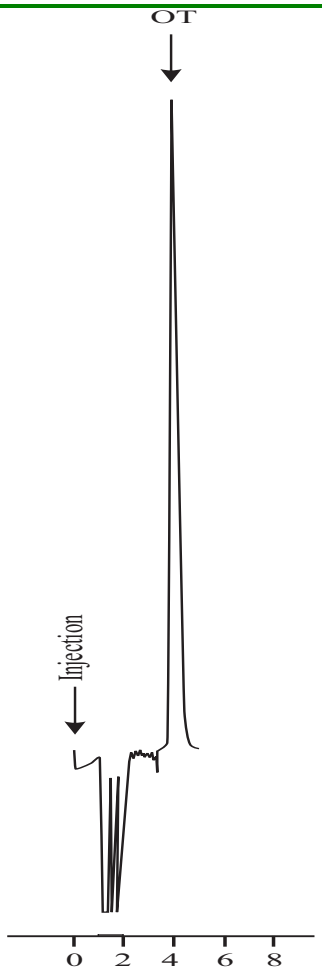


Figure 2.19 *Typical chromatogram of OT under the optimised conditions for assessing the in vitro release of OT*

2.9 REVALIDATION OF THE ANALYTICAL METHOD

The revalidation of analytical procedures is necessary if process variables, that may affect the results obtained using that method, are introduced. In the pharmaceutical industry, such variables may include changes in the source of raw material or drug substance, manufacture of the raw material by a different synthetic route or a change in composition of the drug product. Revalidation should be performed to ensure that the analytical procedure maintains its characteristics and specificity and to demonstrate that the analytical procedure continues to identify the strength, quality, purity, and potency of the drug substance and/or drug product. The degree of revalidation of an analytical method depends on the nature of the change(s) made to the method [231]. A change in the range, wavelength of detection and the volume of injection and HPLC system used and the absence of internal standard in these studies required revalidation of the analytical procedure.

2.9.1 Linearity

The linearity of the method was tested within the concentration range that was expected to occur during *in vitro* dissolution testing of OT in hydrophilic hydrogel systems that were to be developed. The calibration standards were prepared as described previously in § 2.3.2 and linearity was assessed in the same manner as described in § 2.5.2. The calibration curve shown in Figure 2.20 was produced by injecting OT samples in the range 0 – 0.50 IU/ml (n = 5).

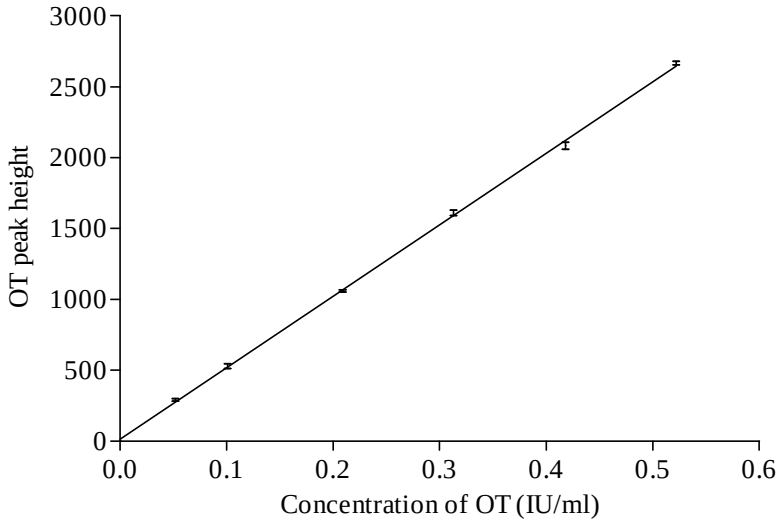


Figure 2.20 *Calibration curve for OT in the concentration range 0 – 0.50 IU/ml*
$y = 5044x + 13.52$, $r^2 = 0.9993$

The calibration curve following revalidation was y = 5044*x* + 13.53, with a linear regression coefficient of 0.9993, indicating that the method is linear.

2.9.2 Precision

Intra-assay and inter-day precision of the analytical method were tested during revalidation as previously described in § 2.5.3.

2.9.2.1 Intra-assay Precision (Repeatability)

The repeatability of the methods was determined as described previously in § 2.5.3.1 and this was determined at three different levels, *viz.*, 0.15 IU/ml, 0.25 IU/ml and 0.35 IU/ml and analysis was performed in triplicate (n = 3). The results of these studies are shown in Table 2.11. Intra-assay Precision (Repeatability)

Table 2.11 *Intra-assay precision for OT in the concentration range 0 – 0.50 IU/ml*

Concentration of OT (IU/ml)	OT Peak height	Standard deviation	% RSD
0.15	798.4	6.3008	0.79
0.25	1327.8	8.5264	0.64
0.35	1893.2	12.3369	0.65

Table 2.12 summarises the precision of the calibration curve data depicted in Figure 2.20.

Table 2.12 *Intra-assay precision data for the calibration curve (0 – 0.50 IU/ml) shown in Figure 2.20*

Concentration of OT (IU/ml)	OT Peak height	Standard deviation	% RSD
0.00	0.0	0.0000	0.00
0.05	291.6	7.7974	2.67
0.10	528.6	17.6153	3.33
0.20	1059.4	7.9875	0.75
0.30	1610.6	17.9527	1.11
0.40	2083.4	25.7740	1.23
0.50	2666.8	12.0291	0.45

The low % RSD values of < 5% that were obtained in precision studies indicate the repeatability of measurements made using this analytical method. It was however noted that as the concentration decreased, there was a decrease in the precision of the method, indicated by higher % RSD values. The resultant % RSD values were slightly higher than those that were obtained during the validation of the analytical method for dosage forms described in § 2.5.3.1 because of the absence of an internal standard in the analysis. In addition, lower concentrations of OT were used in the optimised method compared to the analytical method for dosage forms,

viz., 0 – 0.50 IU/ml compared to 0 – 12.0 IU/ml, respectively and therefore the precision obtained was higher.

2.9.2.2 Inter-day Precision (Intermediate Precision)

Intermediate precision was assessed as previously described in § 2.5.3.2, and was determined at three (3) levels, *viz.*, 0.15 IU/ml, 0.25 IU/ml and 0.35 IU/ml, in triplicate (n = 3) for three (3) days. The results of inter-day studies are summarised in Table 2.13. The method was found to be reproducible over several days as indicated by the low % RSD values that were obtained.

Table 2.13 *Inter-day precision for OT in the concentration range 0 – 0.50 IU/ml*

Concentration OT (IU/ml)	Day1		Day 2		Day 3	
	Peak height	% RSD (n = 3)	Peak height	% RSD (n = 3)	Peak height	% RSD (n = 3)
0.15	798.4	0.79	805.8	0.89	788.4	1.09
0.25	1327.8	0.64	1352.5	0.87	1308.8	0.98
0.35	1893.2	0.65	1907.4	0.52	1882.5	0.53

2.9.3 Accuracy

The accuracy of the method was determined as described previously in § 2,5.4 and this was determined at three (3) different levels, *viz.*, 0.15 IU/ml, 0.25 IU/ml and 0.35 IU/ml. Each sample was analyzed in triplicate (n = 3) and the results are summarised in Table 2.14, which shows a % Bias of less than 5% for the concentrations studied.

Table 2.14 *Accuracy for OT in the concentration range 0 – 0.50 UI/ml*

Concentration of OT (IU/ml)	Determined concentration (IU/ml)	% RSD	% Bias
0.15	0.1521	0.79	3.11
0.25	0.2614	0.64	0.15
0.35	0.3781	0.64	3.59

2.9.4 Limits of Quantitation and Detection

Different methods can be used to determine the LOQ and LOD as previously described in § 2.5.7 and in this case, the LOQ and LOD were determined using the signal to noise ratio method. The LOQ which gave a signal to noise ratio of 10:1 was determined to be 0.03 IU/ml with a % RSD = 6.01 (n = 6). The concentration that gave a signal to noise ratio of 3:1 was found to be 0.01 IU/ml with a % RSD = 10.89 (n = 6) and this was considered to be the LOD.

2.9.5 Range

The ICH [233] recommends that the range for dissolution testing must be ±20 percent over the specified range. The method was found to linear in the concentration range 0.03 – 0.5 IU/ml.

2.10 CONCLUSION

The successful application of analytical methods in the pharmaceutical sciences for different purposes including the quantitation of drugs in dosage forms for quality control purposes, determination of drug plasma for pharmacokinetic studies or generating *in vitro* release profiles, primarily depend on the use of an optimised and validated analytical method. The success of a separation in RP-HPLC is important in ensuring that the method is optimised for the detection of all relevant compounds.

Different analytical columns have different properties because of the variation in stationary phases and the careful selection of a column for an analytical procedure is important to ensure that the interaction between an analyte and a stationary phase is optimal. The use of silica columns with a C_{18} support backbone for the separation of molecules is well documented in the literature. For peptide separations, the hydrophobic interior of such stationary phases allows for the successful retention of these entities with acceptable retention times.

The use of ACN as an organic modifier is appropriate in comparison to the use of MeOH. With a polar molecule such as OT, the retention of OT decreased with an increase in ACN content and a concentration of 20% v/v ACN in a phosphate buffer was found to be optimal. Although the use of ion-pairing reagents to improve the separation in peptide mixtures has been advocated, this was not considered essential for OT, as the resultant chromatogram obtained without the use of these modifiers was acceptable. Buffered systems are important in the separation of ionisable and amphoteric molecules such as OT. The pH of a mobile phase affects the distribution between charged entities and therefore impacts on the interaction of the drug with both the mobile and stationary phases, thereby affecting the success/failure of the separation. A pH of 5 was found to result in the best-shaped peaks, with reasonable retention times. The molarity of the buffer affects the retention time of an analyte and molarity was optimised to produce sharp peaks for OT. The careful selection of an internal standard is advocated and resolution between the analyte of interest and the internal standard is important in order to allow accurate quantitation of the two compounds. MHB was selected as an appropriate internal standard thatg was well resolved from the OT peak.

The validation of a method is essential in the pharmaceutical sciences. The linearity of a method allows for the successful generation of a calibration curve to allow for accurate quantitation of a molecule of interest. The coefficient of determination obtained for this method (R^2 = 0.999)

ensured that interpolation of data within the range specified is reliable and accurate. The range used for a calibration curves must be relevant to the application of the method. For the determination of OT in dosage forms the most relevant range was found to be between 0.3 –12 IU/ml.

Much lower concentrations are expected when *in vitro* release tests are conducted and therefore the range was modified accordingly. The precision of the optimised method ranged between 0.45 – 3.33% RSD therefore indicating that the method is reproducible and that day-to-day variations that would affect the response of the detection of analyte are minimal. The accuracy was appropriate and ranged between 0.54 – 3.83% Bias and 0.15 – 3.11% Bias for the dosage form analysis and *in vitro* release methods, respectively. In early formulation studies it is critical to ensure that changes in formulations that are reflected in the *in vitro* release profile are depicted accurately, and therefore appropriate decisions with regard to optimisation of dosage forms are made, as and when is necessary or required.

The method that was optimised was sensitive, accurate, and precise. It was found to give a linear response in the desired concentration range. The method is therefore suitable for its intended use and therefore applicable in the analysis of OT from in pharmaceutical dosage forms and can be applied to the assessment of *in vitro* release of drug from hydrogel systems.

CHAPTER 3

PROTEIN AND PEPTIDE FORMULATION

3.1 INTRODUCTION

3.1.1 Advances in Protein and Peptide Therapeutics

The full sequencing of the human genome [237,238] has increased understanding and provided insight into the causes of genetic disease and possible drug therapies, including the use of endogenous proteins and peptides to cure or alleviate genetic disorders. High-throughput expression of recombinant proteins is required to ensure that proteins and peptides are produced rapidly and efficiently for therapeutic and research purposes [239].

The use of proteins and peptides in therapeutics is gaining recognition and acceptability, as these agents appear to offer therapeutic advantages compared to small conventional molecules. The selectivity, affinity, and specificity to target proteins and lower toxicity profiles of proteins and peptides compared to chemical entities, as well as the ability to produce effective and potent activity, are some of the major reasons for research advances in the development of protein and peptide drug delivery systems [240-242]. In addition, proteins and peptides have the ability to cure disease as they elicit biochemical changes *in vivo* by interacting with receptors and enzymes [242]. Bioactive peptides play an important role in the control of genetically linked diseases such as for example glucagon-like peptide-1 (GLP-1) which is important in the control of diabetes mellitus [243,244] and ghrelin, which is vital in the control of obesity [245,246]. Furthermore, the involvement of proteins and peptides in enzymatic catalysis, immuno-protection, control of growth and differentiation processes, enhances the applicability of proteins and peptides in therapeutics [242]. Despite advances in production and the potential utility of peptides and proteins in clinical therapeutics, there are many formulation challenges that must be overcome in order to ensure safe and effective delivery of these entities.

Initially, proteins used for therapeutic indications, including human insulin and human growth hormone were ‘simple replacement proteins’ with identical amino acid sequences to specific endogenous proteins and were administered clinically to mimic endogenous levels *in vivo* [247]. However, current trends in biotechnology indicate that modifications in protein sequences have been made in order to produce therapeutically superior molecules that are tailored to achieve

therapeutic goals. Modifications that have been made include mutations in primary sequences of the molecules, alteration of the molecule by covalent bonding to chemical groups or in the glycocomponent of proteins [247].

There are numerous protein and peptide agents currently available on the market for therapeutic indications and many others currently undergoing clinical trails [248]. There have been exciting developments in the approval of biopharmaceutical agents, including recombinant proteins, monoclonal antibodies, and nucleic acid-based drugs, with over 165 agents currently registered on the European and North American markets [248]. Of particular interest is the approval of the first non-parenteral protein formulations, *viz*., Exubera® (Nektar Therapeutics, San Carlos, CA, USA), an inhalable form of recombinant insulin and Fortical® (Fairfield, NJ, USA), an oral form of recombinant calcitonin [248]. Table 3.1 lists some of the protein therapeutic agents that have been approved for human use and indicates the manufacturer and therapeutic indication.

Table 3.1 *Some examples of proteins and peptide formulations registered [248]*

Product	**Company**	**Indication**
Advate® (octocog-α recombinant human factor VIII)	Baxter (Leverkusen, Germany)	Haemophilia A
Exubera® recombinant human insulin)	Pfizer (New York, NY, USA) and Aventis (Kent, UK)	Diabetes mellitus
Serostim® (recombinant human growth hormone)	Serono Laboratories (Geneva)	AIDS-associated catabolism and wasting
Viraferon® (recombinant interferon-α-2b)	Schering-Plough (Kenilworth, NJ, USA)	Hepatitis B, C
Procrit® (recombinant human erythropoietin)	Ortho Biotech (Bridgewater, NJ, USA)	Anaemia
Follistim® (follitropin-β, recombinant human follicle stimulating hormone)	NV Organon (West Orange, NJ, USA)	Infertility

Commercial production of proteins and peptides must be highly efficient and economical, producing purified macromolecules relatively quickly and in acceptable quantities. A variety of techniques may be used for production of proteins and peptides, each with their own advantages and disadvantages. High-throughput cloning is one means of protein and peptide production, where the gene for a therapeutic protein/peptide to be cloned is transferred to appropriate vectors to allow expression of the gene in that vector, resulting in the production of the protein of choice [239]. Expression systems for the production of recombinant proteins using for example *Escherichia coli*, are the simplest means of producing proteins. It is therefore used extensively for high-throughput production of therapeutic proteins and peptides [239,248], with nine (9) of the thirty-one (31) therapeutic proteins approved since 2003 being produced using this method [248]. Mammalian cell lines are also used for production of recombinant proteins

and although this method is technically complex and more expensive compared to the use of *Escherichia coli* in expression systems and seventeen (17) of the therapeutic proteins approved for use since 2003 are produced in this way [248]. Cell-free expression systems using cellular lysates containing the necessary biochemical components for transcription and translation of DNA templates *in vitro* [239] may also be used for production of therapeutic peptides and proteins. Synthetic procedures are more applicable to the production of smaller peptides such as OT as described in § 1.2.3.

3.1.2 Challenges in Protein and Peptide Delivery

3.1.2.1 Introduction

The success of protein and peptide formulations depends on the understanding by the formulation scientist of various manufacturing and process factors that affect the physico-chemical stability of proteins/peptides and the biological processes that affect the *in vivo* performance of these molecules [240]. The large molecular size, low permeation rates through biological membranes and surface activity of proteins are some of the primary causes of instability of proteins in formulations [242]. Important considerations are the physical and chemical instability, immunogenicity, and pharmacokinetic profiles of proteins and peptides. Production and manufacturing strategies to overcome the inherent instability of these entities *in vitro* and *in vivo* are necessary to ensure the success of protein and peptide formulation development and production and for the achievement of desired therapeutic outcomes.

Universal challenges in protein and peptide formulation include stability of the preparation and potential impact of the drug delivery system on protein conformation in formulation development, manufacturing, and the post-application processes [249]. Furthermore, the secondary and tertiary structures of proteins are important in eliciting biological responses and these must be conserved in pharmaceutical dosage forms, during the manufacturing processes, transportation, and long-term storage [250].

3.1.2.2 Physical and Chemical Instability of Proteins and Peptides

Physical instability of proteins and peptides refers to non-covalent modification of the molecule and involves changes in the secondary, tertiary, and quaternary structures of the molecule, including denaturation, adsorption to surfaces, aggregation, and precipitation [251]. Chemical instability of proteins involves processes where chemical modification by formation or destruction of covalent bonds occur, resulting in modified molecular entities being formed [251].

The sensitivity of protein structure and conformation to external conditions causes many formulation challenges, with respect to handling, formulation aspects, manufacturing, and storage issues. Conformational changes in protein structure may arise because of the aforementioned processes affecting protein and peptide stability *in vitro*, causing aggregation and ultimately precipitation and therefore impact negatively on the integrity and success of protein formulations [240,250,252,253].

The specific conformation of a protein is a result of protein folding, which is dominated by hydrophobic and electrostatic interactions, hydrogen bonding, intrinsic propensities, and van der Waal's forces [250]. Protein conformations are unstable and pharmaceutical processes are likely to impact on protein conformations. Such processes include shear forces, shaking, temperature, pH, and protein concentration [250,253]. The conformation of a protein is also dependent on the size of a protein and number of domains in the structure. Multi-domain large proteins are more susceptible to small changes in environmental conditions compared to their smaller molecular weight counterparts, resulting in aggregation [240].

Aggregation can be considered a physical process where protein monomers become coupled forming larger units, which may be insoluble and are often bio-inactive as they are unable to cross biological membranes to facilitate intracellular action or interact with membrane receptors [250,251]. Although aggregation is a predominantly physical process, it may also occur via covalent bond formation forming cross-links between protein molecules or altering the tendency to cause aggregation of proteins in solution [252]. Aggregation may result in formation of soluble or insoluble aggregates and the nature of the specific protein and conditions to which the protein is exposed will affect the type of aggregate formed. It is therefore important that the formulation scientist is aware of the specific conditions that cause aggregation for a particular protein, and whether they are prevalent during the formulation and manufacturing process and procedures [253]. The primary structure of a protein may play an important role in aggregation, with more hydrophobic proteins having a greater tendency to aggregate than their hydrophilic counterparts [254]. The secondary structure is also an important determinant of aggregation, where β-strands have a greater propensity to result in aggregation compared to α-helices, by forming coiled-coil contacts [255].

Shear induced by vortexing results in partitioning of proteins to the air/water interface, encouraging partial unfolding of the protein structure on exposure to air [256]. The use of non-

aqueous solvents [250] and the concentration of protein [257] may also promote aggregation of proteins in solution.

Other factors that affect the stability of protein formulations include drying processes [258], medium for reconstitution of freeze-dried formulations [259] and pharmaceutically relevant conditions, which mimic *in vivo* conditions to which the protein is exposed, such as temperature (37 °C) and humidity [260], as well as the storage conditions [261].

Proteins tend to be stable within specific temperature ranges and generally, protein stability decreases with increasing temperature either by denaturation or by catalysis of chemical reactions [253,262].

The pH of the solution to which the protein is exposed is another factor that alters protein conformation by affecting the aggregation behaviour of proteins, or catalysis of chemical reactions that may result in denaturation [253,262]. Most proteins tend to be stable within a narrow pH range and it is therefore imperative that in formulation development, protein stability is adequately monitored and/or controlled.

Proteins have the potential to adsorb onto surfaces, thereby reducing *in vitro* concentrations. The exposure of proteins to materials to which they can adsorb in formulation manufacture and to packaging and filters must be avoided as adsorption impacts negatively on biopharmaceutical formulation stability [250,251]. Salts, metal ions and chelating agents that may be used in formulations are also important considerations as they have the potential to affect the overall conformation and stability of protein molecules [263].

Proteins are susceptible to chemical instability that often results in degradation of the molecules by hydrolysis, deamidation, isomerisation or racemisation, oxidation, and formation or breakage of disulphide linkages, thereby resulting in the formation of degradation products [250,251]. Chemical instability does not always result in the biological inactivity of proteins, since the site of chemical change may not be crucial for the interaction of the molecule with the target receptor(s) and therefore a biological response may still be observed [264].

The use of appropriate analytical techniques is essential to monitor changes in protein stability. Capillary electrophoresis [265] and HPLC [266] have been used to monitor protein degradation, while differential scanning calorimetry (DSC) [267], fluorescence [268] and nuclear magnetic

resonance (NMR) [269] have been used to detect changes in protein folding. The aggregation behaviour of proteins has been monitored using light scattering mass spectroscopy [270].

Biopharmaceutical molecules such as proteins and peptides may be contaminated with biological substances such as viruses [240]. However, the inactivation of viral contaminants requires heat treatment at 60 °C, which causes denaturation of many biological proteins [253]. It is therefore imperative that protein formulations be protected against denaturation by controlling ionic strength and pH using buffer systems, or by addition of stabilisers such as Tween 20 [271] to limit aggregation.

The stability of protein formulations *in vitro* can be improved through modification of the protein, addition of pharmaceutical adjuvants and the control of environmental conditions [253]. To prevent protein folding, formation of disulphide bonds and salt bridges, mutations in regions involved in local folding, have been introduced to stabilise and cause rigidity in these regions, thereby stabilising proteins [251,272].

Additives such as salts of ionic compounds may be included in protein formulations to improve stability, as salts are able to bind to specific ionic sites and prevent denaturation of proteins in formulations [251]. Polyalcohol materials such sugars and glycerol, stabilise proteins and reduce denaturation through selective solvation of protein surfaces at low additive concentrations. Water particles surround the protein surface in order to exclude hydrophobic additives and prevent exposure of the hydrophobic interior of proteins to the additives, thereby stabilising a formulation [251].

Detergents and amphiphiles such as polysorbate 20 [273], Pluronic® F68 and Brij 35 [274] reduced the aggregation of proteins due to shear forces by competing with proteins at the air/water interface, thereby stabilising protein formulations. However, the effects of the detergents may not be adequate in preventing aggregation in long-term stationary storage of protein formulations [275]. Cyclodextrins have also been found to be effective in controlling protein aggregation in pharmaceutical preparations for pharmaceutically relevant proteins such as insulin [276,277]. Cyclodextrins prevent folding of proteins by binding to aromatic residues in the protein chain, stabilising the protein in an unfolded state and reducing the rate of protein folding [278]. Lyophilisation or freeze-drying of proteins and peptides removes water from the protein, leaving the protein in an unreactive anhydrous or almost anhydrous state [240] and increases the stability of protein and peptide formulations [279,280]. To improve the shelf life

of protein pharmaceuticals, a formulation must be at the right pH and ionic strength and a stabiliser (e.g. sugars, polyethylene glycol) must be selected to ensure an acceptable shelf-life is obtained [253].

3.1.2.3 Biological Instability of Proteins and Peptides

Proteins and peptides often have short half-lives *in vivo* because of degradation by protease enzymes in the systemic circulation, liver metabolism and rapid renal clearance especially for lower molecular weight (< 5 kDa) proteins [241]. In addition, proteins and peptides are unable to enter intracellular spaces, as they cannot penetrate lipophilic cell membranes, limiting their use in controlling intracellular processes *in vivo* [241].

A further complication with regards the use of therapeutic proteins, is their ability to induce immunogenic effects *in vivo* [240,281] by inducing T and B-lymphocyte activity resulting in antibody production and the consequent rapid elimination of therapeutically administered proteins and peptides. Factors that influence antibody formation *in vivo* include protein structure, immuno-modulatory effects of the protein, formulation composition, presence of contaminants and impurities, route, dose, frequency and duration of administration [281]. Prior to the advent of recombinant biotechnology allowing the use of human proteins in therapy, animal proteins were used which often resulted in activation of an immune response against therapeutic proteins thereby limiting *in vivo* use of the molecules [281]. Conversely, the use of recombinant human proteins has resulted in a reduced incidence and severity of immunogenic effects as patients are expected to be more tolerant of endogenous proteins as opposed to exogenous molecules. However, there have been reports of immunogenic effects and reduced clinical efficacy with long-term recombinant interferon therapy for multiple sclerosis [282,283] and pure red-cell aplasia following recombinant erythropoietin therapy [284]. The consequences of immunogenic effects in therapy include interference with the pharmacokinetic parameters of the molecule of interest and may result in a diminished therapeutic response and consequent therapeutic failure, or precipitation of an immune response, resulting in an attack on related endogenous proteins with a potential fatal outcome [281].

Strategies to improve the *in vivo* pharmacological and pharmacokinetic profiles of proteins and peptides have been proposed, and include PEGylation, acylation, or mutation of the amino acids of a primary sequence in a protein. However, it is important that biological efficacy be retained and that there is no induction of immunogenicity because of modifications made to proteins and/or peptides [240].

Chemical modification by covalent binding of proteins and/or peptides to polyethylene glycol (PEG), known as PEGylation [285-289] is a strategy to increase the circulating half-life of the molecule and to reduce the immunogenicity of proteins and/or peptides. The conjugation of proteins and peptides with PEG occurs by reacting functional groups such as amino, cysteine or carboxyl groups of proteins and peptides with modified PEG molecules. Either a single large molecular weight PEG molecule (30 – 40 kDa) or several smaller molecular weight entities (5 kDa) can be attached to the protein or peptide molecule [241]. PEG modification is advantageous and modifies protein and peptide pharmacokinetic profiles and biodistribution of the molecules *in vivo* [285]. The increment in size following conjugation results in lower renal clearance and a resultant increase in circulating half-life, reducing the need for frequent dosing of protein and peptide formulations that would otherwise be rapidly cleared [285]. Attachment of PEG molecules to proteins or peptides also masks charged and glycosylated sites on protein surfaces and shields antigenic and immunogenic epitopes of proteins, reducing immunogenic responses [290,291]. Furthermore, PEGylation results in reduced phagocytosis by the reticulo-endothelial system and liver cells and reduction of protein degradation [290]. However, the attachment of a PEG moiety to proteins and peptides may result in diminished biological responses *in vivo* as the PEG molecule may interfere with protein/peptide and receptor interaction, thereby warranting the need for extensive testing of several PEG-protein/peptide derivatives [241]. PEGylated protein products approved for human use include Pegasys (Hoffman-La Roche, Nutley, NJ, USA) and PegIntron (Schering-Plough (Kenilworth, NJ, USA), which are PEGylated interferon N-2α and PEGylated interferon N-2β, respectively.

Alternatively, N-terminal modification by acetylation or glycosylation or C-terminal amidation [292] may be performed in an attempt to improve the biopharmaceutical profile of proteins and peptides. The introduction of unnatural amino acids into primary protein or peptide sequences at vulnerable sites reduces proteolytic degradation of proteins and peptides, thereby potentially increasing the therapeutic efficacy of the molecules [290,292].

Acylation is the chemical attachment of fatty acids to exposed residues on protein surfaces and increases the affinity of proteins for serum albumin, thereby increasing protein binding, and prolonging circulation half-life of the molecule [293,294]. This has been successfully used to produce a long acting insulin analogue, insulin determir (Novo Nordisk, Bagsværd, Denmark) which is available on the market. Another strategy that can be used to increase the molecular weight of peptides is by genetic fusion of a protein to the Fc domain of human gamma immunoglobulin (IgG), thereby reducing the renal clearance of the protein [295].

The hydrophilic nature of peptides hinders penetration of these entities into cells and therefore one strategy to improve cellular permeability has been to develop cell-penetrating peptides [296,297]. Peptides are attached to synthetic molecules or naturally occurring cationic peptides or amino acid sequences containing basic amino acids that are proline rich and are able to cross lipophilic cell membranes, thereby shuttling the desired peptides into intracellular spaces [296,297].

An analogue approach has been proposed to enhance the bioavailability of insulin following subcutaneous administration [298,299]. Insulin exists as individual monomers in the human body, but in pharmaceutical dosage forms, insulin tends to associate into dimers and hexamers resulting in low penetration of the insulin through biological membranes because of the large molecular weight and size of the dimers and hexamers. These polymeric units must dissociate before absorption from the site of injection, causing a delay in absorption and ultimately, a delayed onset of biological action. Mutation of amino acid residues important in the self-association of insulin molecules results in a reduction in the tendency of insulin to self-associate promoting the prevalence of insulin monomers or dimers that are capable of penetrating biological membranes easily [300]. The onset of action is therefore accelerated and insulin lispro (Eli Lilly, Indianapolis, IN, USA) and insulin aspart (NovoRapid®) (Novo Nordisk, Bagsværd, Denmark) with rapid time-action profiles are available on the market.

3.2 MECHANISMS AND OBJECTIVES OF CONTROLLED DRUG DELIVERY IN PROTEIN FORMULATIONS

3.2.1 Introduction

Controlled drug delivery defines methods that can be used to modulate the release of a molecule from a dosage form in order to obtain a desired release pattern that is specific to an indication or for the clinical use of a molecule [301].

There are two types of controlled release that can be achieved *viz*., temporal and distribution control [302]. Briefly, the aim of temporal control systems is to deliver a drug molecule over an extended or for a specific period during treatment, into the systemic circulation. The benefits of temporal release are exemplified in Figure 3.1, which shows plasma drug concentrations following use of frequent injections, administered every six (6) hours compared to the use of a controlled release injection formulation that is only administered once.

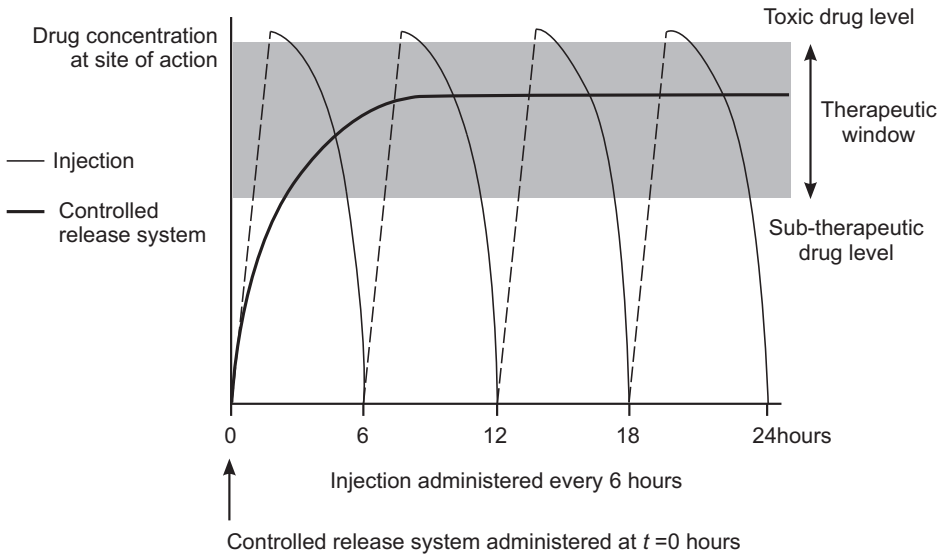


Figure 3.1 *The drug concentrations following the administration of multiple injections compared to a controlled release temporal formulation, (redrawn from [302])*

The therapeutic window of a drug defines the drug plasma concentration range in which therapeutic efficacy is achieved. Concentrations that are above and below the maximum and minimum therapeutic levels result in side effects and toxicity, or no therapeutic benefits, respectively [303]. The advantages of using temporal delivery systems are that plasma concentration levels are maintained within the therapeutic range and there are reduced incidences of toxicity or therapeutic failure [301].

Distribution control systems aim to localise drug action by restricting absorption of a therapeutic agent or drug into the general systemic circulation, with the primary advantage of decreasing adverse effects in systemic regions, whilst maintaining high drug concentrations at the site of action [302]. Distribution systems can be particularly useful for the administration of toxic drugs such as cytotoxic agents for cancer chemotherapy, thereby reducing systemic adverse effects. A diagrammatic illustration of a distribution control system, with plasma concentrations for both the systemic region and the localised site is depicted in Figure 3.2.

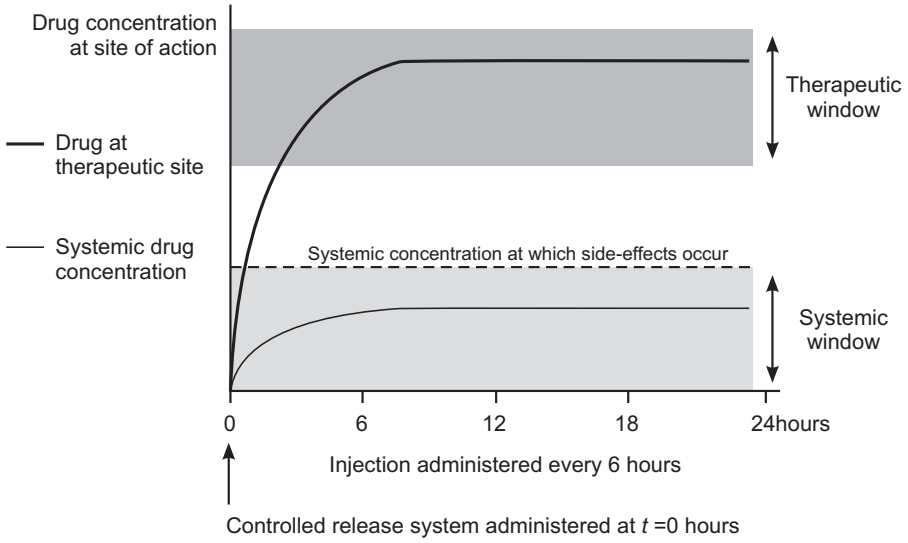


Figure 3.2 *Drug plasma concentrations from a distribution control system showing plasma concentrations in the systemic circulation and at the site of action, (redrawn from [302])*

The advantages of controlled release systems in drug delivery include improved efficacy and reduced toxicity as the level of drug/therapeutic molecule is maintained within the therapeutic range. Furthermore, improved patient compliance is likely due to reduced dosing frequencies and eventually culminates in a better chance of therapeutic success [302].

3.2.2 Objectives in Controlled Protein Delivery

The objective of current and innovative drug delivery developments is to deliver drugs that facilitate maximum therapeutic performance, decrease undesirable or untoward side effects, as well as increase patient compliance. Innovative drug delivery systems for proteins and peptides focus on drug delivery that is both controlled and safe, in order to maximise the therapeutic potential of biopharmaceutical molecules.

Therapeutic proteins and peptides often have short half-lives resulting in rapid elimination of a molecule from the body. In order to achieve therapeutic success, it is desirable to obtain continuous drug release over extended periods to maximise the therapeutic potential of the protein or peptide [249,304]. This is particularly important when using proteins that require frequent administration and where the use of a sustained release formulation would reduce the frequency of administration that is required to maintain the blood protein levels at therapeutic levels.

The use of controlled release systems for the delivery of proteins and peptides may also be beneficial where the molecule must be released in pulses as opposed to continuous release. An example of this requirement is in the treatment of diabetes, where the continual release of insulin from a delivery device would result in severe hypoglycaemic effects. Therefore, the development of an insulin delivery system that only releases the protein when required would be beneficial for the management of insulin-dependent diabetes. Furthermore, the inherent instability of proteins and peptides to enzymatic degradation and the possible production of antibodies *in vivo*, has been noted and therefore the formulation of proteins and peptides as distribution controlled delivery systems would be valuable to reduce the incidence of adverse effects [301].

3.3 ROUTES OF PROTEIN DELIVERY

3.3.1 Introduction

Different routes of administration for peptides and proteins have been described and reported in the literature. These vary according to the specific protein in a delivery system and the therapeutic indication of that protein. The desired therapeutic goal is important to consider in the formulation development stages, depending on the desired release rate, be it controlled, sustained, pulsatile, or immediate release. The required duration of action to achieve a therapeutic goal must also be considered in the formulation development process. Approaches to solving protein delivery issues include the use of oral, transdermal, nasal and ocular routes of delivery as well as the parenteral route.

3.3.2 Oral Delivery

3.3.2.1 Introduction

The oral route of drug delivery is the most favoured route for the administration of medicinal agents and is the most widely used method for drug delivery [240,242]. However, the primary challenge with respect to oral delivery of proteins and peptides is the inherent instability of these agents to digestive enzymes and pH in the gastro-intestinal tract (GIT) which result in protein degradation. Moreover, the low permeability of proteins and peptides across the gastric and intestinal mucosa limits the feasibility of the use of this route for protein delivery [242].

Barriers to the oral absorption of proteins and peptides may be described as physical, chemical and enzymatic [305]. The physical barriers to absorption of proteins and peptides include the large molecular size and hydrophilic nature of these macromolecules, which renders them unable to traverse aqueous channels and tight junctions between epithelial cells and cross lipophilic epithelial cell membranes in the GIT, respectively [305].

pH induced degradation in the GIT refers to acid induced degradation in the stomach, where larger protein and peptide molecules are broken down to their constituent amino acids and smaller peptide units such as dipeptides and tripeptides. In addition, the stability and conformation of proteins and peptides is dependent on environmental pH and changes in pH in the GIT from acidic pH values in the stomach to less acidic and more alkaline pH values in the lower GIT, results in changes in conformation and folding of proteins and aggregation. These factors reduce the therapeutic potential of orally administered proteins and peptides [305].

The GIT is rich in enzymes that degrade proteins and peptides by hydrolysis of peptide linkages or by chemical modification of the molecules by oxidation, reduction, or phosphorylation [305]. The small intestine contains the largest surface area for drug absorption, but also contains the largest number of hydrolytic enzymes secreted from the pancreas, mucosal cells and from the brush border of epithelial cells [305], to which proteins and peptides are susceptible.

Advances in formulation technology, stabilisation of proteins and peptides in the GIT and the possibility in increasing permeability across the gastric mucosa have resulted in a corresponding increase in the oral bioavailability of these molecules from as low as 1% to between 30 – 50% for some proteins [242].

However, it is important to note that technologies used in oral delivery systems must not affect the overall integrity of the intestinal lumen or other endogenous biological systems since this may result in serious interactions with other drugs that may be concomitantly administered [306].

Whilst the improvement in the bioavailability of therapeutic proteins and peptides may offer obvious advantages to improving therapeutic outcomes, an increase in the absorption of drugs may also have detrimental effects for agents with a narrow therapeutic range. Variability in absorption of drugs from the GIT may be a result of age or genomic factors and concurrent pathophysiological conditions that may compromise the structure of the intestinal mucosa. As a

result, increased absorption may cause high plasma levels of a therapeutic agent and result in fatal effects, such as for example high plasma levels of insulin resulting in excessive hypoglycaemia causing coma and death [306,307]. It is therefore important that the safety and efficacy of oral biopharmaceutical dosage forms, in addition to pharmacokinetic parameters of proteins, such as bioavailability and distribution be well established [306]. The effects of long-term administration of excipients such as permeation enhancers or enzyme inhibitors on the integrity of the GIT and systemic physiology must also be evaluated if any formulation is to be used chronically [307].

3.3.2.2 Chemical Modification

Absorption of a molecule across the intestinal mucosa is a function of the lipophilicity of that molecule. The degree of lipophilicity allows a molecule to partition into the lipid epithelial cell membranes and be transported into and through intracellular spaces [308]. However, the hydrophilic nature of the majority of proteins and peptides limits their GIT absorption and therefore some formulation strategies have focused on increasing the lipophilicity of protein and peptide molecules.

Lipidisation of molecules describes the covalent conjugation of a hydrophobic moiety/compound to proteins and peptides which results in an increase in the lipophilicity of the proteins and peptides [307]. An example of lipidisation is the synthesis of palmitoyl derivatives of insulin that have increased the absorption of insulin in the GIT as compared to unmodified insulin [309]. However, there is also a likelihood of reduced bioactivity of molecules that is associated with such chemical modifications and therefore, the synthesis of reversible modified molecules has been investigated. In such cases, lipidisation is reversed *in vivo* but results in improved epithelial absorption and stability [310].

PEGylation results in the increased stability of proteins because of protection of the molecules from proteolytic degradation and has been investigated for improving the bioavailability of orally administered insulin [311].

The addition of novel functional groups to protein and peptides molecules that allow for receptor recognition or use of a delivery carrier system may also be undertaken in an attempt to improve the biopharmaceutical profiles of orally delivered peptides and proteins [242,306]. The main purpose for the addition of novel functionalities to proteins and peptides is to improve absorption by manipulation of endogenous transport-carrier molecules recognised in the GIT

and therefore allow for the shuttling of therapeutic proteins and peptides across GIT mucosa. Examples of associated protein transport mechanisms include membrane transporters and receptor-mediated endocytosis that recognise and internalise their specific ligands attached to macromolecules, and therefore ferry them across the intestinal lumen [306].

Conjugation of insulin with transferrin increases insulin permeability across the GIT mucosa through receptor mediated transcytosis, which normally results in the absorption of transferrin [312,313]. In addition, it has been proposed that the conjugation of insulin with transferrin also protects insulin from enzymatic degradation in the GIT [313]. Transferrin has also been conjugated to recombinant granulocyte colony-stimulating factor, where efficacy of the conjugate was found to last for up to three (3) days as compared to only one (1) day, following subcutaneous administration of the macromolecule in its native form [314].

3.3.2.3 Formulation Strategies

Addition of protease inhibitors to protein formulations has been investigated with some success for improving the oral bioavailability of proteins and peptides, although the success of protease inhibitor use requires a full understanding of enzyme specificity for a particular protease inhibitor [315]. Examples of protease inhibitors include aprotinin for trypsin and chymotrypsin inhibition and amastatin, bestatin and boroleucine with inhibitory effects for aminopeptidases [315]. To improve the oral bioavailability of insulin, sodium glycocholate, camostat mesilate and bacitracin are effective in the large intestine, although it has been reported that the activity of these molecules was limited when used in the small intestine due to numerous enzymes present in that region of the GIT [316]. Soybean extract contains proteolytic enzyme inhibitors and has been used to improve insulin bioavailability [317]. *In vitro* studies have indicated that soybean extract reduces the degradation of insulin when exposed to simulated intestinal and gastric fluid, thereby making soybean extracts potentially useful in formulations for the oral administration of insulin and other proteins [317].

Absorption enhancers may also be used to improve the oral bioavailability of proteins and peptides from the GIT [305]. Absorption enhancers may function in different ways and include agents that disrupt the integrity of the epithelial mucosa by opening tight junctions, decrease mucus viscosity, and promote leakage of proteins through membranes or are able to shuttle proteins and peptides via specific membrane protein transporters [305]. Examples of permeation enhancers include chelators, surfactants, bile salts, fatty acids, and their derivatives [305].

In addition, practical approaches to enhancing peptide and protein bioavailability include the use of enteric coating for protection of biopharmaceutical molecules from pepsin digestion in the stomach. The coating prevents the release of a protein/peptide in the stomach, but allows for the rapid release of drug in duodenum thereby providing a higher concentration of therapeutic proteins and peptides at the epithelial surface and promoting faster absorption of the molecule into the systemic circulation [306]. Other strategies for absorption enhancement include a temporary decrease of the local pH in the small intestine, adhesion to mucous membranes allowing longer contact times with the mucosa, as well as colon targeting where there are minimal proteolytic enzymes [306]. In most cases, a combination of different approaches to oral delivery often results in the successful development of a formulation for oral use.

3.3.2.4 Particulate Delivery Systems

The primary advantage of particulate delivery systems is their ability to protect protein molecules against enzymatic degradation and to provide a pathway for the transfer of macromolecules across epithelial cell walls. Examples of particulate delivery systems include hydrogels, microparticles, nanoparticles and lipid based systems such as liposomes and solid lipid nanoparticles [306].

Solid lipid nanoparticles are prepared from solid lipids, have a mean photon-correlation spectroscopy diameter ranging between 50 – 1000 nm, and are enclosed in a surfactant layer [318]. Solid lipid nanoparticles may be likened to emulsions that contain oil droplets, but instead a solid lipid phase as opposed to a liquid is used in their manufacture [318]. The advantage of lipid-based particles is their inability to entrap hydrophilic proteins and peptides efficiently and therefore promote drug stability and rapid release [306]. Colloidal lipid nanoparticles coated with chitosan have been recommended for the oral delivery of peptides, using salmon calcitonin as a model drug [319]. Lipid nanoparticles improved the bioavailability of molecules by increasing permeability across the GIT mucosa and potentially increasing blood residence time through modification of the biodistribution of drug *in vivo*. A combination of lipid nanoparticles with chitosan, a bioadhesive polymer that also increases transmucosal absorption of compounds and the stability of nanoparticles in simulated gastric and intestinal contents, is likely to result in an increased bioavailability as reported for salmon calcitonin [319]. In addition, chitosan coated nanoparticles have been shown to increase the permeability in Caco-2 cell monolayers by reducing the transepithelial electric resistance of the cell layer. This effect results in sustained release of calcitonin following the administration of calcitonin

containing chitosan coated nanoparticles [319]. A similar technology using chitosan to coat liposomes was also used for an oral calcitonin delivery system [320].

Lectins are a structurally diverse class of proteins with the ability to bind carbohydrates and glycosylated sites in epithelial cell membranes of the GIT mucosa, facilitating drug transport across cellular membranes [321]. Lectin-modified solid lipid nanoparticles have been investigated as carriers for oral administration of insulin [321,322]. Lectin-modified solid lipid nanoparticles were able to protect insulin from degradation by digestive enzymes *in vitro* and *in vivo* results indicated an improved oral absorption of insulin from these delivery systems [322].

The formulation of insulin-transferrin conjugates with pH sensitive complexation hydrogels prepared from UV initiated free radical polymerisation of methacrylic acid (MAA) and methoxy-terminated poly(ethylene glycol) monomethacrylate (PEGMA) consisting of cross-linked polyMAA grafted with PEG chains was found to increase insulin-transferrin bioavailability [323]. The enhanced bioavailability of insulin is a result of an increased GIT residence time by adherence of the formulation to the GIT mucosa and inhibition of insulin degradation in the GIT [323]. pH sensitive complexation hydrogels may be considered suitable for oral delivery of proteins or peptides, as they are able to respond to changes in pH in the GIT thereby protecting biopharmaceutical agents from degradation. Reversible formation and dissociation of polymer chains in pH responsive systems is the principal mechanism by which drugs are protected in these systems. At acidic pH values, such as those that occur in the stomach, polymer chains interact strongly, entrapping proteins and peptides within the network, thus protecting them from acid induced degradation. However, at higher pH values (pH > 5.2) the polymer networks dissociate because of ionisation of the acidic functional groups, causing repulsion of the polymeric chains and subsequent release of the entrapped macromolecules [324]. Furthermore, complexation hydrogels are able to enhance the transport of macromolecules, including insulin [324,325], across the intestinal mucosa increasing their potential applicability as oral protein and peptide delivery systems.

Multiple water-in-oil-in-water (w/o/w) emulsions are more complex systems than simple oil-in-water (o/w) or water-in-oil (w/o) emulsions and in these systems, drops of the dispersed oil phase contain smaller dispersed water droplets that consist of the same phase as the continuous water phase [326]. These systems are intrinsically thermodynamically unstable systems and the inherent instability limits their use as drug delivery systems, which must be stabilised using appropriate combinations of surfactants. Cole *et al*. [326] reported using complexes of

poly(acrylic acid) and hydrophilic poloxamers, such as PF-127 in the aqueous phase in combination with lipophilic surfactants to prepare stable emulsions for oral protein delivery. The described method was used for the delivery of insulin and was shown to be suitable for either rapid onset or sustained action preparations depending on the type of complex formed between poly (acrylic acid) and PF-127 [326].

3.3.2.5 Site Specific Delivery in the GIT

The ileum is an attractive site for macromolecular absorption since it has the capability and mechanism that allows for the absorption of large molecular entities. The uptake systems include the ileal uptake pathway suitable for cobalamin absorption that uses a receptor mediated transcytosis mechanism, which allows the absorption of cobalamin bound to an intrinsic-factor, by the intrinsic-factor receptor [305]. Conjugation of peptides to cobalamin is a possible mechanism of increasing the oral absorption of peptides and proteins and has been reported to effectively bind conjugates to the intrinsic-factor receptor, thereby allowing absorption of peptide entities [327].

The colon has markedly reduced enzymatic activity as compared to other regions of the GIT, resulting in reduced degradation, in addition to a prolonged residence time, making the colon an attractive site for targeting protein and peptide absorption [305]. The drawbacks of the large intestine for targeted delivery are that it has a smaller surface area/volume ratio compared to the small intestine and has fluctuating pH that is dependent on the type of food that is consumed [305]. An example of colon specific delivery systems include the use of pH responsive systems that respond to varying pH values in the GIT, allowing for the release of a drug in the alkaline environment of the colon [324,328] as described in § 3.3.2.4.

3.3.3 Nasal Delivery

3.3.3.1 Introduction

The nasal route of delivery has been investigated as an alternative non-invasive route for the administration of proteins [329] as this route permits painless self-administration of drugs thereby potentially improving patient compliance and acceptability. Due to the impracticality and challenges associated with achieving success for oral protein delivery and the unpopularity of parenteral delivery due to the pain associated with injections, the nasal route is a potentially feasible and attractive avenue for protein delivery. The primary advantages of nasal delivery are that absorption can be highly efficient because of the large surface area for absorption, a thin

diffusion pathway to the blood stream exists, and there is less proteolytic activity in the nasal mucosa as compared to the GIT [330]. In addition, there is no hepatic first-pass metabolism as therapeutic agents are absorbed directly into the systemic circulation [331]. Furthermore, rapid absorption and onset of action that occurs following nasal delivery is comparable to that obtained following the intravenous administration of drugs, because of the rapid absorption and rich vasculature of the nasal tissues [332].

The main challenges to success of intranasal administration of proteins and peptides are the low bioavailability of these agents due to a low mucosal permeability because of the hydrophilic nature of proteins and peptides and enzymatic degradation in the nasal tract [333]. Additionally, the large molecular weight of proteins render delivery through this route a pharmaceutical challenge [334] and the efficient physiological elimination mechanism and nasal mucociliary clearance of proteins and peptides from the nasal tract further reduce bioavailability of agents delivered using the nasal route [335,336]. The volumes used in nasal delivery systems are limited to between 25 – 150 μl per nostril [332] and it is therefore important that in formulation development, dosage strength is considered, with particular regard to the volume of formulation to be administered. In addition, pharmacokinetic parameters including bioavailability from the nasal tract must be evaluated. Formulation developments in nasal delivery systems have therefore focused on a means to increase the bioavailability of proteins and peptides by a variety of techniques.

3.3.3.2 Formulation Strategies

Strategies to improve the bioavailability of peptides and proteins in the nasal cavity have included the use of a combination of a surfactant (laureth-25) and a mucolytic agent (N-acetyl-L-cysteine), which was used in a delivery system for salmon calcitonin [337]. The mucolytic agent decreases the viscosity of mucus in the nasal tract, allowing for rapid surfactant diffusion into the epithelial membrane, where it increases membrane fluidity and permeability to proteins and peptides [337]. The use of the mucolytic agent (N-acetyl-L-cysteine) in a powder formulation using ethylcellulose as a water insoluble inert excipient was reported to increase the nasal bioavailability of calcitonin in rats and dogs [337] and a faster absorption rate for the molecule was observed as compared to subcutaneously administered calcitonin. In addition, the combination did not result in irritation of the nasal mucosa therefore suggesting the potential utility of N-acetyl-L-cysteine in combination with ethylcellulose for protein and peptide delivery.

Absorption enhancers have been investigated as a means of improving the nasal bioavailability of drugs from nasal formulations. One such example is sodium glycocholate, a bile salt, which may act by interaction with membrane lipids and enzymatic inhibition and opening the tight junctions between epithelial cells, thereby increasing the transport of macromolecules across the epithelium [331]. The use of sodium glycocholate in combination with glycofurol resulted in increased nasal absorption of a model peptide, Peptide T in rabbits [331]. Additional absorption enhancers that have been investigated include chitosan [338,339], which may increase the permeation of proteins due to its bioadhesive effects, causing a reduction in mucociliary clearance of nasally administered proteins. In addition, chitosan is reported to have the ability to temporarily widen tight epithelial junctions *in vivo* [338,339]. Furthermore, cationic chitosan molecules are able to interact with the negatively charged mucosal surface and this interaction is enhanced by the use of large molecular weight chitosans, which bind to membranes for extended periods [339]. The use of dimethyl-*β*-cyclodextrin has been proposed as a permeation enhancer for protein/peptide powder formulations [340]. The use of absorption enhancers in nasal formulations has a major drawback of causing local irritation and toxicity that is undesirable in therapy and therefore absorption enhancers that are investigated for nasal use must be rigorously tested for *in vivo* toxicity and for short and long-term effects on the mucociliary system.

Sucrose cocoate, an emulsifier has been investigated as a means for improving the nasal bioavailability of insulin and calcitonin in rats [341] and it was reported that preparations of insulin and calcitonin containing 0.5% sucrose cocoate resulted in a rapid decline in plasma glucose and calcium levels, respectively due to the increased circulatory hormone levels.

Liquid and/or powder formulations may be used for nasal delivery and some studies [335,340,342] have shown that powder formulations are more stable and allow for better absorption and hence higher bioavailability compared to liquid dosage forms. This is primarily a result of the longer duration of contact of the formulation at the epithelial cell surface when powdered formulations are used compared to liquid dosage forms [335]. Dry powders for inhalation containing human parathyroid hormone were prepared by spray drying a blend of parathyroid hormone, lactose, and dipalmitoylphosphatidylcholine and were assessed for *in vivo* bioavailability and safety in rats [343]. A spinhaler device was used to administer the formulation and no acute inflammation was observed for up to 48 hours following a single dose administration, indicating the non-toxic or non-irritancy behaviour of the formulation [343].

Protein and peptide formulations for nasal delivery may also be formulated as jet or ultrasonic nebulisers, metered dose inhalers, and dry powder inhalers [344].

3.3.3.3 Particulate Delivery Systems

The use of colloidal carriers has been investigated as a mechanism for improving the permeability of proteins and peptides across nasal epithelial tissue cells [345]. The potential for use of chitosan as a colloidal carrier for nasal delivery has been investigated, whereby chitosan forms colloidal particles that entrap proteins and peptides and improve their permeation in the lungs [345].

Nanoparticles made of conjugates of poly(DL-lactic acid) with ethylenediamino or diethylenetriamino bridged bis(*β*-cyclodextrin) have been investigated for use in protein delivery systems [346], where bovine serum albumin (BSA) was encapsulated within nanoparticles and have an improved pharmacokinetic profile. Poly(lactic acid) poly(ethylene glycol) nanoparticles [347] and N-trimethyl chitosan nanoparticles [348] have also been investigated as protein and peptide delivery modules that show high loading efficiencies and low cytotoxicity.

3.3.4 Transdermal

3.3.4.1 Introduction

The transdermal route of administration has the potential for use as a non-invasive delivery route for therapeutic agents to the systemic circulation via the skin [349,350]. The primary advantages of this route of delivery, besides the non-invasiveness nature of the route, include a large surface area that is accessible for absorption and avoidance of GIT degradation and hepatic first-pass metabolism, which may lead to an increased bioavailability of therapeutic agents administered via this route [349,350]. However, the skin's outermost layer, the *stratum corneum*, is lipophilic and impenetrable to many therapeutic agents [349,350] and therefore limits the applicability of transdermal delivery for hydrophilic compounds such as proteins and peptides.

To improve the potential of this route of delivery for agents, including proteins and peptides several techniques that compromise skin structure to increase permeability but do not cause significant clinical damage to the skin, have been investigated [351]. Techniques such as iontophoresis [352], electroporation [353,354], laser treatment [355], and microneedles [351]

have been reported to improve the permeability of the skin to macromolecules such as proteins and peptides. Non-invasive methods offer a less painful way of drug delivery compared to the conventional parenteral route and therefore would have the advantage of better patient acceptability [351].

3.3.4.2 Transdermal Patches

Transdermal patches are one of the primary means of delivering molecules systemically via the skin, although their use is limited to low molecular weight (< 500 Da), highly lipophilic molecules that are able to permeate the *stratum corneum* and for potent drugs that are able to produce biological effects at low doses [350]. These factors affect the utility of transdermal patches for the delivery of peptides and proteins, which have larger molecular weights, and are usually hydrophilic in nature.

An interesting example of a transdermal protein and peptide delivery system is the Macroflux® transdermal patch that is able to deliver biopharmaceutical agents to a patient with minimal discomfort, in a controlled and reproducible manner [356,357]. Macroflux® transdermal patches have a titanium micro-projection array that is affixed to a polymeric adhesive platform. The total array may take up an area of up to 8 cm^2 and the density of micro-projections may be up to 300 micro-projections/cm^2, with each micro-projection being < 200 μm in length [356]. Each of the micro-projections is coated with drug and when the patch is applied to the skin, the micro-projections penetrate through the *stratum corneum* into the epidermis, from where the drug is absorbed into the systemic circulation. The advantage of this system is that it can produce a high rate of absorption promoted by high local concentrations of drug around the micro-projections and the large surface area provided by the array of micro-projections [356,357].

3.3.4.3 Iontophoresis

Iontophoresis refers to the application of a small constant electrical potential or field across the skin to allow for permeation of both charged and uncharged molecules across the skin into the systemic circulation [352]. The amount of drug carried across the skin barrier depends on the current, duration of application and surface area of skin exposed to the drug [352]. This method has been investigated for the delivery of insulin [358,359] and calcitonin [360].

The primary advantage of iontophoresis in protein and peptide delivery is the ability to control the kinetics of delivery of the therapeutic agent, by controlling rate of release into the skin. This approach permits the use of a personalised approach to achieving appropriate therapeutic

outcomes. The flexibility in drug delivery means that dosing can be altered as a function of deterioration or improvement of a condition. In cases in which bolus inputs are required following basal delivery, tight control of drug delivery and optimised therapy can be achieved using iontophoresis [352]. Additionally, iontophoresis allows for an immediate onset and rapid offset of action once the applied current is terminated and iontophoresis is therefore suitable for emergency purposes [352]. Moreover, iontophoresis allows for the individualisation of therapy depending on the specific drug to be administered and the desired therapeutic profile *in vivo*. For example, iontophoresis can be used to deliver an agent using pulsatile and/or continuous release or by mimicking the endogenous peptide/protein cycles [352].

Iontophoresis has been demonstrated to deliver insulin in animals [361], but in humans, it is more difficult to administer insulin in appropriate therapeutic doses. The transport of large molecular weight proteins such as insulin is a challenge when using iontophoresis as large proteins are unable to permeate through the intact *stratum corneum* [352]. Iontophoresis has been successfully applied to the administration of smaller proteins and peptides such as calcitonin [360] resulting in pharmacological activity *in vivo*.

Modifications to improve iontophoresis as a drug delivery technique include the use of poly(lactic-co-glycolic acid) (PLGA) nanospheres where the peptide/protein is encapsulated in nanospheres and then delivered across the skin using iontophoresis [362]. Triptorelin delivery was reported to have no burst effect in the release profile, allowing for sustained and predictable peptide delivery via this system.

3.3.4.4 Microneedles

The use of microneedles allows the creation of large transport pathways to permit the diffusion of molecules into the systemic circulation via the skin. The pathways that are created are larger than the dimensions of the molecule that is intended to be delivered and therefore this technique provides a possible alternate route for macromolecule delivery [351]. Studies to determine the utility of microneedles as a delivery system indicated an increased permeability of several orders of magnitude and the permeability of macromolecules such as insulin and BSA following use of microneedles was enhanced [363,364]. The use of microneedles therefore permits an alternate approach to the use of invasive parenteral delivery for proteins and peptides.

An attractive feature for the use of microneedles in therapy is the painless manner by which macromolecules may be delivered to the systemic circulation. Microneedles penetrate only to

between 10 – 20 μm into the *stratum corneum* that contains no nerve endings, therefore offering the premise of painless drug delivery [351]. The painless nature of microneedles delivery has been investigated in a clinical trial [365] where subjects reported no pain following the insertion of microneedles.

Microneedles can be either solid or hollow and each type has specific advantages. The use of solid microneedles allows different approaches to increase the efficiency of molecule delivery. The 'poke with patch' approach uses microneedles to produce openings in the skin structure to increase the permeability of a drug and subsequent application of a patch containing the drug results in an improved bioavailability. Iontophoresis can be applied to deliver the drug more efficiently, otherwise diffusion is the main mechanism by which drug passes into the skin [351]. In the 'coat and poke' approach, microneedles are coated with drug prior to insertion into the skin, thereby allowing drug to be delivered to the body during the insertion process, with no drug reservoir required to ensure delivery occurs [351]. Alternatively, the 'dip and scrape' method can be used, where microneedles are dipped into a drug solution and then scraped across the skin, leaving the drug-embedded in the microabrasions created by the microneedles [351]. Hollow microneedles permit the incorporation of drug into a hollow space within the needle, allowing for active fluid flow on application of microneedles, thereby permitting faster release rates compared to the use of solid microneedles [351].

Microneedles have been assessed for use as insulin delivery systems in hairless diabetic rats [366], where solid microneedles were injected into the skin using a high velocity injector. A solution of insulin was placed on top of the needles for a period of 4 hours, during which there was an observed steady decline in blood glucose levels in the rats. These results indicate the therapeutic potential of microneedles for insulin delivery and their ability to elicit a pharmacological response *in vivo*. Hollow microneedles have also been shown to be effective in reducing blood glucose levels following application into the skin of diabetic hairless rats [364].

3.3.5 Ocular Delivery

3.3.5.1 Introduction

The ocular route of delivery may be used for the systemic delivery of proteins and peptides, as this route allows for the rapid absorption of agents from the eye into the systemic circulation and permits the avoidance of hepatic first pass metabolism [332,367]. Instillation of formulations into the lower conjuctival sac can result in systemic absorption [368].

The bioavailability of macromolecules via this route is expected to be lower than that for smaller drug molecules because of their molecular dimensions, hydrophilicity, and susceptibility to peptidase degradation in different tissues in the eye. One of the key disadvantages to the use of the ocular route for protein and peptide administration is that large sized molecules are likely to result in itching in the eye and lachrymation, causing a reduction in the bioavailability of therapeutic agents, which are washed out of the eye by tears [332,367]. Furthermore, there is an increased potential for irritation of the eye and the possible need for frequent instillation or application of the ophthalmic preparations [332]. The dynamics of the lachrymal drainage system limits the utility of this route of delivery of agents intended for systemic absorption as it rapidly drains lachrymal fluids and any other instilled solutions from the pre-corneal area into the nasal cavity and throat. Drainage of eye fluid results in rapid elimination and reduced bioavailability of therapeutic agents administered via the ocular route [368]. Examples of macromolecules for which absorption through the eye into the systemic circulation has been observed include enkephalinamide [369], inulin [369,370], and insulin [371,372].

3.3.5.2 Formulation Strategies

The use of insulin solutions for ocular delivery has been investigated [373-375] but bioavailability of insulin was limited due to the rapid clearance of topically administered solutions from the eye. Failures in these formulations, indicates the need for use of permeation enhancers or alternate formulation optimisation strategies to increase absorption of ocularly administered proteins and peptides. The use of permeation enhancers is limited compared to the transdermal route because of the sensitivity of the eye to different chemical entities. An issue when using permeation enhancers for ocular delivery includes the potential toxicity to the ophthalmic tissues following repeated and long-term application of products [376].

Examples of agents that have been used include the sodium salts of bile acids such as taurodeoxycholate, taurocholate, glycocholate and deoxycholate salts which increase the permeability of insulin [372,377], showing the potential use of bile salts for application to the eye. Other examples of permeation enhancers include BL-9 [371,372], saponin [378,379], Brij 78 [380].

Sucrose cocoate has been investigated as a means to improve the ocular absorption of insulin [341] in rats, where it was observed that an insulin preparation containing 0.5% sucrose cocoate resulted in increased insulin plasma levels with a corresponding decline in plasma glucose levels. Tetradecylmaltoside, a long chain alkyl glycoside was found to promote calcitonin

absorption, albeit to a lesser extent than that observed following the nasal administration of calcitonin [381].

Ocular formulations may also be prepared as viscous solutions, suspensions, and using liposomes, in an attempt to increase the bioavailability of ocularly administered drugs [368]. Increased bioavailability would be expected with increased viscosity of formulations because of the longer contact time and reduced clearance of the formulation in and from the eye.

3.3.5.3 Particulate Delivery Systems and Intraocular Implants

Liposomal delivery systems have been evaluated for the ocular administration of insulin [371,382] with the primary intention of sustaining and controlling the rate and extent of insulin release. The *in vitro* results indicate a sustained release could be achieved with these formulations and *in vivo* studies in rabbits resulted in a decrease in plasma glucose levels [382]. The comparison of therapeutic effectiveness of liposomal formulations and solution of insulin solution with or without permeation enhancers showed that the liposomal formulation produced a prolonged glucose lowering effect *in vivo* as compared to the solutions [371].

The utility of an ocular insert for sustained release of insulin using Gelfoam®, which is an absorbable gelatine sponge, as the insulin carrier, was investigated for sustained release of the protein [383]. One of the advantages of this technology for ophthalmic administration of drugs is that when Gelfoam® absorbs the aqueous components of tears, it becomes soft and pliable and is comfortable in the eye [383]. Brij-78 was used as an absorption enhancer and the result indicated that the use of an insert resulted in a duration of action of approximately 10 hours as compared to 0.9 hours for an insulin solution when 1 mg of insulin was administered, indicating the potential usefulness of the device for the controlled delivery of the insulin [383]. Brij-78 as a permeation enhancer was found to improve the therapeutic efficacy of insulin using Gelfoam® devices and prolongs the duration of action of ocular inserts compared to insulin solutions [380]. Gelfoam® has aslo been found to be effective for insulin delivery when formulated with acetic acid in the absence of a surfactant, with the primary advantage of reducing eye irritation that may occur because of inclusion of surfactants in ophthalmic preparations [384-386].

3.3.6 Parenteral Delivery

3.3.6.1 Introduction

The parenteral route of drug delivery particularly via the intravenous, intramuscular, and subcutaneous routes, is currently the most accessible and viable means of protein delivery [367]. The major drawback of protein and peptide therapy administered in this manner is the rapid clearance of proteins and peptides from the systemic circulation as reported in § 3.1.2.1. Consequently, multiple injections that are often unacceptable to patients must be used to achieve the desired therapeutic outcomes. Strategies for parenteral formulation therefore focus on improving the pharmacokinetic parameters of proteins and peptides, thereby reducing the number of injections that must be administered to achieve the desired therapeutic outcomes [387]. Different dosage forms have been investigated for parenteral protein and peptides delivery with differing levels of success.

3.3.6.2 Emulsions

The use of water-in-oil (w/o) emulsions may result in pharmaceutical preparations with sustained release profiles for therapeutic agents, decreasing the need for frequent administration compared to conventional parenteral dosage forms [388]. In addition, increased stability of proteins and peptides is possible when using w/o emulsions since hydrophilic proteins and peptides are sequestered into the aqueous, dispersed phase and the oil phase protects the components of the aqueous phase from enzymatic degradation [388].

Oils and surfactants for use in emulsions for parenteral delivery systems must be non-toxic and should cause minimal muscle irritation or necrosis. Biocompatible emulsion systems include w/o emulsions prepared from a medium chain length triglyceride derived from fractionated coconut oil as the oil phase and polyglycerol polyricinoleate and sorbitan monooleate as surfactants located in the lipophilic phase [388]. Such systems have been reported to have a sustained release effect with an absorption half-life of up to at least 8 hours, with an associated high retention of the system at the intramuscular injection site. After 24 hours following injection of the preparation, 76 – 83% of the original amount in the dosage form was present at the site of injection, indicating the potential of w/o emulsions for use as sustained release delivery systems for proteins and peptides [389].

A w/o emulsion that has been investigated for use as a potential depot for the sustained release of a model polypeptide, aprotinin [390]. The system that was reported to be stable *in vitro* and

in vivo with resultant peak plasma concentrations occurring at 12 hours is comprised of fractionated coconut oil, triglycerol polyricinoleate-6, and Span 80 as the surfactants.

A challenge in the use of emulsion systems for the controlled delivery of proteins and peptides is the possible discrepancy between the resultant *in vitro* and *in vivo* data that are derived from experiments. Inconsistencies may be a consequence of an interaction between surface-active proteins such as albumin and emulsion systems or the *in vivo* degradation of lipid phases of emulsions by lipase enzymes, which alters the composition of emulsions resulting in an unpredictable release profile of drug from these systems [390]. Furthermore, emulsions used as parenteral formulations are limited by the lack of long-term stability and as a result, may fail in controlling drug release over extended periods of time [367]. It has been reported that protein formulations made from w/o emulsions may result in changes in the protein conformation placed in that formulation and that changes in the distribution of secondary structure elements, *viz.*, the α-helix and β-sheets in insulin and growth hormone for example [391].

3.3.6.3 Microparticles and Nanoparticles

The classification of microparticles and nanoparticles is based on their respective colloidal sizes. Nanoparticles may be defined as submicron (< 1 μm), ranging in size from 10 – 1000 nm. These colloidal systems are often, but not always made of polymeric materials [392,393]. Nanoparticles may be distinguished into nanocapsules, which are vesicular systems, and in which a drug is confined within a cavity surrounded by a membrane or nanospheres into which a drug is dispersed in a monolithic system comprised of a porous or solid polymeric matrix [392,393]. Microparticles may be defined as polymeric entities that are sized between 1 – 1000 μm [394] and may be separated into two (2) types, *viz.*, microcapsules that are micrometric reservoir systems or microspheres which are micrometric matrix systems [394].

Microspheres developed for parenteral delivery of proteins and peptides can be biodegradable or non-biodegradable although the use of non-biodegradable microspheres is limited by the toxicity of polymeric materials remaining in the body for long periods and the associated difficulty in removing implanted systems. Recent research has focused on the development of biodegradable microspheres that require no surgical intervention for removal. In addition, release rates from non-biodegradable microspheres are not constant and therefore difficult to predict [395] warranting extensive investigations into the use of biodegradable systems.

Microspheres for parenteral use have mainly been prepared from PLGA [396-400], although other materials such as monomethoxypoly(ethylene glycol)-b-poly(DL-lactide) copolymer (PELA) have also been used [401]. Nanoparticles that have been investigated for the sustained release of proteins and peptides have also been prepared from PLGA [402] with copolymerisation with poly(γ-glutamic acid) (γ-PGA) and L-phenylalanine ethylester (L-PAE) [403] amongst other polymeric systems.

Marketed examples of protein and peptide microsphere formulations include Lupron Depot®, a sustained release depot suspension of leuprolide acetate, Decapeptyl®, an injectable depot of triptorelin and Nutropin Depot®, a monthly subcutaneous preparation for administration of recombinant human growth hormone, which has subsequently been withdrawn from the market [404].

Major challenges in the use of microparticles for protein formulations include *in vitro* protein instability during encapsulation and instability *in vivo* during clinical use, resulting in unpredictable and unreliable release rates from formulations. Some studies have shown aggregation of the proteins during the encapsulation process for growth hormone [398], lysozyme [405], and ribonuclease [406]. *In vivo* instability was found to result in incomplete protein release for proteins such as insulin-like growth factor-I [399] and basic fibroblast growth factor [407]. As a result, encapsulation of the proteinaceous drug into microparticles must be carefully evaluated and optimised and the *in vivo* behaviour of the protein and peptide formulations prepared from such microspheres must be thoroughly investigated. The use of microspheres may also be disadvantageous in therapeutic applications since wide gauge needles are often required to administer the dosage form resulting in excessive pain at the injection site [408].

Another reported strategy for ensuring the sustained release of proteins includes the use of PLGA microspheres, which are porous when recombinant human growth hormone was loaded into the microspheres, but were made non-porous after protein loading resulting in the controlled and predictable release of the protein delivered using such systems [398]. The advantages of this drug delivery system include the method of preparation that allows for high encapsulation efficiency and high protein loading, without alteration to the protein structure.

PLGA nanospheres were reported to release BSA in a sustained release manner [402]. Factors important in the optimisation of nanoparticle delivery systems were reported to be the method of

preparation, length of the polymer used for the preparation of the microspheres and the presence of surfactants in the microsphere composition for modulating protein release. The nanospheres that were produced were reported to produce an initial burst effect, followed by a sustained release profile for the model protein [402].

Ovalbumin was successfully loaded onto or into nanoparticles prepared by copolymerisation of poly(γ-glutamic acid) (γ-PGA) with L-phenylalanine ethylester (L-PAE) by surface immobilisation and encapsulation [403]. The results of the copolymerisation indicated that the nanoparticles that were produced could be preserved by freeze-drying without adverse effects on the integrity of the delivery systems. Furthermore, cytotoxicity testing indicated that no relevant cell damage was caused by the nanoparticles and thereby indicating the potential utility of these systems as protein delivery modules [403].

Sucrose acetate isobutyrate has been evaluated as an additive in the preparation of microspheres [409] and was found to increase protein encapsulation efficiency and to retard the release rate of lysozyme due to a reduction in the degradation of PLGA microspheres. Surfactants have also been evaluated as a means of improving the stability of microspheres [397] and a comparison of poloxamer 188, polysorbate 20, and sorbitan monooleate 80 showed that the surfactants reduced the effective diameter and the shape of the microspheres and the encapsulation efficiency of proteins was reduced. The release rate of insulin increased in the presence of surfactants as compared to when no surfactants were added. In addition, there were no short or long-term effects on the conformation of insulin observed in the study. Polysorbate 20 at a 3% w/v concentration was found to be the most appropriate surfactant for the formulation of insulin microspheres [397]. The addition of zinc carbonate to microspheres prepared from PLGA [399] was reported to reduce the burst effect that is often observed when microsphere preparations are used to deliver proteins.

3.3.6.4 Liposomal Systems

Liposomes are self-assembled phospholipid vesicles with colloidal dimensions [410]. Liposomal systems and modifications thereof have been investigated as drug carrier systems for the parenteral delivery of proteins and peptides [410,411]. Liposomes are made from natural non-toxic, non-immunogenic lipid molecules that form vesicles and are able to entrap drug molecules in their aqueous interior or the membrane structure of the liposome [410,412]. The primary advantage of using liposomal delivery systems for the delivery of proteins and peptides

is that liposomes have an aqueous environment in which the encapsulated proteins can be sequestered and protected from degradation by proteolytic enzymes [412].

Liposomes are amphiphilic, possessing both polar and non-polar segments, and are self-aggregating forming different types of aggregates depending on temperature, molecular shape of the lipid segments and the conditions (concentration and ionic strength) of the aqueous/lipid environment to which they are exposed. The different types of aggregates that may be formed include micelles, inverse, prolate, inverse prolate and oblate micelles and unilamellar or multilamellar vesicles [410].

DepoFoam™ technology is comprised of multivesicular liposomes with multiple non-concentric aqueous chambers that are surrounded by a lipid membrane network [413,414]. Formulations of proteins prepared using the DepoFoam™ technology have high loading capacity and encapsulation efficiency and minimal chemical/conformational changes in the loaded macromolecules were observed. In addition, the liposomes that were formed were found to have narrow size distribution and were primarily spherical particles. The utility of the technology was assessed by *in vitro* and *in vivo* release testing of liposomes containing macromolecules such as insulin, leuprolide and enkephalin. The compounds were released over a sustained period, varying from a few days to several weeks and sustained *in vivo* therapeutic effects were observed in the test animals [414].

A combination of polymeric and liposomal systems has resulted in optimised delivery systems designed to overcome the shortcomings of liposomes and polymeric systems as delivery vehicles for proteins and peptides. Individually, liposomal and polymeric systems are often unstable and have biocompatibility issues respectively. The combination systems are referred to as carrier-microencapsulated liposomal systems, where drug-containing liposomes are encapsulated into microparticles [415]. Polymers that have been used for these purposes include dextran [416], and alginate [415,417] and the polymeric systems protect liposomes from degradation and increase stability as well as drug loading and encapsulation efficiencies by up to 95%, compared to the use of microcapsules or liposomes individually [415]. There was little chemical change in BSA by the formulation process used to prepare liposome-encapsulated microcapsules. Furthermore, the potential of sustained release was demonstrated by the prolonged release of BSA over a period of two (2) weeks [415]. A similar technology for protein delivery has been described and uses a liposome-containing horseradish peroxidase

encapsulated with fibrin and demonstrated an increased stability of the protein within the system [412].

3.3.6.5 Hydrogel Systems

Hydrogels are cross-linked hydrophilic polymers that form three-dimensional networks that are capable of absorbing large amounts of water or biological fluids [418]. Biocompatibility and limited toxicity, easy manipulation and solute permeability, are some of the key reasons for the application of hydrogel systems as drug delivery devices in controlled release technologies. Other advantages of hydrogels for drug delivery include the biodegradable nature of some hydrogels, high protein loading and encapsulation efficiency, easy scale up to produce large quantities of useful formulations, and their stability on storage [418]. In addition, hydrogels are easily injectable, with good drug diffusion properties, and may be designed to form *in situ* gelling systems for drug delivery. However, hydrogels are noted for their poor mechanical strength and difficulty in sterilisation and handling due to the fragile nature of some systems in addition to the difficulty in coordinating the release rates of drugs from hydrogel systems making it a challenge to produce reliable and predictable drug delivery systems [418].

Polymeric hydrogels have found application for the delivery of proteins and peptides especially for *in situ* forming depot systems, which have the advantages of ease of administration, are less invasive and painful in comparison to implants [419]. Localised or systemic drug delivery may be achieved and drug delivery may be tailored to specific therapeutic goals using such systems and generally, *in situ* forming hydrogels have relatively uncomplicated fabrication and manufacturing conditions and procedures [420].

In situ cross-linking of polymeric systems may be used for controlled diffusion of protein and peptide drugs, where the formation of cross-linked polymer systems is initiated *in vivo* following administration of the delivery system [420]. However, polymers for *in situ* cross-linking devices must have double bonds and require free radical initiation of the cross-linking process and are damaging to living tissue, hence limiting the applicability of this method for sustaining or controlling protein delivery [420].

Hydrogel materials can be designed to respond to the external environment and those materials that are able to respond in this manner are called environmentally or stimuli sensitive hydrogels and may be referred to as smart gels. The environmentally sensitive nature of the materials is

due to the presence of thermodynamically active functional groups on the polymer chains that result in volume changes of the polymer with changes in the external environment [418].

The use of responsive systems has relevance in biomedical and pharmaceutical applications, allowing for the development of drug delivery systems that can be tailored to release therapeutic agents with changes in the external environment. Environmental triggers that result in volumetric changes in responsive hydrogels include temperature, pH, pressure, electric fields, and glucose, amongst others [418,419]. Responsive systems can further be classified as externally regulated or self-regulated delivery systems, where externally regulated systems respond to external triggers for the pulsed delivery of drugs from a polymeric matrix such triggered by changes in magnetic fields, ultrasonic and/or thermal conditions. Conversely, self-regulated systems allow for negative feedback control of drug release from a polymeric system. Drug release occurs when therapeutic intervention is required and is halted when drug effects are not necessary and triggers for release include, for example pH, enzyme substrate reactions, glucose levels and competitive binding [421].

3.3.6.5.1 Thermo-Sensitive Hydrogel Systems

Thermo-responsive *in situ* forming hydrogels undergo changes in solubility with changes in the temperature of the environment to which the hydrogel is exposed [418,420]. The critical solution temperature of a thermo-responsive gel is the temperature at which the systems undergo a dramatic change in the solubility of the gel and exhibit such changes by swelling or shrinking. Thermo-sensitive hydrogels can be classified as positive or negative temperature-sensitive systems. Positive thermo-sensitive hydrogels have an upper critical solution temperature (UCST) above which the aqueous solubility of hydrogel increases and they undergo swelling, but below which the solubility of the gel is low and the hydrogel shrinks. Conversely, negative thermo-sensitive hydrogels have a lower critical solution temperature (LCST), above which the solubility decreases and they undergo shrinking with increases in temperature above the LCST [422,423]. Examples of thermo-responsive hydrogels include poly(N-isopropylacrylamide) (PNIPAAm), poly(N,N-diethylacrylamide) and poly(N-isopropylacrylamide-co-butylmethacrylate) the chemical structures of which are shown in Figure 3.3.

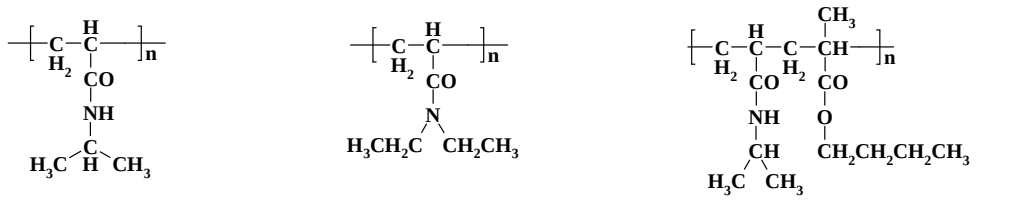
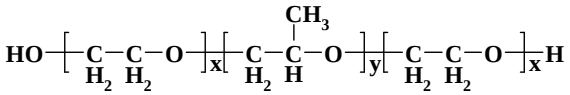


Figure 3.3 *Chemical structures of some temperature-sensitive polymers [424]*

Thermo-responsive gels are often characterised by the presence of many hydrophobic functional groups such as alkyl groups on the polymeric backbone [424]. PNIPAAm is the prototype thermo-responsive gels and has been the most extensively used and studied material. PNIPAAm has an LCST close to body temperatures and forms a stiff gel at approximately 32 °C, and therefore has potential to form depot delivery systems at body temperature. However, the biomedical use of this polymer for sustained release is limited due to the cytotoxicity and non-biodegradable nature of the material necessitating surgical removal of dosage forms if this gel is used [420]. In addition, cross-linking agents and monomers used in synthesis of PNIPAAm and its derivatives are not biocompatible and therefore they have limited human use [424].

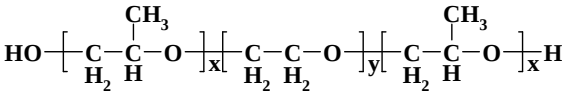
Copolymers are synthesised by the polymerisation of more than one type of monomer and block copolymers occur when the monomers used to synthesise the polymer occur as blocks of various lengths that are covalently bound together [425]. In a block copolymer, the blocks are arranged such that alternating hydrophobic and hydrophilic units occur in the backbone. Block copolymers made of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) possess reversible thermo-responsive behaviour, converting from sol to gel with increases in temperature [425,426]. PEO-PPO block copolymers have been investigated as drug delivery systems since high concentrations of PEO-PPO block copolymers exist as stiff gels at body temperatures, but are viscous fluids at low temperatures therefore having the potential for the formulation of depot systems for the sustained delivery of proteins or peptides. PEO-PPO block copolymers are commercially available as Pluronic® and Tetronic® [427]. Pluronic® copolymers have been approved by the Food and Drug Administration (FDA) for use in pharmaceutical products, including for parenteral dosage forms [428]. The molecular structures of these block copolymers are shown in Figure 3.4.

A



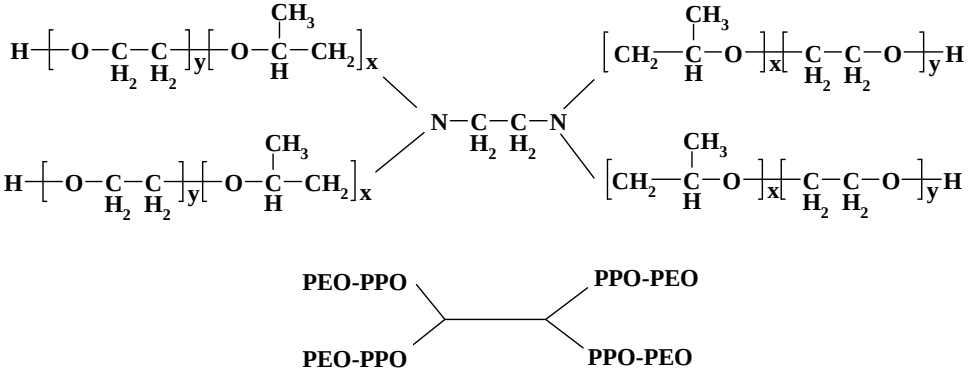
PEO-PPO-PEO

B



PPO-PEO-PPO

C



D

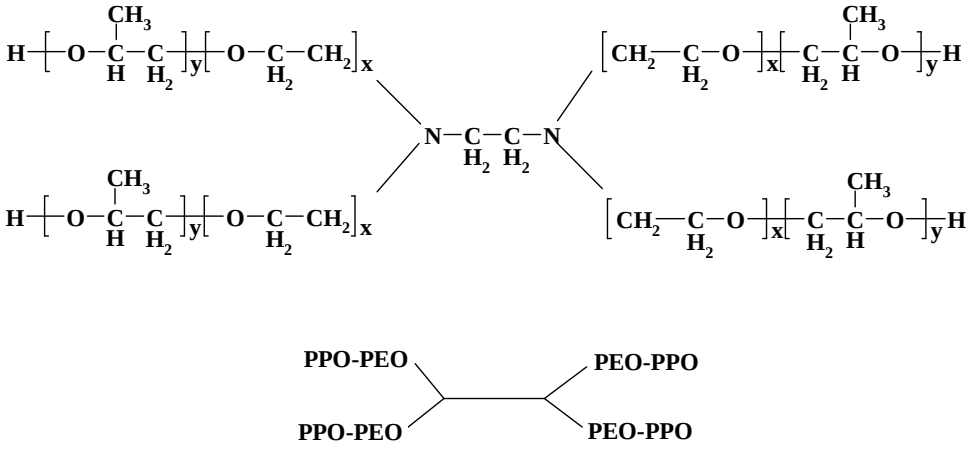


Figure 3.4 *Polymer structures of several block copolymers: A, Pluronic®, B, Pluronic® R, C, Tetronic® and D, Tetronic® R [424]*

ReGel® is an ABA triblock copolymer comprised of poly(lactide-co-glycolide) A blocks, and poly(ethylene glycol), B blocks [429,430] that have a range of gelation temperatures depending on the ratio of PLGA and PEG in the copolymer, the molecular weight of the copolymer and concentration of the copolymer in solution. At low temperatures, the copolymers exist as viscous solutions, but on injection, a stiff gel is formed which has the potential for depot formation and sustained release of drugs formulated in the gel [429,430]. No chemical reaction

is required for the formation of the gel and conversion of the gel to the sol state is achieved by reducing the temperature of the system to below the transition temperature of the gel polymer [429,430].

Polymeric materials with a LCST undergo a decline in aqueous solubility with increasing temperatures and possess moderately hydrophobic functional groups or contain a mixture of hydrophilic and hydrophobic functional groups. At low temperatures, hydrogen bonding between the hydrophilic portion of the molecule and water molecules dominates and the polymer is hydrated and swollen. However, hydrogen bonding decreases with increasing temperatures and hydrophobic interactions between the polymer chains become more dominant resulting in a decrease in the aqueous solubility of the gel. Inter-chain interactions result in the polymer becoming more compact and the material shrinks in size [424] and therefore forms a potential depot for sustained drug release. This swelling behaviour is known as inverse or negative temperature dependence [424].

If the polymeric chains in a hydrogel system are not covalently cross-linked, then sol to gel transitions may be observed with increases in temperature instead of the swelling-shrinking transitions as previously described. Block copolymers such as the Pluronics® and ReGel® undergo such temperature-induced transitions [424].

PNIPAAm hydrogels have been evaluated for use as protein carriers including for BSA and insulin as model proteins [431]. It was observed that the rate of release was dependent on the type and concentration of cross-linker used in the system, thereby indicating the necessity for careful evaluation of hydrogel systems for the controlled release of protein molecules. In addition, the size of the protein was also found to be important in determining the rate and extent of release from the hydrogel matrix, where insulin which has a smaller molecular weight as compared to BSA showed a faster rate and more complete extent of release [431].

ReGel® has been evaluated for the *in vitro* and *in vivo* release of insulin [432] and the results of these studies indicated that no burst effect occurred and pharmacological effects were observed for a period of 15 days after which approximately 90% of the insulin in the delivery system had been released.

Thermo-sensitive biodegradable hydrogels prepared by the polymerisation of Pluronic® triblock copolymers and ε-caprolactone were investigated as a sustained release carrier for insulin and

BSA [433]. It was reported that the resultant *in vitro* release rates were related to the molecular weight of the protein, with larger proteins being released at slower rates than the smaller sized proteins. From these studies, it was concluded that the hydrogel system investigated was suitable for use as a potential vehicle for the delivery of proteins and peptides.

3.3.6.5.2 pH Sensitive Hydrogel Systems

pH responsive hydrogels possess a large number of acidic (e.g. carboxylic and sulphonic acids) or basic functional groups (e.g. ammonium salts) that can accept or release hydrogen ions and are known as polyelectrolytes. Alteration of the pH of the environment in which the hydrogel is dispersed results in a change in the state of ionisation of the hydrogel resulting in the material either swelling or shrinking [328,424]. Swelling of pH responsive gels occurs because of ionisation of acidic or basic groups and as the degree of ionisation increases, there is an increase in the repulsion of the polymer chains resulting in swelling of the hydrogels [434,435]. Hydrogels with acidic functional groups tend to swell at pH values above the pK_a of the polymer and basic polyelectrolytes swell at pH values below their pK_b values [423]. Examples of pH-responsive polymers include poly(acrylic acid) and poly(*N,N'*-diethylaminoethyl methacrylate) and their molecular structure is shown in Figure 3.5, in addition to the ionisation reactions of the materials in acidic and basic media.

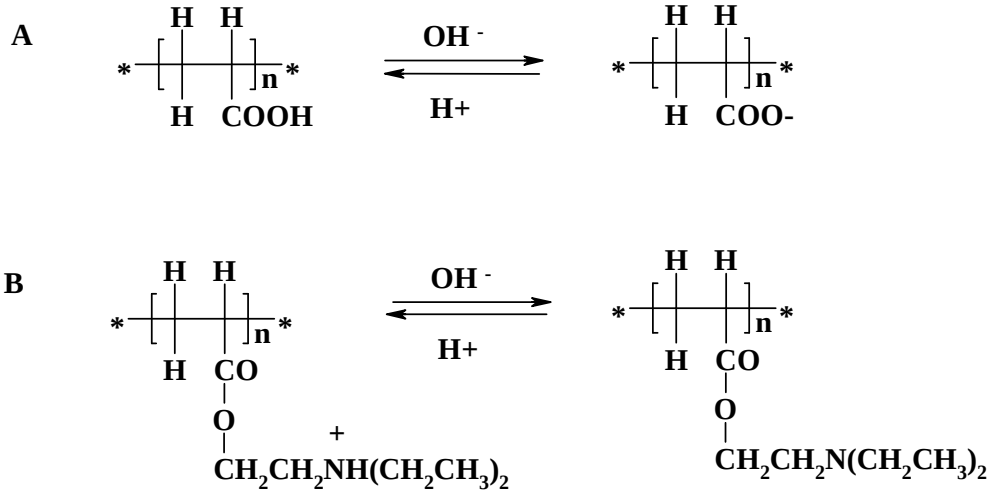


Figure 3.5 *pH responsive ionisation of polyelectrolyte polymeric systems, A, poly (acrylic acid) B, poly (N, N'-diethylaminoethyl methacrylate [424]*

The ionisation of polyelectrolytes is more complex in comparison to that of monoacids or monobases as electrostatic forces exist between adjacent similarly charged functional groups

[424]. The use of pH responsive systems is often applied to oral delivery systems where the delivery system is exposed to a wide pH range following administration.

3.3.6.6 Sterilisation

Parenteral formulations must be sterilised prior to their use *in vivo* and sterilisation techniques that may be used for such purposes include membrane filtration, gamma irradiation, autoclaving, ethylene oxide sterilisation and high hydrostatic pressure sterilisation [436]. The selection of the most appropriate means of sterilisation for each of the different parenteral dosage forms developed is an important consideration during development studies in order to ensure that the integrity of a formulation is not compromised.

Membrane filtration is a technique that physically removes contaminants from a pharmaceutical preparation by passing the formulation through a membrane [436]. Filtration has an advantage that it does not cause denaturation or alteration in the physicochemical and conformational properties of proteins and peptides in dosage forms. However, the use of this sterilisation method is limited when the dosage form is particulate in nature such as microparticles and nanoparticles > 200 nm in size and which will be retained on a filter [436]. Furthermore, proteins are surface active and can potentially be adsorbed onto membrane filter media resulting in a reduction in the effective concentration of the protein in solution [250,251].

Autoclaving at high temperatures (120 °C) is a very effective method of sterilisation of parenteral products [436]. However, high temperatures may cause degradation of proteins and may result in the instability of polymeric materials or other excipients used in formulation and preparation of dosage forms and it is thus important that the stability of API and excipients be investigated in autoclave studies conducted at relevant temperatures.

Gamma irradiation is often used for the sterilisation of parenteral products with the advantage of a high penetration power and the isothermal nature of the process that allows for the sterilisation of heat sensitive materials such as proteins, without denaturation but with effective elimination of microorganisms [436]. In addition, gamma irradiation allows for the homogenous sterilisation of preparations that may also be sterilised in their packaging thereby preventing further contamination prior to use [436]. Despite the advantages of gamma irradiation as a sterilisation procedure it has been found to cause degradation of irradiated proteins by the formation of free radicals [437,438] and may compromise the integrity of excipients by initiating free radical induced chemical reactions in polymeric materials. To stabilise proteins

from degradation by ionising radiation, additives such as *o*-vanillin have been reported to prevent radiation-induced degradation of proteins such as porcine somatotropin [437].

An alternative method for the sterilisation of biopharmaceutical products is the use of supercritical carbon dioxide, which has the advantages of being non-reactive and is able to penetrate substances readily whilst maintaining protein conformations in pharmaceutical preparations. This method may also be used to sterilise protein/peptide formulations in their final packaging [439]. Furthermore, the application of supercritical carbon dioxide sterilisation rapidly inactivates bacterial endospores making this method both appealing and applicable to the sterilisation of parenteral biopharmaceutical products [439].

3.3.6.7 Syringeability and Injectability

Syringeability refers to the characteristics of a parenteral formulation during the withdrawal of the formulation from its packaging and includes characteristics such as foaming potential, ease of withdrawal, and accuracy of dose measurement [367]. Injectability refers to the properties of a parenteral formulation during the actual administration of that formulation to a patient and includes the force required for the injection, qualities of aspiration, clogging and the nature of product fluid flow [367]. Parenteral delivery systems must be both syringeable and injectable to allow for the simple and reproducible administration of a drug product.

3.3.6.8 Packaging

Biopharmaceutical parenteral preparations are commonly presented as single-dose vials containing either a solution or a lyophilised cake intended for reconstitution prior to administration of the product [440].

The importance of an appropriate packaging configuration for biopharmaceutical products is obvious since the packaging must ensure that formulations are maintained in a stable state, protected from light, chemical contaminants and other potential sources of instability that may ultimately compromise the integrity of the formulation. The compatibility between a biopharmaceutical product and packaging must be established as packaging may introduce contaminants into formulations [440].

Packaging materials for pharmaceutical products may contain metal ions, plasticisers, and chemicals that may leach into a product contained therein. The conformation of proteins is important for biological safety and efficacy and the presence of these materials may result in

chemical and/or physical instability resulting in aggregation of proteins as discussed in § 3.1.2.2. In addition, low concentrations of proteins and peptides are often in dosage forms and therefore, any chemical reactivity between the packaging and the biopharmaceutical product would result in a decrease in the effective concentration of the therapeutic agent in a product and therapeutic failure if that batch is used [440]. Moreover, proteins and peptides have a tendency to adsorb onto the surfaces of packaging materials, thereby further reducing the effective concentration of the proteins in the biopharmaceutical preparation [440]. Lyophilised proteins are also susceptible to instability reactions because of inappropriate packaging conditions and in cases, for example where a seal does not fit tightly to the primary package, moisture may enter the formulation resulting in deactivation of the protein.

Silicone oil is a commonly lubricant for stoppers for parenteral vials, but often causes nucleation of proteins around the oil droplets resulting in deactivation of protein and peptide molecules [440]. It is therefore imperative that the design of biopharmaceutical dosage forms include compatibility assessments with potential packaging materials and these investigations must not be neglected and should be investigated over an extended period of time in order ensure the delivery of safe and effective protein and peptide formulations.

3.4 CONCLUSION

The successful formulation of proteins and peptides into sustained release dosage forms has the potential advantage of improving therapeutic outcomes for patients with less frequent painless administration of these agents. The importance of understanding the physicochemical and biopharmaceutical properties of proteins must not be neglected. These properties and factors have been discussed in Chapter 1, *vide infra*, for OT. Knowledge of the physicochemical stability of OT is important in formulation development to ensure that the molecule is not exposed to any factors that would otherwise result in degradation or aggregation of the protein. OT is unstable at pH extremes as described in § 1.3.2, exhibiting a maximum stability at a pH of between 3 and 5. In any formulation process, OT must not be exposed to any environment with pH values outside of this range, as this would result in degradation of OT. Furthermore, the final formulation developed for OT must also be buffered to within this pH range to ensure maximal stability of the molecule.

The biopharmaceutical parameters of OT are important in designing an appropriate dosage form for the peptide, whilst ensuring that OT is delivered in a safe and effective manner.

Considerations about current clinical administration guidelines are important to ensure that the right dose of OT is delivered at the correct rate and these considerations have been discussed in § 1.4.6.

A review of literature regarding the current trends and technologies in delivery of protein and peptide macromolecules was critical in considering alternate routes of delivery and dosage forms for OT. The selection of an appropriate dosage form was based on the suitability of that method for OT delivery, ease of administration, and comfort for the patient, ease of manufacture and/or preparation of the dosage form. Ensuring that OT was retained in a stable form was a priority in the decision making process. Other important factors that were considered were the expense of the manufacturing process and assessment methods for the delivery system. These factors were deemed vital in the decision-making process, since medicines must be affordable and novel delivery systems must offer distinct advantages over currently available therapies. Each of the different routes of protein delivery discussed herein was considered as a potential method of delivery for OT.

The oral route of administration would be the most ideal for administration of OT for the prevention of post-partum haemorrhage. However, OT is unstable and undergoes extensive degradation when exposed to gastric pH and enzymes in the GIT as documented in § 1.5.1 and therefore administration of OT in its native form, would be of no therapeutic use. Different strategies to improve OT stability were considered and one possible alternative was the modification of the hydrophilic peptide by conjugation with lipophilic groups, via an acylation reaction. Chemically, this is a relatively straightforward reaction, but modification of the molecule requires full identity characterisation, percent yield determinations and the exact positions of modification, which requires expensive instrumentation and can be labour, resource, and time intensive. Moreover, chemical modification may result in diminished biological efficacy of the molecule as modification may occur at a position that is necessary for molecule-receptor interaction and biological activity.

Similarly, although modification with PEG offers distinct advantages to the pharmacokinetic stability of proteins and peptides following both parenteral and oral delivery as described in § 3.1.2.3 and § 3.3.2.2, respectively, the challenges highlighted with the acylation of OT made this non-viable for OT formulation development. In addition, the attachment of PEG requires the reaction of PEG derivatives with reactive groups such as amino, sulphydryl and carboxyl groups on the molecule. The chemical structure of OT shown in Figure 1.2 reveals that the

peptide does not have a free carboxyl or sulphydryl group and that it has only a free amino terminal group that may be modified. However, the amino terminal functionality is from a cysteine residue that forms a disulphide bridge and therefore there is a likelihood of steric hindrance occurring during reactions with PEG derivatives and this may alter the conformation of OT and render it inactive.

Site-directed delivery of OT into the colon using complexation hydrogels was considered, as metabolism of OT in this region of the GIT is not as extensive as that in the stomach and small intestine as discussed in § 1.5.1. However, the method of manufacture for complexation hydrogels is complex, diminishing the appeal of attempting to deliver OT via this route of administration.

Transdermal delivery of OT was also considered and the use of a patch, such as Macroflux® described in § 3.3.4.2 was considered an attractive option and would be easily applied in the third stage of labour. However, the complication of optimising and characterising the *in vitro* and *in vivo* release of OT from these systems was a challenge. Microneedles were also disregarded for the same reasons and the use of iontophoresis for OT delivery for prevention of post partum haemorrhage was found to be impractical for this particular indication.

Nasal delivery is a currently used technique for OT administration for promotion of lactation and the development of OT using this route of delivery has the advantage that pharmacokinetic parameters of OT using this route are well established. The use of a nasal solution of OT would be limited by the resultant short duration of action of the molecule, although modification by using colloidal carriers and bioadhesive polymers may overcome this limitation. However, the production of these systems requires a full characterisation of the particles used in the delivery system and therefore was not considered for OT delivery.

Ocular delivery of OT has been demonstrated demonstrated [441], but was not considered as a practical alternative for OT administration due to the discomfort expected from instilling eye drops after labour and possible reduced efficacy because of lachrymation.

The parenteral route was therefore considered the most feasible means of OT delivery. Nanoparticles and microparticles offer the advantage of extended release preparations that are capable of releasing drug for periods ranging from days to weeks. However, such a long duration of action was not desirable for use in the prevention of post partum haemorrhage using

OT. Liposomal systems offer an aqueous phase for the encapsulation of hydrophilic proteins and peptides that may reduce enzymatic degradation, but the inherent instability of liposomes, limits their potential usefulness in protein delivery.

Hydrogels were considered as a possible carrier system for the administration of OT, with the primary advantage of being biocompatible. Although the low mechanical strength of most hydrogels may limit the duration and extent of release, this was considered as a positive factor as this would allow development of a dosage form that would release the drug over a relatively short period. Thermo-sensitive hydrogels were regarded as the best potential drug delivery matrices for OT as they permit the administration of a cold preparation via a needle, but forms a potential depot for sustained release *in vivo*. NPIAAm was considered for this application, although the usefulness is limited by the known cytotoxicity of the polymer.

The Pluronic® block copolymers were evaluated as a potential delivery depot for OT. Some solutions of Pluronic® block copolymers are able to form gels as described and may be used for the sustained delivery of proteins and peptides. Furthermore, the FDA has approved Pluronic® block copolymers for parenteral use and therefore toxicity data has been evaluated making these polymers potentially useful for sustained drug delivery. Pluronic® block copolymers are also commercially available and therefore, little composition characterisation is required, as the copolymers must conform to international manufacturing standards. Pluronic® block copolymers were therefore characterised according to ultraviolet and infrared spectroscopy, critical micelle concentration, gelation temperatures and viscosity among other properties and these studies are fully described in Chapter 4, *vide infra*.

CHAPTER 4

THE CHARACTERISATION OF PLURONIC® F127 AS A POTENTIAL VEHICLE FOR THE PARENTERAL DELIVERY OF OXYTOCIN

4.1 INTRODUCTION

The characterisation of Pluronic® F127 (PF-127) as a potential vehicle for the parenteral delivery of OT was performed as part of preliminary studies in the development of a delivery system for the peptide and to assess whether OT release from the dosage form was predictable. Information from characterisation studies would be valuable for predicting the behaviour of the vehicle during the manufacturing process, following storage as well as during administration of the dosage form *in vivo*. The impact of these processes on the integrity of the dosage form can be predicted from the data generated during preliminary studies. In addition, the polydisperse nature, possibility of homopolymer and diblock polymer impurities, as well as batch-to-batch variability characteristic of polymeric materials such as PF-127 warrants particular investigation of the batch to be used in the formulation of a dosage form [442,443].

4.2 PLURONIC® COPOLYMERS

4.2.1 Introduction

The non-proprietary name for the Pluronic® group of copolymers is poloxamer. They are also known commercially as Lutrol®, Monolan®, Supronic®, Synperonic® or as poloxalkol, poly(ethylene-propylene) glycol copolymers and poly(oxyethylene)-poly(oxypropylene) copolymers [428]. They are a series of commercially available high molecular weight synthetic block copolymers of ethylene oxide and propylene oxide [10,11,426]. The general formula for the poloxamer block copolymers is depicted in Figure 4.1.

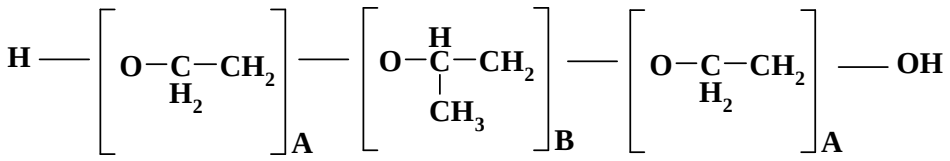


Figure 4.1 *The general formula for Pluronic® copolymers*

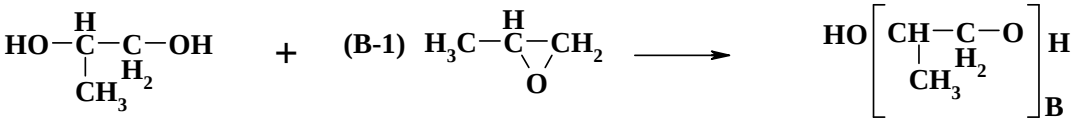
They are known as ABA block copolymers where the poly(propylene oxide) (PPO) block (B) is sandwiched between two poly(ethylene oxide) (PEO) blocks (A). The PPO block is hydrophobic and the PEO block is hydrophilic, thereby creating amphililic poloxamers that possess surfactant properties and that are self-associating in melts and solutions [425].

Variations in the lengths and proportions of the PPO and PEO units generate a wide variety of poloxamers with different physicochemical properties and applications [425-427]. They are used primarily in pharmaceutical applications as solubilising or emulsifying agents for parenteral preparations of poorly soluble drugs and in the formation of fat emulsions. In syrups and elixirs, they are used for clarifying purposes and in suppositories and ointments; they may be used as wetting agents [428]. Poloxamers have been investigated as micro-containers for drugs where a drug is solubilised in micelles [444,445] and can increase the penetration of drugs through tissues such as the blood brain barrier [446]. In addition, they have been shown to potentiate the action of anticancer agents in cancerous cells [447]. There has also been a long-standing interest in the use of poloxamers in the development of sustained release formulations for parenteral, topical, and oral delivery of drugs [448-454].

4.2.2 Synthesis

The synthesis of poloxamer block copolymers is carried out under conditions of elevated temperature and pressure, with the use of a basic catalyst such as sodium or potassium hydroxide. A summary of the synthetic procedure for the production of these polymers is shown in Figure 4.2. Propylene oxide is slowly added to propylene glycol to produce a poly(oxypropylene glycol) derivative of the desired weight, depending on the specific poloxamer copolymer block required (Step 1). This produces the middle block of the copolymer (B as shown in Figure 4.1). Ethylene oxide is then added slowly to the polymer until the final molecular weight that is required, is obtained (Step 2). Finally, the catalyst is neutralised by the addition of phosphoric acid to produce a final pH of approximately 7 [426].

Step 1



Step 2

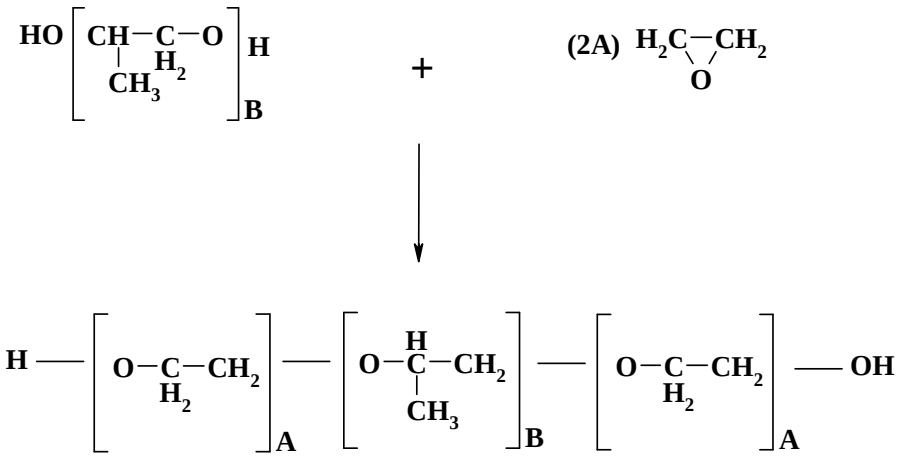


Figure 4.2 *The synthesis of Pluronic® copolymers [426]*

4.2.3 The Pluronic® Grid

Different poloxamers of differing molecular weight and proportions of PEO and PPO can be produced using the synthetic procedure described Figure 4.2. As a result, polymers of different properties with regard to their physical state and nature at room temperatures and pressures are produced. This has resulted in the development of the Pluronic® grid, which characterises the different poloxamers with respect to their molecular weight and the ratio of PEO to PPO [425,426]. The Pluronic® grid is summarised in Figure 4.3.

Copolymers shown in the Pluronic® grid that occur along vertical lines have the same PEO: PPO ratio and those shown along the same horizontal lines have the same PPO block lengths. The naming of the poloxamer polymeric materials is based on the Pluronic® grid. The use of a letter denotes the physical state of the polymer, *viz.*, L for liquid, P for paste and F for flakes. The initial one (1) or two (2) digits indicate the molecular weight of the hydrophobic PPO block and the last digit denotes the proportion of PEO multiplied by 0.1 [425,426].

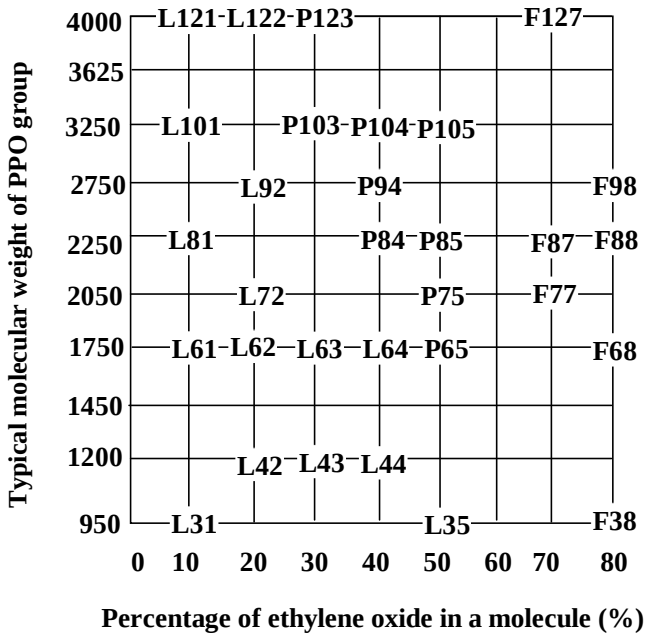


Figure 4.3 *Pluronic® grid adapted from [425,426]*

4.2.4 Gel Formation Properties of Poloxamers

Some poloxamers are able to form gels depending on the molecular weight and proportion of PEO present in the copolymer. The lower molecular weight polymers are not able to form gels in aqueous media at any concentration, but as the molecular weight and proportions of PEO increase, there is an increased tendency of the polymer to form gels. Higher concentrations of the lower molecular weight copolymers are necessary for gel formation, for example, Pluronic® F88 (PF-88) forms a gel at 40% w/w and Pluronic® F108 (PF-108) forms a gel at 30% w/w, whereas PF-127 forms a gel at a concentration of only 20% w/w [426].

PF-127 exists as a viscous liquid at low temperatures (2 – 8 °C) and forms a stiff gel when warmed and the temperature at which this occurs depends on the concentration of gel former [425,426]. The thermo-gelling behaviour of PF-127 means that at low temperatures, the sample is free flowing, allowing relatively easy administration of a PF-127 based formulation using a syringe and a needle. The stiff gel that is formed at body temperatures would produce a potential depot for sustained delivery of drugs such proteins and peptides *in situ*; PF-127 is therefore a potentially useful copolymer for the development of a thermo-responsive parenteral delivery system.

4.3 PLURONIC® F127

4.3.1 Physicochemical Properties

PF-127 has a nominal molecular weight of approximately 12600, but the weight ranges between 9840 – 14600 [10,11,428], with an approximate weight percent of PEO of 70%, (73.2±1.7%) [10]. PF-7 is free flowing with a melting point between 52 – 57 °C [428]. It is freely soluble in water, ethanol, and propan-2-ol and practically insoluble in light petroleum [11,428].

4.3.2 Toxicity

The toxological properties of the poloxamer block copolymers have been studied [426]. Poloxamers are considered only slightly toxic and their toxicity decreases as the molecular weight and the proportion of PEO in the polymeric backbone increases. PF-127, which contains 70% of PEO is considered to be non-toxic with a reported very high LD_{50} [426], the highest dose/concentration at which 50% of the test animals survive after the administration of a single dose of the copolymers.

Chronic studies, where the poloxamers were administered to animals for a long period also indicated the non-toxic nature of these copolymers and no adverse effects were observed in tissues and organs. Three (3) generation toxicology studies, where the offspring of test animals dosed with PF-127 were observed and monitored for any adverse effects, showed that the block copolymers did not result in any adverse effects that could be attributed to the dose of the poloxamers received by the first generation test animals [426].

It is reported that the poloxamers, including PF-127 are considered safe, non-irritating, and non-sensitising when applied in 5% and 10% w/v concentration to the eyes, gums, and skin [428]. Following intravenous administration for fourteen (14) days, of up to 0.5 g/kg/day of poloxamer in rabbits and dogs, no serious adverse effects were noted [428]. In a longer-term study, of up to two (2) years, rabbits were fed 3% or 5% w/w of poloxamer in food and the test animals did not show any obvious signs of toxicity, although at a dose of 7.5% w/w, there were indications of retardation in the growth rate of the animals [428]. Furthermore, poloxamers do not cause haemolysis after exposure of human blood cells for a period of eighteen (18) hours at 25 °C [428]. The non-toxic nature of PF-127 has resulted in its inclusion in the FDA Inactive Ingredients Guide, including for use in parenteral preparations [428].

The suitability of PF-127 as a vehicle for intramuscular use has been evaluated in rabbits [455] and the toxicity was evaluated by monitoring the morphology of muscle tissue and by monitoring creatine phosphokinase levels following single and multiple doses of the polymer. PF-127 administration causes a similar muscle irritability that is comparable to traditional parenteral vehicles such as peanut oil and Cremophor® EL, therefore making it a potentially viable vehicle for the parenteral administration of drugs [455].

Although PF-127 has generally been considered non-toxic, with no serious reported adverse effects, Wout *et al.* [456] showed that it can result in a sustained hypercholesterolamia and hypertrigylceridaemia over a period of four (4) days in rats, following intraperitoneal administration of a 30% w/w solution of PF-127 at a dose of 1.5 g/kg. These changes are related to alterations in the biological activity of lipid metabolising enzymes [456]. Blonder *et al.* [457] showed the dose dependant effects of PF-127 on hyperlipidaemia and after single injections of lower doses ($\leq$ 27.5 mg/kg) in rabbits, there was no alteration in the lipid metabolism in those animals. Despite the induction of hyperlipidaemia following the use of PF-127, the use of the gel as a controlled delivery matrix for drugs would result in only low doses of poloxamer being administered, and as such would not result in these untoward effects [457].

4.4 EXPERIMENTAL

4.4.1 Reagents and Materials

PF-127 was donated by BASF (Ludwigshafen, Germany) and was used as received. Nujol® was obtained from UniLab (Redmont, WA, USA). Cobaltous nitrate was obtained from Merck Chemicals (Modderfontein, South Africa) and ammonium thiocyanate was purchased from Sigma-Aldrich (St Louis, MO, USA). Carbonyl free ethyl acetate and acetone were obtained from Burdick and Jackson (Muskegon, MI, USA) and Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa), respectively. Absolute ethanol was obtained from Saarchem-Holpro Analytic (Pty) Ltd, (Krugersdorp, South Africa). All chemicals were used as received without further modification and were at least of analytical reagent grade.

4.4.2 Methods

4.4.2.1 Preparation of PF-127 Solutions

The preparation of different solutions of PF-127 solutions was undertaken using the cold method as previously described by Schmolka [426]. The solutions of PF-127 were prepared by weight (% w/w) and commences by weighing appropriate amounts of PF-127 flakes into a 100 ml beaker. The flakes were added to chilled HPLC-grade water (5 °C) slowly over a period of about 2 – 3 min with gentle stirring using a Labcon^{TM} MSH 10 model (Labmark, Maraisburg, South Africa) magnetic stirrer. The solution was then placed in a refrigerator for a period of approximately 24 hours or until all the PF-127 had dissolved and the solution was clear on visual inspection. Figure 4.4 summarises the cold method.

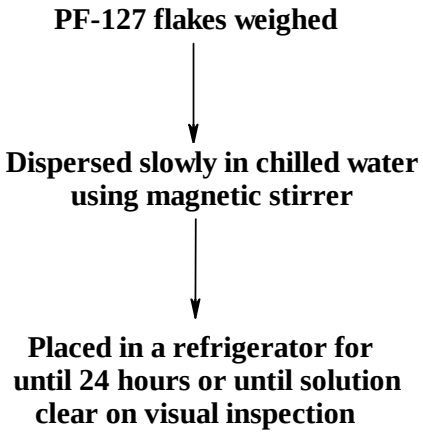


Figure 4.4 *Cold method for PF-127 gel prepration*

4.4.2.2 Preparation of Stock Solutions

A 1% w/v solution of PF-127 was prepared by accurately weighing 1 g of PF-127 flakes which were dispersed as described in Section 4.4.2.1 in approximately 70 ml of chilled HPLC-grade water in a 100 ml beaker and placed in a refrigerator until the solution was clear on visual inspection. The solution was then placed in a 100 ml A-grade volumetric flask and made up to volume with HPLC-grade water to obtain a final concentration of 1% w/v. The stock solution was used to prepare calibration standards, by serial dilution, of PF-127 with resultant concentrations of 0.01%, 0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4% w/v using A-grade glassware.

Approximately 10 g of PF-127 flakes were accurately weighed and dispersed in approximately 70 ml of chilled HPLC grade water at 5 °C as described in § 4.4.2.1 and placed in the

refrigerator until the solution was clear on visual inspection. The solution was then placed in a 100 ml A-grade volumetric flask and made up to volume with HPLC grade water to obtain a final concentration of 10% w/v. The 10% w/v stock solution was used to prepare standards with concentrations 0.00001%, 0.00002%, 0,00005%, 0.0001%, 0.0002%, 0.0005%, 0.001%, 0.002%, 0.005%, 0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.5%, 1%, 2% and 5% w/v, by serial dilution using A-grade glassware and ensuring that a minimum of 1 ml was used at each dilution.

4.4.2.3 Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of a 0.5% w/v PF-127 solution and a combination of a 0.5% w/v PF-127 solution and 200 IU/ml OT were obtained using a GBC UV/VIS 916 spectrophotometer (GBC Scientific Equipment Pty Ltd, Dandenong, VC, Australia). The spectrum was recorded in the wavelength range 200 – 600 nm, at a scan speed of 600 nm/min and data interval of 0.800 nm.

4.4.2.4 Infrared Spectrum

The infrared (IR) absorption spectrum of PF-127 and a mixture of PF-127 and OT, was obtained using a FT-IR Spectrometer Spectrum 2000 (Perkin Elmer Instruments LLC, Shelton, CT, USA) over the range 4000 – 400 cm^{-1}, using Nujol® to suspend the poloxamer and the mixture.

4.4.2.5 Validation of an Analytical Method for the Quantitation of PF-127

An analytical method reported to be suitable for the quantitation of PF-127 [458] was validated by assessing linearity, accuracy, and precision. A stock solution of PF-127 and the calibration standards were prepared as described in § 4.4.2.2.

In order to analyse the samples of PF-127, 3 g of cobaltous nitrate and 20 g of ammonium thiocyanate were dissolved in a 100 ml A-grade volumetric flask with HPLC-grade water to produce cobalt thiocyanate. 200 µl of the relevant Pluronic® standard, 100 µl of cobalt thiocynate, 200 µl of ethyl acetate and 80 µl of absolute ethanol were then added to a 1.5 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and centrifuged for 1 minute at 14500 g in a Model 5414 Eppendorf Centrifuge (Eppendorf AG, Hamburg, Germany). After centrifuging, dark blue sediment formed at the bottom of the centrifuge tube and two (2) upper layers were formed above the sediment. Thereafter, the two (2) upper layers were carefully aspirated so as not to disturb the sediment. The sediment and tube were washed with 200 µl ethyl acetate until the ethyl acetate that was aspirated was colourless. The sediment was then

dissolved in 2 ml acetone and the absorbance measured at 328 nm using a Model 1240 UV-VIS Spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) [458]. All determinations were performed in triplicate (n = 3).

4.4.2.6 Measurement of Surface Tension

The measurement of surface tension was achieved using the Du Noüy Tensiometer Version 2.0 (Cambridge Instrument Co. Ltd, London and Cambridge, UK). The solutions for measurement were prepared by serial dilution of a stock solution of 10% w/w PF-127 and the solutions prepared for testing were prepared in the concentration range 10^{-5} – 10% w/v PF-127, as described in § 4.4.2.2. Surface tension measurements were carried out in triplicate (n = 3) in an ambient environmental temperature of 19 °C that was monitored using a mercury thermometer for the duration of the experiment.

4.4.2.7 Determination of Transition Temperatures for PF-127 Solutions

The measurement of the gelation or transition temperatures for various concentrations of PF-127 solutions was conducted according to the method described by Gilbert *et al.* [459]. Aliquots (2.5 ml) of PF-127 solution of concentrations 18%, 20%, 22.5%, 25%, 27.5%, 30% and 32.5% w/w were prepared as described in § 4.4.2.1 and placed into 10 X 75 mm Kimble Borosilicate Glass Culture tubes (Division of Owens-Illinois Perrysburg, OH, USA). The solutions were placed in a water bath at 5 °C and the temperature of the water bath was increased by 1 °C in incremental steps and the temperature was allowed to equilibrate for 15 min prior to the assessment of gelation. Gelation was considered to have occurred when the test tube was rotated by 90° and no movement of the meniscus occurred. A second transition temperature was considered to have occurred if the solutions became flowable at another temperature after the initial formation of the gel. The assessment of gelation was performed in triplicate (n = 3).

4.4.2.8 Measurement of Viscosity

Viscosity measurements were performed using a Brookfield Digital Viscometer Model DV-I+ (Brookfield Engineering Laboratories, Inc, Stoughton, MA, USA). The viscometer was calibrated using a 5000 cP viscosity standard (Brookfield Engineering Laboratories, Inc, Stoughton, MA, USA) in a 100 ml beaker and a correction factor was determined. PF-127 solutions were placed in a water bath and allowed to equilibrate for at least 30 min before viscosity readings were taken at the different temperatures, *viz* 5, 10, 15, 20, 25, 30 and 37 °C. The viscosity of the relevant solution was measured in a 100 ml beaker with an internal diameter of 44.5 mm. Disc spindles were used to measure the viscosity before gelation occurred

and T-bars spindles were used to assess viscosity after gelation had commenced. In addition, a Helipath Stand (Brookfield Engineering Laboratories, Inc, Stoughton, MA, USA) was used to facilitate viscosity measurement after the gel had formed. Measurements were performed in triplicate (n = 3).

4.4.2.9 Gravimetric Analysis of PF-127 Solutions of Different Concentrations

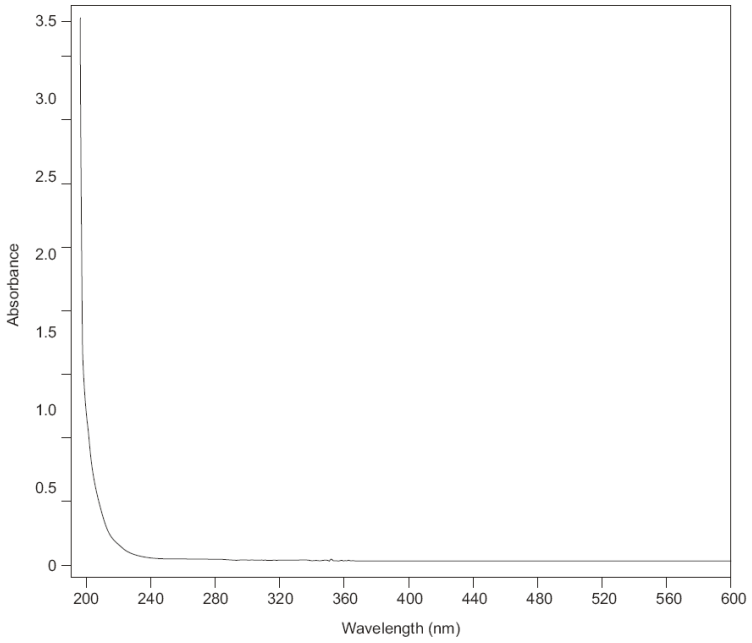
Gravimetric analysis of three different concentrations of PF-127, *viz*. 20%, 25% and 30% w/w, solutions was performed by accurately weighing approximately 2 g of the previously manufactured gel solutions as described in § 4.4.2.1 into moulds with a single open end. The mould dimensions were 23.6 X 11.0 X 8.0 mm (LxWxD) and weighing was undertaken using a Mettler-Toledo Model AG135 electronic balance (Mettler-Toledo, Inc. Columbus, OH, USA). The gels were allowed to set at 37 °C in a convection oven for a period of 15 min prior to the dissolution test. The gels were then placed into a USP Apparatus 1 dissolution bath (Hanson Research Cooperation, Chatsworth, CA, USA) set at 25 rpm and a bath temperature of 37 $\pm$ 0.5 °C. Samples were removed from the basket at 30, 50, 90, 120, 180, 240, 300, 360, 420 and 480 min, and weighed on a Mettler-Toledo AG135 electronic balance (Mettler-Toledo, Inc. Columbus, OH, USA) after gently blotting dry using filter paper, after which they were replaced and the test was continued. These studies were conducted in triplicate (n = 3).

4.5 RESULTS AND DISCUSSION

4.5.1 Ultraviolet Absorption

The UV absorption spectra of a 0.5% w/v solution of PF-127 and of a combined solution of OT (100 IU/ml) and PF-127 (0.25% w/v) are shown in Figure 4.5. PF-127 has no significant absorbance in the specified region, as poloxamers do not contain chromophores (Figure 4.5a). The spectrum obtained for the combined solution of OT and PF-127 (Figure 4.5b) is similar to that reported in Figure 1.5 indicating that it is unlikely that an interaction exists between the drug and copolymer that may alter the chemical nature of the molecules and OT biological activity, *in vivo*.

A



B

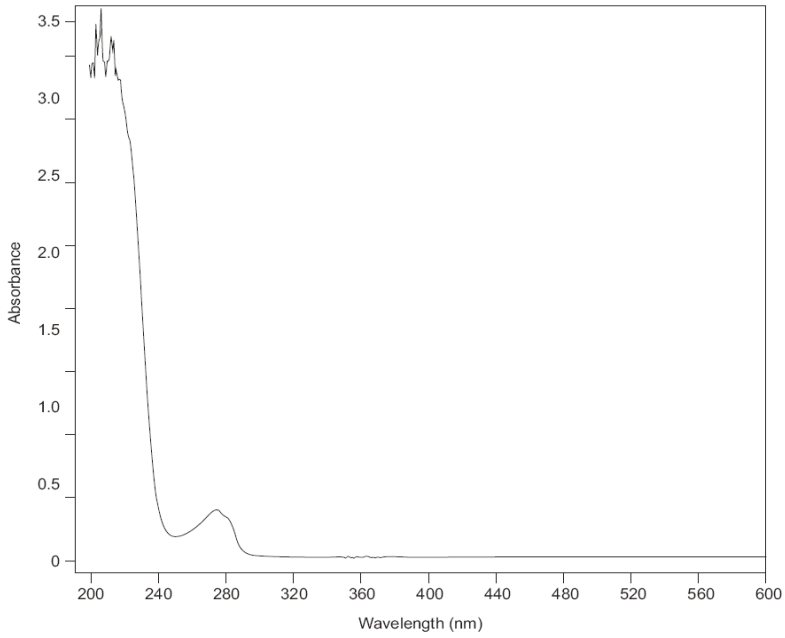


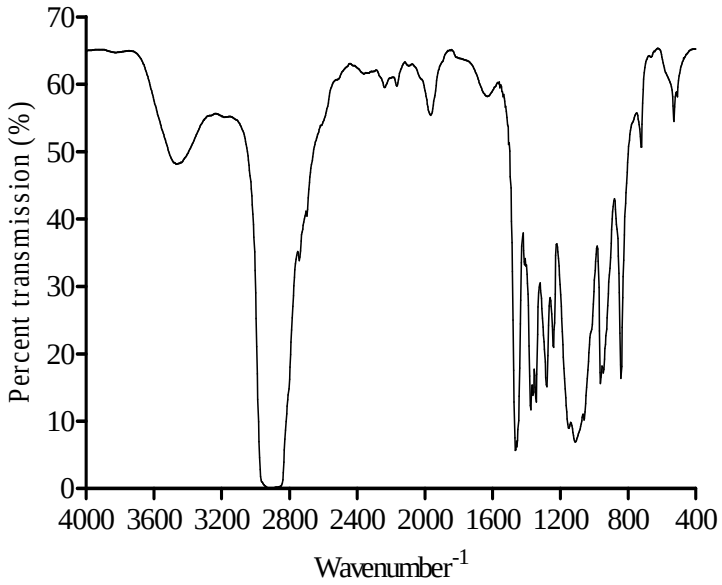
Figure 4.5 *UV absorbance spectrum for PF-127 (0.5% w/v) solution (A) and for PF-127 (0.5% w/v) combined with OT solution (200 IU/m)l (B)*

4.5.2 Infrared Spectrum

The IR spectrum of PF-127 is shown in Figure 4.6. Poloxamers are characterised by repeating units of ethylene oxide, with ether linkages between each of the units. Poloxamers also have C-H bonds and a terminal hydroxyl group and the infrared spectrum indicates the presence of these functional groups. The medium intensity broad band at around 3500 cm^{-1} is attributed to the presence of a hydroxyl group and the broad intense peak that occurs between 3000 – 2850 cm^{-1} is due to C-H bond frequencies, which are present in both the poloxamer molecule and liquid paraffin that was used to produce a sample for analysis. In addition, the wide bands of frequencies that occur between 1400 – 800 cm^{-1} are also due to the presence of C-H bond [29].

The incidence or occurrence of an interaction between PF-127 and OT is considered minimal on investigation of the infrared spectra reported in Figure 4.6. An interaction between two compounds would be observed in the infrared spectrum by the appearance of frequencies different to those observed when considering IR spectra of pure OT or PF-127. The spectrum shown in Figure 4.6b reveals no incidence of new frequency bands when compared to both the spectra of OT and PF-127 shown in Figures 1.5 and 4.6a respectively, indicting no interactions between the compounds.

A



B

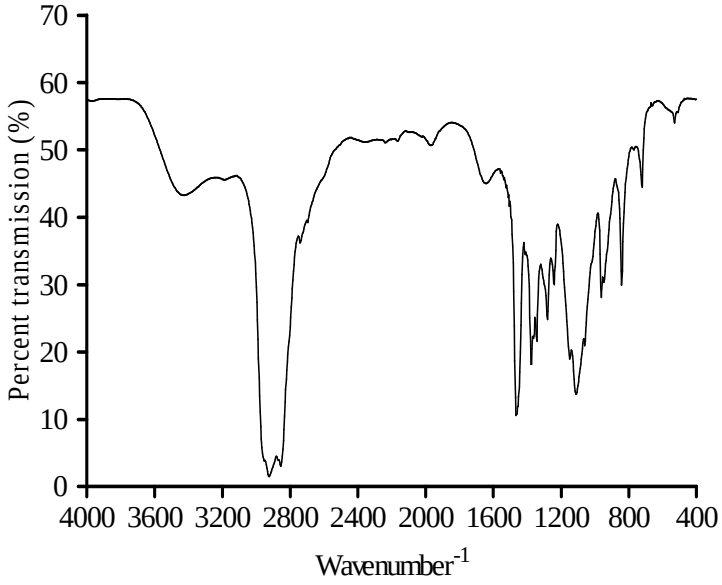


Figure 4.6 *Infrared absorption spectra of PF-127 (A) and for PF-127 combined with OT (B)*

4.5.3 Analytical Method for PF-127

4.5.3.1 Introduction

An analytical technique for the quantitation of dissolved poloxamer is valuable for establishing the mechanism(s) of drug release from gels [460]. The use of an accurate analytical tool was reported by Yang *et al.* [460] and that had been adapted from a method reported by Ghebeh *et al.* [458].

4.5.3.2 Poloxamer Assays

A method for the approximation of the amount of PF-127 dissolved in a receptor medium has been described [450]. An aliquot of the receptor medium was removed at specific intervals, the aqueous component was evaporated and the amount of PF-127 dissolved at these times was determined gravimetrically from the residues that remained after ensuring that complete evaporation of the aqueous media had been achieved [450].

Most commercial non-ionic surfactants such as PF-127 do not have chromophores that would be useful to enable quantitation by methods such as HPLC with UV or UV spectrophotometry [461] (See Figure 4.5a). The development of titrimetric and spectrophotometric analytical methods for poloxamers depends on the formation of complexes between polyether groups in the polymer and large cationic materials [461]. However, the main challenge with respect to assay development is the lack of reactivity of the polyether groups in poloxamers, which require vigorous reaction conditions to facilitate interaction [458].

Spectrophotometric techniques are used routinely for the quantitation of non-ionic surfactants such as poloxamers, despite sensitivity to interference [461]. The main challenge in the use of spectrophotometric assays for the quantitation of non-ionic surfactants include complicated calibration methods as the development of colour complexes depends on the molar concentration of the surfactant, length of the ethoxy chains and the particular hydrophobic component of a molecule [461]. In addition, the use of arbitrary standards for calibration may result in calibration errors, because a standard may not yield the same molar complex as a sample [461].

Analytical methods described in the literature for the accurate quantitation of poloxamers are the Wickbold [462], potassium tetrakis (4-halophenyl) borate [463], iodine assay [464], and cobalt thiocyanate methods [458,465,466].

The Wickbold method [462] results in the formation of a complex between the poloxamer and tetraiodobismuthate and the resultant precipitate is dissolved and titrated with pyrrolidone dithiocarbamate. The method requires the use of specially designed apparatus for concentrating samples [462]. The titration of poloxamer with potassium tetrakis (4-halophenyl) borate provides an alternate method of analysis in which the end point of the titration is determined by an indicator for excess tetrakis (4-halophenyl) borate [463]. Both these methods are described as tedious and time consuming, and were therefore not used for the quantitation of poloxamer in a reported study [458]. At an acidic pH, iodine reacts quantitatively with Pluronic F-68 giving a brown solution of which the optical density may be measured using spectrophotometric detection. This method however is non-specific and side reactions of the oxyethylene chain with other compounds may occur and the method is susceptible to interference from other compounds [464].

The cobalt thiocyanate method is the most commonly used method for the quantitation of non-ionic surfactants [461]. The method has been used for the quantitation of poloxamers [465,466] and was optimised by Ghebeh *et al.* [458] to increase both the sensitivity and reproducibility of the method to allow the use of a smaller sample volume as compared to other methods. The method is based on the formation of a tetrathiocyanatocobaltate (II) complex with the polyether linkages in the polymer and which can be extracted from an aqueous phase into organic solvents [458,461]. The complex that is formed, precipitates after centrifuging and is soluble in acetone, which is used to dissolve the complex after washing [458] as described in § 4.4.5.7. The intensity of the colour that is developed is proportional to the poloxamer content of the system being tested [458]. The structure of the complex is postulated to be due to hydrogen (H^+), ammonium ($NH4^+$) or cobalt (M^+) ions, which complex with the oxygen atoms in the ether linkages to form oxonium ions. These in turn are able to react with anionic entities such as thiocyanate ions (X^-) [466], as shown in Figure 4.7.

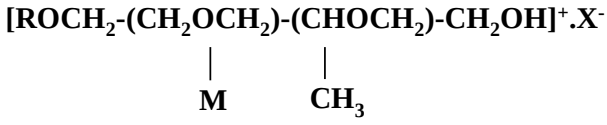
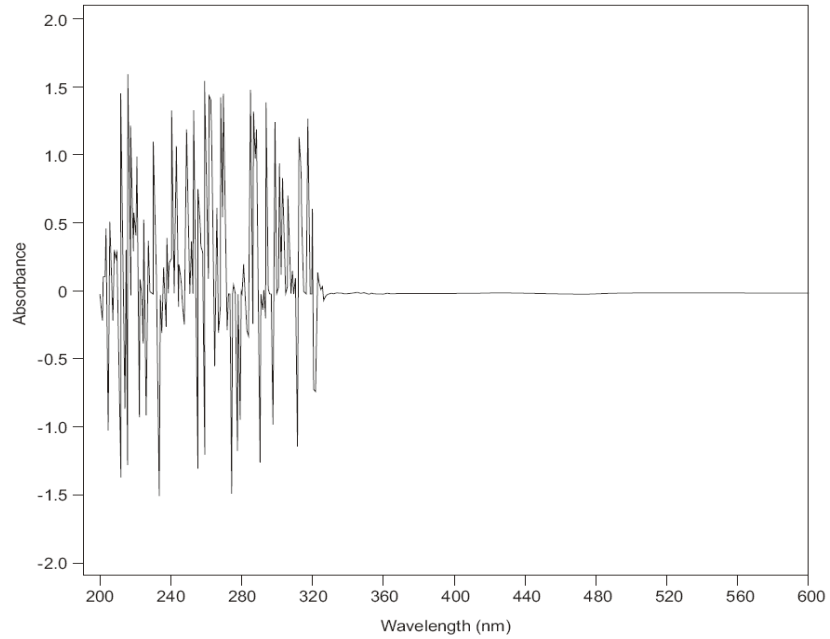


Figure 4.7 *Structure of tetrathiocyanatocobaltate (II) complex with poloxamers [466]*

The coloured complex that is formed has an absorption spectrum in the UV region, with sharp absorption occurring at a wavelength of 328 nm [458,461] as shown in Figure 4.8, which shows the absorption spectrum of acetone baseline (A) and the complex (B).

A



B

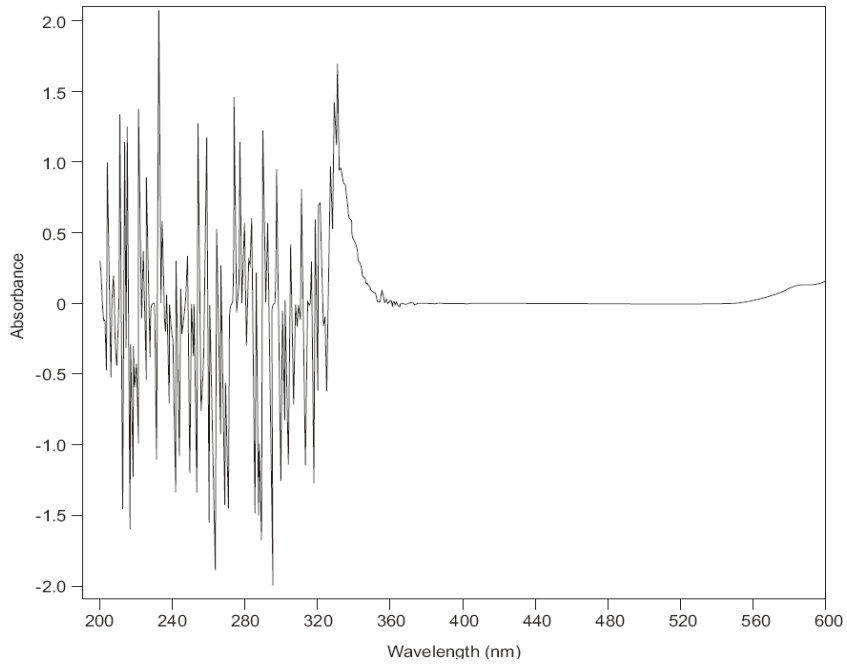


Figure 4.8 *UV spectrum of acetone solution (baseline) (A) and tetrathiocyanatocobaltate (II) complex with poloxamers (B)*

The parameters investigated during validation studies included an assessment of linearity and range, precision (intraday and interday) and accuracy. The linearity was determined in order to ensure that the method used was indeed linear for the concentration range to be studied. The intra-day precision (repeatability) was determined to assess the degree of scatter among the individual sample points and inter-day precision was determined at three (3) levels within the concentration range to assess the reliability of the method on analyses carried out on different days. The limits of quantitation (LOQ) and detection (LOD) were not determined for these particular studies because the amount of gel former that would be present for the use of the analytical method is greater than what would be expected to be the LOQ and LOD.

4.5.3.3 Linearity and Range

The calibration curve for PF-127 in the concentration range 0.01 – 0.4 % w/v revealed that the analytical method was linear in the concentration range studied as observed from the coefficient of determination ($r^2 > 0.99$) and the calibration curve is shown in Figure 4.9.

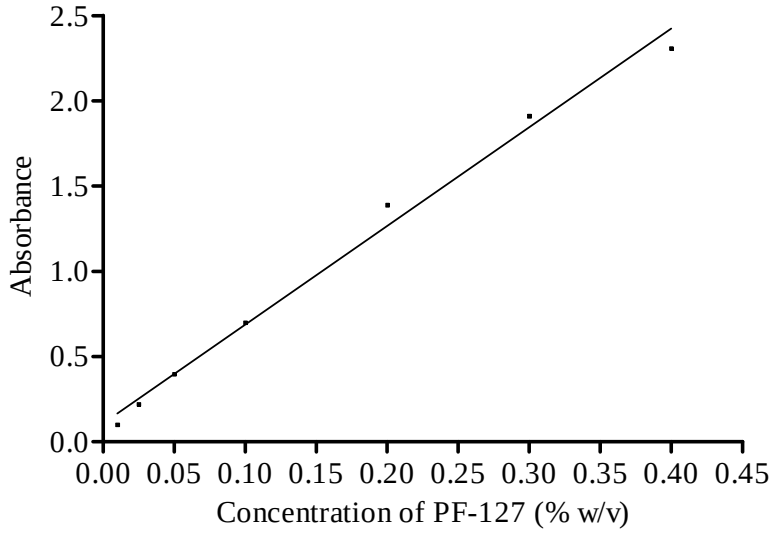


Figure 4.9 *Calibration curve for PF-127 in the concentration range 0 – 0.40% w/v*
$y = 5.790 + 0.1069$, $r^2 = 0.9916$

4.5.3.4 Intra-assay Precision (Repeatability)

The precision of an analytical method is a measure of the degree of scatter or agreement among individual data values, when the method is applied to multiple samples [232]. The repeatability data determined from the calibration curve shown in Figure 4.9, reported as the % RSD are summarised in Table 4.1.

Table 4.1 *Intra-assay precision for PF-127 for the calibration curve (0 – 0.40% w/v) shown in Figure 4.9*

Concentration of PF-127 (% w/v)	Absorbance	Standard deviation	% RSD
0.010	0.101	0.00195	1.92
0.025	0.222	0.00083	0.38
0.050	0.397	0.00356	0.90
0.100	0.699	0.00231	0.33
0.200	1.390	0.00327	0.24
0.300	1.912	0.00644	0.34
0.400	2.309	0.00879	0.38

The precision of the method was considered very good, ranging between 1.92% for the lowest concentration and 0.23% for higher concentrations.

4.5.3.5 Inter-day Precision (Intermediate Precision)

The interday precision of the method was determined at three (3) levels *viz.*, 0.075%, 0.15%, and 0.35% w/v over a period of three (3) days. The measurements were performed in triplicate (n = 3) and the results are summarised in Table 4.2.

Table 4.2 *Inter-day precision for PF-127 in the concentration range 0 – 0.40% w/v*

Concentration	Day 1		Day 2		Day 3	
(% w/v)	Absorbance	% RSD	Absorbance	% RSD	Absorbance	% RSD
0.075	0.518	0.211	0.520	0.724	0.540	0.242
0.150	1.071	0.304	1.039	0.690	0.998	0.719
0.350	2.095	0.401	2.135	0.435	2.165	0.370

The interday precision of the method was found to be very good with low % RSD less than 1% and thus the method can be considered to be reliable on a day-to-day basis.

4.5.3.6 Accuracy

The accuracy of the method was determined at three concentrations in the range 0 – 0.4% w/v, *viz.* 0.075%, 0.15%, and 0.35% w/v in triplicate. The results for the accuracy studies are summarised in Table 4.3.

Table 4.3 *Accuracy for PF-127 in the concentration range 0 – 0.40% w/v*

Concentration of PF-127 (% w/v)	Absorbance	% RSD	Predicted concentration (% w/v)	% Bias
0.075	0.540	0.242	0.0748	0.309
0.150	0.998	0.719	0.154	2.604
0.350	2.165	0.370	0.355	2.571

The analytical method for PF-127 was found to be accurate, yielding % Bias values below 5% at all levels.

4.5.4 The Critical Micelle Concentration of PF-127

4.5.4.1 Introduction

Block copolymers such as poloxamers possess surface and bulk activity and therefore may be considered to have surfactant properties [467]. At the surface of solutions, poloxamers have been shown to reduce surface tension [468,469] and can form micellar aggregates in the bulk solution. Furthermore, the formation of micelles by poloxamers has been established previously [470,471].

PF-127 is composed of two dissimilar entities that result in the formation of aggregates in aqueous and organic media [468,472-474]. The micelles are composed of a hydrophobic core, largely comprised of PPO blocks surrounded by a hydrated corona of PEO blocks. There is however evidence to suggest that the hydrophobic core does not completely exclude PEO units and water when in solution [442,475].

The driving force for micellisation is provided by the hydrophobic PPO block component of the polymer. In comparison for alkyl surfactants, that also form micelles, the hydrophobic portion must be considerably larger than the alkyl chains of the alkyl surfactants to result in aggregation and formation of micelles [442]. The unimers and corresponding micelles of PF-127 and other poloxamers are therefore much larger than those formed by non-ionic alkyl surfactants. The radius of Pluronic® micelles is reported to be between 60 – 120 Å as compared to 20 – 30 Å of those formed by the alkyl surfactants [442].

The type of micellar aggregates that are formed depend on the molecular weight and size of the copolymer blocks, the composition of the solvent and the temperature of the environment in which the materials are used. A study of the phase behaviour of triblock copolymers has shown that the type of aggregates formed, depends on the temperature of the environment and the percentage composition of the polymer backbone [476].

The formation of micelles is an important consideration when studying the behavioural characteristics of PF-127 gels. The formation of micelles determines the gel formation characteristics and the viscometric properties of the copolymer and therefore cannot be separated from these properties.

The critical micelle concentration (CMC) is the concentration at which amphiphilic entities such as surfactants start forming thermodynamically stable aggregates known as micelles in the bulk solution [467,477]. The formation of micelles by amphiphilic triblock copolymers is a more complex process than that which occurs when low molecular weight surfactants aggregate, forming micelles. In contrast to the low molecular weight surfactants, there is no distinct concentration at which the micellisation process commences and therefore a range may be used to define the CMC of these materials [425].

Predictions with regard to the distribution and pharmaceutical availability of drugs and other excipients such as preservatives is important, and can be inferred from knowledge of the existence or absence of micellar aggregates in pharmaceutical preparations. The presence of micelles may also become important when formulating poorly soluble drugs, the solubility of which will be increased following solubilisation in the micelles [467].

A variety of different techniques can be used for the determination of micelles in block copolymer solutions and other surfactant systems. Such techniques include the measurement of changes in the equivalent conductivity, osmotic pressure, interfacial and surface tension, density change, and detergency of solution in which potential micelle forming excipients are incorporated [477]. The use of different techniques for determining the CMC for poloxamers has resulted in the reporting of conflicting results as different methods have different levels of sensitivity to the polydisperse nature of triblock copolymers. In addition, the presence of appreciable amounts of diblock copolymer impurities and free PPO chains, which are difficult to remove, have also contributed to the variability of these results [425,442]. Ultrasonic velocity and light scattering techniques [478-480] in addition to sound velocity [480] have been used to confirm the presence of micelles. Surface tension [469,473,479-481] and dye solubilisation [472,479,480] are however, the most commonly used methods to determine the CMC of poloxamers and have been found to have good correlation to each other [425].

4.5.4.2 The Surface Activity of PF-127

Surface-active substances are adsorbed at the air/water interface when in solution [467,477]. The hydrophilic portion of a surfactant is oriented towards the aqueous component of a system whereas the hydrophobic region projects out of the aqueous solution into the air. The presence of surfactants at the air/water interface affects the surface and interfacial tension of the vehicle [467,477] and typically an increase in the concentration of a surfactant results in a decline in the surface tension of a solution. This phenomenon shows an increase in the activity of the surfactant in solution under constant coverage of the air/water interface by a surfactant. A

continuous decrease in surface tension occurs as a result of the presence of surfactant materials until a plateau is reached, after which, further increases in the concentration of surfactant does not result in any changes in the surface tension. The concentration at which this occurs is known as the CMC [467,477,481]. Amphiphilic triblock copolymers are adsorbed at the air-water interface and show the ability to decrease the surface tension of water [481].

The surface tension of PF-127 solutions was measured as described in § 4.4.2.6. A calibration factor was then calculated using Equation 4.1 from the surface tension of water at the temperature studied, 19 °C, 75.5 dynes/cm compared to the surface tension of water at 20 °C (72.75 dynes/cm). A calibration factor is important for the correction of the measurement of surface tension as surface tension varies with temperature [467].

$$C = \frac{Surface\,tension\,of\ water\,at\,ambient\,temperature\,(72.75\,dynes/cm)}{Scale\,reading\,of\ distilled\ water\,(75.50\,dynes/cm)} \quad \textbf{Equation 4.1}$$

Surface tension readings were multiplied by a correction factor to determine the true value. Figure 4.10 depicts the surface tension changes for PF-127 solutions (n = 3) over a concentration range of 0.00001 – 10% w/v of PF-127. The plot shows surface tension versus the logarithm of concentration of the polymer in solution and the % RSD for all calculated surface tension values ranged between 0.20 and 1.85 % RSD.

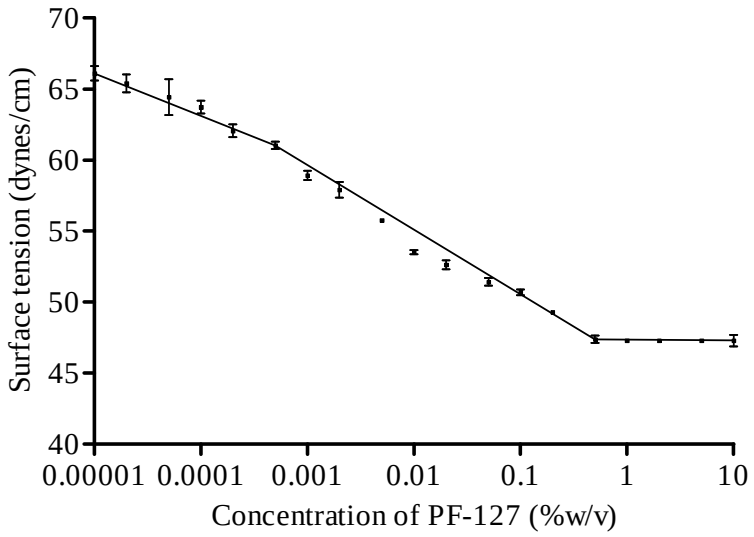


Figure 4.10 *Semi logarithmic plot of PF-127 concentration versus surface tension of PF-127 solutions*

At low concentrations of PF-127 (10^{-5} – 10^{-3} %w/v), there is a decrease in the surface tension of the solution in a linear manner over several concentration decades. Thereafter a change in the slope of the line occurs at approximately 0.001% w/v PF-127, after which there is further decline in the surface tension as the concentration of PF-127 in solution increases, until the surface tension reaches a plateau at a concentration of 0.5% w/v of PF-127. The surface tension remains constant despite further increases in the concentration of the copolymer in solution. The results generated in these studies for PF-127 are in agreement with results for the same copolymer that have been studied and reported in the literature [468,469,481], where two changes the slope of the surface tension curve were observed. The final surface tension obtained was approximately 40 mN/m for PF-127 at 25 °C [468] and the first change in surface tension occurred at a concentration of approximately 0.001% w/v of the poloxamer [468,469,481]. The surface tension at the second change is also in agreement with the value obtained by Wanka *et al.* [468] who observed a second change in the slope at a concentration of approximately 0.4% w/v of PF-127.

The presence of two changes in the slope of the surface tension readings has led to some confusion regarding the interpretation of the CMC and understanding of what occurs in the micellar systems that are formed. In a comprehensive study, Alexandridis *et al.* [481] examined the results obtained from their surface tension measurements and other related information in order to understand the conformation of the copolymers at the interface and their structure in the bulk solution, so as to elucidate the process of micelle formation and structure more clearly.

Alexandridis *et al.* [481] proceeded to investigate the origin of the first change in the slope of the graph of logarithm of Pluronic® concentration versus surface tension. In earlier studies [482,483], this value was attributed to the CMC, but further analysis of the second change in the curve lead to the conclusion that the actual CMC occurred at the second change in the curve. It was thought that the first change in the slope was a result of the polydisperse nature of the poloxamer and the presence of synthetic and other hydrophobic impurities [425]. However, Alexandradis *et al.* [481] argued that the polydisperse nature of the copolymer would result in a poorly defined and obscure single decline in the surface tension and a decrease at the CMC value as shown by some earlier findings [484]. This is contrary to the surface tension behaviour changes observed for copolymers such as the poloxamers. In addition, the presence of impurities would result in a decrease at the CMC prior to the surface tension increasing slightly to reach a plateau [484]. Prasad *et al.* [469] proposed that the presence of the first change was due to the formation of monomolecular micelles, whereas Alexandridis *et al.* [481] argue that

the presence of monomolecular micelles is unlikely. This is because the concentration at which the first change occurs is very low and there is no apparent driving force to promote the formation of compact structures.

A recent explanation in the surface behaviour of poloxamers is that the first change in the surface tension slope observed is due to a modification in the configuration of PF-127 at the air/water interface, occurring at low bulk concentrations of poloxamer. However, the concentration of PF-127 at the air/water interface is significant resulting in conformational changes of the poloxamer at the interface. At very low concentrations of $< 10^{-6}$% w/v, the copolymers are adsorbed at the air/water interface in an extended configuration and the surface tension decreases with increasing poloxamer concentration. Concentrations in the region of 10^{-6}% – 10^{-3}% w/v result in constant coverage of the surface at the interface and the PEO-PPO-PEO copolymers are adsorbed at the air/water interface as an inverted U and PEO chains at the air/water interface and hydrophobic PPO blocks projecting into the air. The surface tension continues to decrease as the concentration of the poloxamer at the air/water interface increases. At a bulk concentration of approximately 10^{-3}% w/v of poloxamer, when the surface is fully covered with surfactant, there is a structural transition that occurs and the layer at the surface becomes more compact, expelling water and subsequently results in PEO segments projecting into the aqueous medium or folding around the PPO. Consequently, more PEO-PPO-PEO copolymers can fit onto the air/water interface and causes the adsorbed layer to become thicker and therefore the surface tension continually decreases, but at a slower rate than previously observed. At the CMC, micelles are formed in the bulk of the solution, the activity at the air/water interface remains the same, and there is no further decline in the surface tension as the concentration is increased, although there is an increase in the number of micelles that are formed in the bulk medium [481].

The change in the surface tension curve at higher concentrations has been considered to be the CMC for poloxamer block copolymers. The reason for this phenomenon is that there is agreement between the value for the CMC that was obtained from dye solubilisation studies and the concentration at which the second change in the slope occurs [472]. Therefore, there is the presence of well-defined polymolecular micelles with a hydrophobic interior that are capable of solubilising hydrophobic molecules in these systems [472]. In addition, the CMC is considered to occur at the second change in surface tension because of the plateau in the profile that is observed after this concentration and which is characteristic at concentrations that are greater than the CMC. Surfactants typically behave in this manner and after the CMC, there is usually no further decline in the surface tension [467].

4.5.5 The Sol-Gel-Sol Transition Temperatures of PF-127

4.5.5.1 Introduction

A thermodynamic study of the phase transitions of PF-127 was undertaken as the thermo-reversible nature of PF-127 has been extensively reported in the literature [426,459,485,486]. PF-127 has the ability to undergo reverse thermal gelation [426] and this has led to interest in the use of this copolymer as a thermo-responsive controlled delivery system.

4.5.5.2 The Transition Temperatures of PF-127

The temperatures at which the transition from sol to gel occurs can be referred to as T_m1 and is dependent on the concentration of the copolymer used in the solution being tested. In addition, on further heating, the PF-127 gel state reverts to a sol state and the temperature at which this occurs can be referred to as T_m2 and which is dependent on the concentration of the polymer in solution [485]. As the temperature is increased, the copolymer solutions turn cloudy at a temperature referred to as the cloud point, which is a characteristic of the polymer [485]. The cloud point of PF-127 occurs at temperatures above 100 ˚C. The transition temperatures, *viz.*, T_m1 and T_m2 of PF-127 solutions in the concentration range 18 – 32.5% w/w were determined using a method previously described in the literature [459] and in § 4.4.2.7. Figure 4.11 shows the sol to gel transition for PF-127 solutions in the concentration range studied.

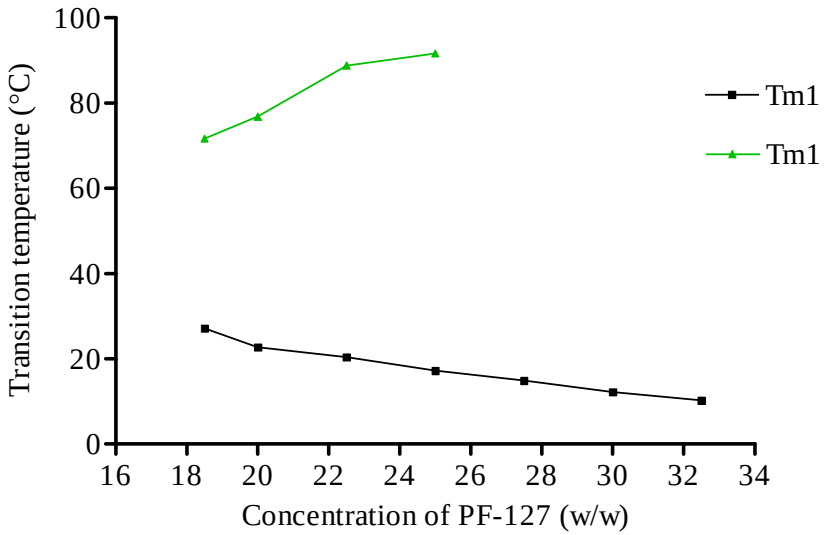


Figure 4.11 *T_m1 and T_m2 transition temperatures for PF-127 aqueous solutions in the concentration range 18 - 32.5 % w/w*

As the concentration of PF-127 is increased, there is a decrease in the transition temperature, T_m1. A solution of 20% w/w PF-127 has a transition temperature of 23 ˚C whereas a solution of 30% w/w forms a gel at only 12 ˚C. This is in agreement with other studies that show that there is a decrease in T_m1 with an increase in the concentration of PF-127 [487]. Furthermore, reported values that were determined for T_m1 for solutions of 20% w/w and 30% w/w were 21.5 ˚C and 10.9 ˚C, respectively. The results in this study are similar to those that have been reported, but because of batch-to-batch variability in the manufacture of copolymers, differences in the sol to gel transition properties can be expected [442,487]. T_m2 is also concentration dependent, and lower concentrations of gel former have lower transition temperatures. Solutions of PF-127 with concentrations of gel former greater than 25% w/w have transition temperatures greater than 100 °C, which cannot be determined using this method.

The presence of micelles in the PEO-PPO-PEO block copolymers has been demonstrated by the surface tension study reported in § 4.5.4. Solutions of PF-127 with concentrations of polymer above 20% w/w have a tendency to form micelles when temperatures increase resulting in the formation of gels and an increased viscosity of the bulk solution. It has been proposed that conformational changes in the methyl groups of the hydrophobic PPO region of the micelles and the motion of hydrophilic components of the polymer result in dehydration of the micelles. Therefore, there is an increase in end chain friction and a resultant increase in the viscosity of the solution culminating in gel formation [471]. This proposition is in agreement with other studies [468,482,488] that show that as temperature increases there is an increase in the molecular weight of the micelles showing an increase in the aggregation number. However, there is no change in the hydrodynamic radius of the micelles indicating that the micelles have a more compact structure at higher temperatures due to dehydration of the micelles as the temperature is increased [488].

Wanka *et al.* [468] described a mechanism for the gelation of PF-127 based on the DLVO-theory and the concept of the dehydration. The overall interaction between aggregates or micelles was found to be repulsive in nature, although there is an attractive Van Der Waal's interaction between such aggregates. The repulsive interaction between the aggregates is due to steric stabilisation of the PEO chains and the attractive interaction is more than likely due to the Van Der Waal's forces present between the PPO chains. The dehydration of the interior PPO chains would result in greater Van Der Waal's forces with a resultant increase in the attractive forces between the aggregates thereby promoting gelation [468]. The basis of this theory also

provides an explanation for gelation at increased temperatures following the dehydration of the core of the micellar structures.

The kinetics of the sol to gel-transition are an important consideration with regard to the specific application of gel use *in vivo*. Wang and Johnston [487] studied the kinetics of poloxamers using a pulse shearometer. The rate of gelation was established for solutions of 20% and 30% w/w solutions of PF-127 and it was evident that the rate of gelation occurred at a faster rate when higher concentrations of copolymer were used in solution. This implies that the concentration of the gel would not only affect the overall release rate of a drug from these systems but would also affect the initial burst effect, if any exists. It may therefore be concluded that after an intramuscular injection of a solution of low PF-127 concentration the gelation process would be slower for these lower concentration solutions as compared to higher concentration solutions, thereby making the active principle immediately available. This phenomenon would result in a higher burst effect from the gel and might be an important consideration with regard to the clinical use of the specific therapeutic agents included in these novel delivery systems [487].

4.5.5.3 The Effect of Additives on the Transition Temperatures

The effect of additives on the transition temperatures of PF-127 is an important consideration when developing formulations in which PF-127 is used as the rate-retarding polymer. For example, buffers salts may be added to formulations for several purposes, including but not limited, to a means of enhancing the stability and solubility of an API by maintaining a constant pH. The addition of sodium chloride may also be considered important for the formulation of parenteral products to produce a pharmaceutical preparation that has the desired state of tonicity. Excipients do not necessarily have biological activity but improve the performance of a formulation and are usually added to formulations. Evaluation of the impact of additives is also important to ascertain whether any factors that would influence the performance and the integrity of a dosage form exist. Three (3) different concentrations of PF-127 and the impact of addition of potential formulation additives were evaluated and the effect on transition temperatures monitored, and the results are depicted in Figure 4.12.

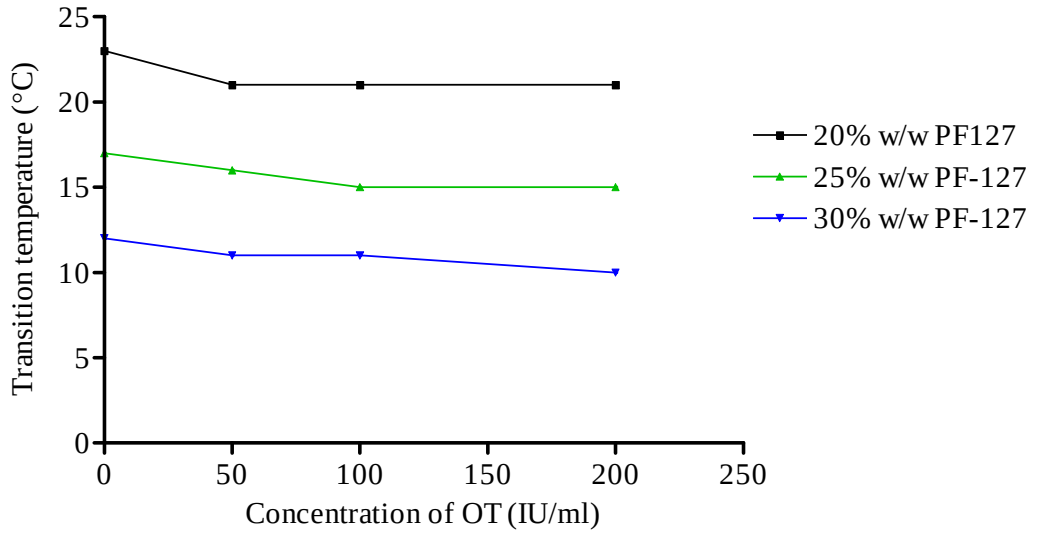


Figure 4.12 *The effect of drug loading on T_m1 for three(3) concentrations of PF-127*

The addition of OT to PF-127 solutions also resulted in a decrease in the transition temperature as shown in Figure 4.12. At lower concentrations of the polymer, it was noted that the drug loading did not affect the extent of the decrease in the transition temperature, however at higher concentrations, such as 30% w/w PF-127, an increase in the drug loading resulted in a greater decrease in the transition temperature of the copolymer.

The results illustrated in Figure 4.12 are in slight disagreement with those reported by Gilbert *et al.* [459] who studied the effects of adding preservatives such as benzoic acid and para-hydroxybenzoate esters to solutions of PF-127. The increase in solute loading resulted in a large decrease in the transition temperature, but these results indicate that OT caused only a slight decrease in the transition temperature. Gilbert *et al.* [459] propose that the hydrophobic solutes bind to the chains of PF-127 and suggest that the para-hydroxybenzoate esters bind to the PEO chains, promoting dehydration and a consequent decrease in the gelation temperature. OT is a hydrophilic entity and does not affect the transition temperatures of PF-127 solutions significantly. It may be that the peptide does not bind appreciably to the polymer backbone and therefore does not interfere with the gelation process as described. However, at higher concentrations of the polymer, there is a possibility that the concentration of polymeric chains may result in steric interactions with the peptide thereby causing a decrease in the transition temperatures observed. Studies [485,486] have revealed that salts such as sodium chloride, sodium sulphate and calcium chloride that have salting-out effects also decrease the gelation

temperature of PF-127 gels. Peptides are known to have a salting-out effect, thereby reducing the effective water concentration in a system and would therefore be expected to cause a decrease in the gelation temperature of PF-127 systems [486].

The impact of additives on gel formation properties in PF-127 solutions was evaluated. The stability of OT is pH dependant as described in § 1.3.2 and therefore OT formulations are prepared with an acetate buffer to maintain the pH at approximately 4 [33]. Furthermore, parenteral formulations for intramuscular administration must be isotonic (0.9% w/v sodium chloride) or they would result in muscle irritation and sodium chloride may be added to OT formulations for this reason [33]. Finally, the formulation of parenteral formulations requires the use of a preservative and chlorbutol is used in OT commercial preparations [33]. The impact of these additives on the transition temperature of PF-127 solutions at three (3) concentrations, *viz*., 20%, 25%, and 30% w/w was investigated and the results are shown in Figure 4.13.

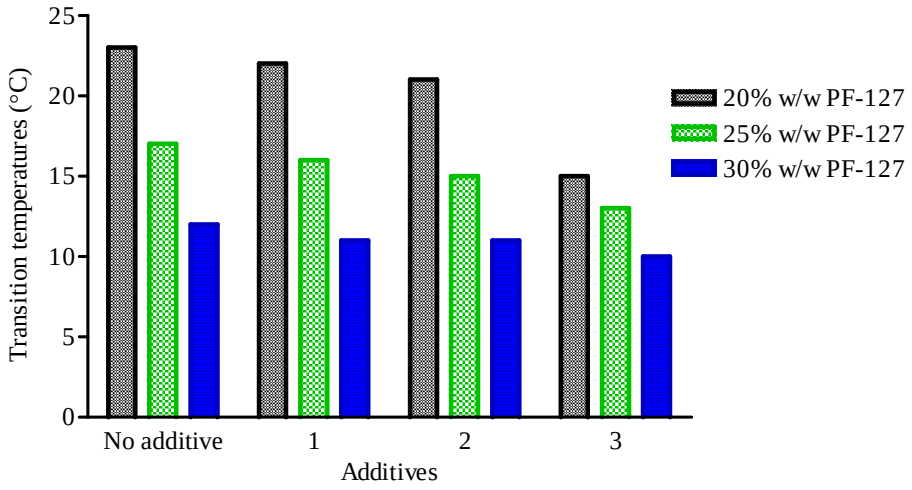


Figure 4.13 *The effect of selected additives on the transition temperatures of PF-127 solutions, where 1: 0.1 M Acetate buffer, 2: 0.5 % Chlorbutol and 3: 0.9 % Sodium chloride*

The addition of salts such as sodium acetate and sodium chloride resulted in a decrease in the transition temperatures for PF-127 whereas the presence of multivalent salts such as magnesium sulphate, aluminium sulphate and trisodium phosphate have been found to prevent gel formation of PF-127 formulations [485]. Sodium chloride and acetate buffer both reduces the transition temperature by a salting-out effect. Chlorbutol may result in a decrease in the transition temperature similar to that caused by para-hydroxybenzoate esters described above, as chlorbutol is also a hydrophobic molecule.

4.5.5.4 Thermodynamic Considerations

The gelation of PF-127 can be described by use of thermodynamic relationships and equations. Vadnere *et al.* [486] performed a physico-chemical characterisation of PF-127. The gelation process of poloxamers can generally be described by use of an equation that shows the dependence of gelation on polymer concentration and this relationship has been described by Eldridge and Ferry [489]. According to the equation, an inverse linear relationship exists between the logarithm of the concentration of gel and the gel to sol transition temperature and the relationship shown in Equation 4.2 can be used to determine the enthalpy of the sol to gel transition for polymeric materials such as PF-127.

$$\ln c = \frac{\Delta H^{o}}{RT_{gel \rightarrow sol}} + K \qquad \textbf{\textit{Equation 4.2}}$$

Where,

c = concentration of the polymer

$\Delta H°$ = enthalpy for the sol-to-gel transition

R = 8.3143 $JK^{-1}mole^{-1}$

T = absolute temperature in Kelvin

K = constant

This relationship was applied to the experimentally determined transition temperatures obtained for PF-127 gels and revealed that a linear relationship exists as depicted in Figure 4.14.

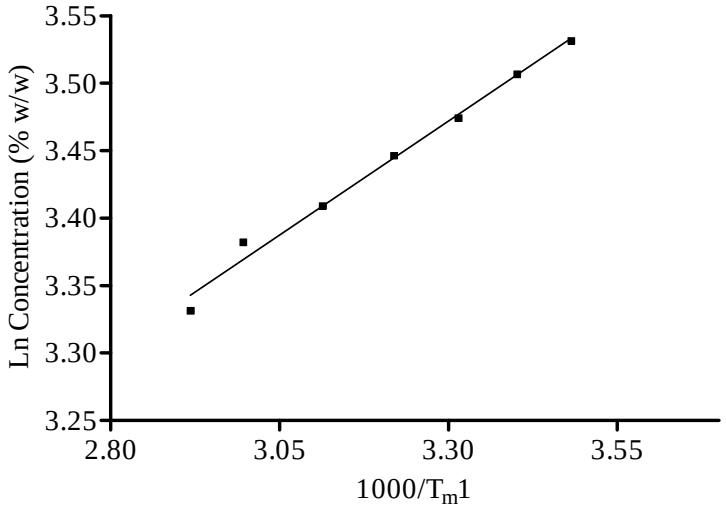


Figure 4.14 *Thermodynamic relationship between the transition temperature, T_m1 and the concentration of the gel former in solution*
$y = 3.015x – 7.1726$, $r^2 = 0.9969$

Enthalpy changes from a reaction process can be estimated from the gradient of the slope of a curve generated using Equation 4.2 [486,489]. The enthalpy change for these experiments was estimated to be 25.07 $kJmol^{-1}$ or 5.99 $kcalmol^{-1}$ and is in agreement with enthalpy change of 9.5 $kcalmol^{-1}$ reported in the literature for PF-127 gels by Vadnere *et al.* [486]. This small positive value for enthalpy is in contradiction with values reported for the gelation of gelatin, which occurs with a large negative enthalpy change ranging between -49 and -73 $kcalmol^{-1}$ favouring the gelation process [489] and a small positive value would be indicative of system in which gelation of the copolymer is not favoured.

Vadnere *et al.* [487] have proposed that the positive enthalpy is accompanied by an increase in the entropy of the system and therefore the gelation of the copolymer is favoured. They proposed a qualitative model to explain the observations with regard to the entropy and which is shown in Figure 4.15.

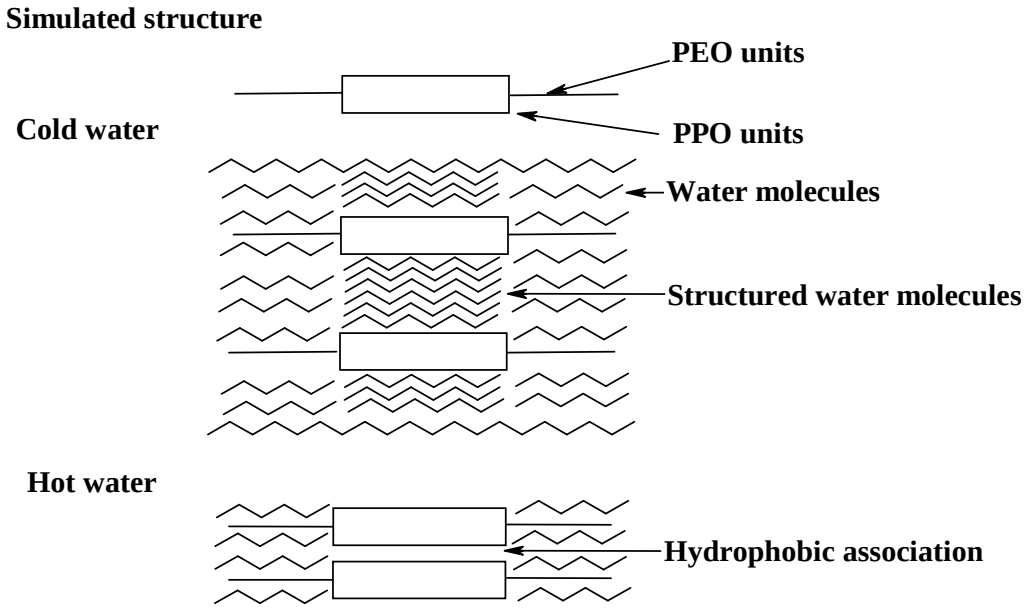


Figure 4.15 *Qualitative model for the gelation process of poloxamer copolymers (adapted from [486])*

In solution, there is a higher order of water around the hydrophobic PPO unit of the copolymer that are structured between adjacent hydrophobic entities. As gelation occurs, the interaction between the hydrophobic units causes water expulsion from the ordered region between the chains, into the bulk solution of lower order. This results in an increase in the entropy of the system and thereby promotes the gelation process, even though a small positive enthalpy change exists in the system that would otherwise negatively influence the gelation process. Positive

entropy would therefore be a driving force for the association of the hydrophobic units. Furthermore, hydrophobic interactions are characterised by endothermic heat changes or positive enthalpy as observed in the gelation process for PF-127 [486].

Yu *et al.* [490] also used a similar relationship to characterise PF-127 gels and obtained an enthalpy value of 28 $kJmol^{-1}$. A linear relationship was observed to exist between the logarithm of concentration of polymer and the inverse of the absolute temperature of the system. Positive enthalpy and entropy values were derived and these are in agreement with the results reported by Vadnere *et al.* [486].

A challenge with respect to the use of PF-127 systems is that the integrity of the gel structures tends to diminish on mixing with aqueous media [491]. This results in rapid dissolution and removal of the gel from the site of a potential depot injection thereby potentially limiting the use of the polymer for long duration sustained release formulations. However, for the application of developing a formulation of OT for prevention of post partum haemorrhage, this was not seen as a deterrent as the targeted duration of delivery from the dosage form was to be 8 hours.

4.5.6 Viscosity of PF-127 Solutions

4.5.6.1 Introduction

The rheological behaviour of a vehicle is an important consideration for quality control purposes and with respect to predicting the release of a drug from a vehicle and ultimately its clinical use. In addition, packaging and storage conditions of the dosage form may be inferred by knowledge of the rheological properties of such systems. The viscosity of PF-127 solutions was studied as a function of the concentration of polymer and environmental temperature.

4.5.6.2 Calibration of the Viscometer

It is recommended that the measurement of viscosity using Brookfield viscometers be carried out using a vessel with an inside diameter of at least 83 mm. A 600 ml low form Griffin beaker is usually used for this purpose as the use of a smaller vessel will result in higher viscosity readings and falsified inflated readings [492]. If a smaller sample volume is to be used, then calibration of the system is necessary to obtain accurate viscosity readings. In these studies as a large sample volume would be required if a 600 ml beaker were used, a 100 ml beaker with an internal diameter of 44.5 mm was selected for use. The viscometer was calibrated prior to use by measuring the viscosity of a 5000 cP Silicone viscosity standard as described in § 4.4.2.8.

The viscosity was found to be 6880 ± 10 cP and a calibration factor of 0.727 was calculated as recommended by Brookfield Engineering Laboratories [492].

4.5.6.3 Viscosity of PF-127 as a Function of the Concentration

The viscosity of solutions of different concentrations was measured and the results are shown in Figure 4.16 in addition to the viscosity of PF-127 solutions determined at different temperatures.

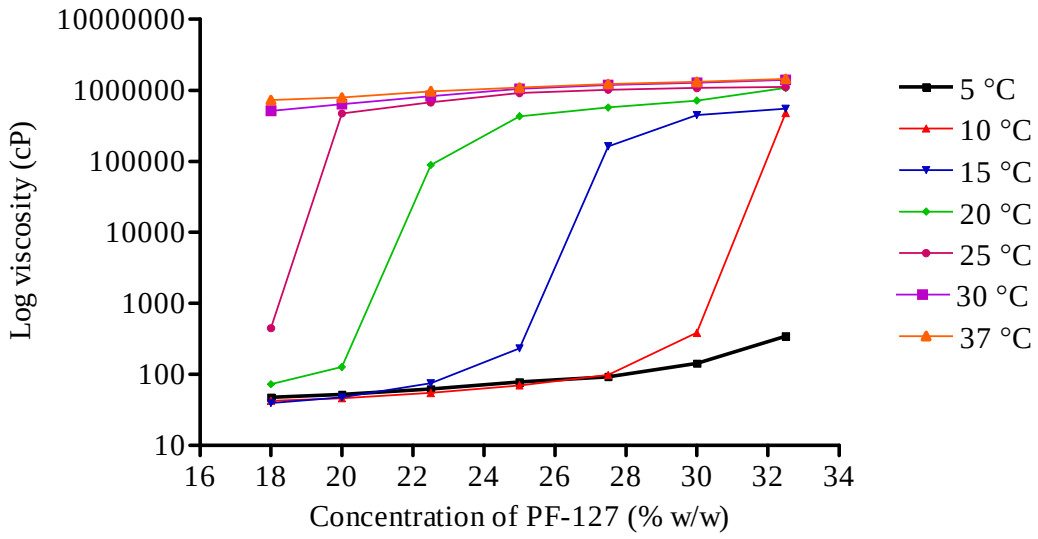


Figure 4.16 *Effect of increasing concentration of PF-127 on the viscosity of aqueous solutions at different temperatures*

As the concentration of PF-127 is increased, there is an increase in the viscosity of the system at all temperatures and these results are in agreement with previously reported studies [493,494]. This increase is more than likely due to the presence of micellar aggregates that are present in these systems. With increasing concentrations of polymer in solution, there is a corresponding increase in the number of micelles formed that are more closely packed, resulting in an increase in the viscosity of the solution [493,495].

A formulation with a lower viscosity would result in the administered formulation spreading extensively into the muscle fibres, resulting in a greater surface area for drug release and a corresponding increase in the rate of drug release, faster absorption into the systemic circulation and resultant device failure. This phenomenon would preclude or limit the use of the more dilute solutions of PF-127 for depot formulations from which sustained drug action is desired [487].

Directly related to the viscosity of these systems is the syringeability and injectability of a product. The administration of viscous solutions with a needle can be difficult and therefore the final viscosity of a formulation to be injected should not be too high, although a high viscosity may be suitable for a long acting depot formulation compared to the lower viscosity formulations. A compromise between the intended duration of action and the impact of viscosity on practical application parameters is therefore necessary. The size of the syringe and needle must be considered in relation to the viscosity and duration of action that is intended to be built into a product and in relation to patient acceptability [496].

4.5.6.4 Viscosity of PF-127 as Function of the Temperature

The viscosity of PF-127 solutions is dependent on the temperature in which the solution is stored or used and therefore the relationship between temperature and viscosity was examined. Close inspection of the curves, for the concentration range 18 – 25% w/w F127 in the temperature range 5 – 20 °C shows an initial decrease in the bulk viscosity as the temperature in increased, after which the viscosity increases. Figure 4.17 shows the impact of the initial warming on the viscosity of PF-127 for a 20% w/w solution as an example of this phenomenon.

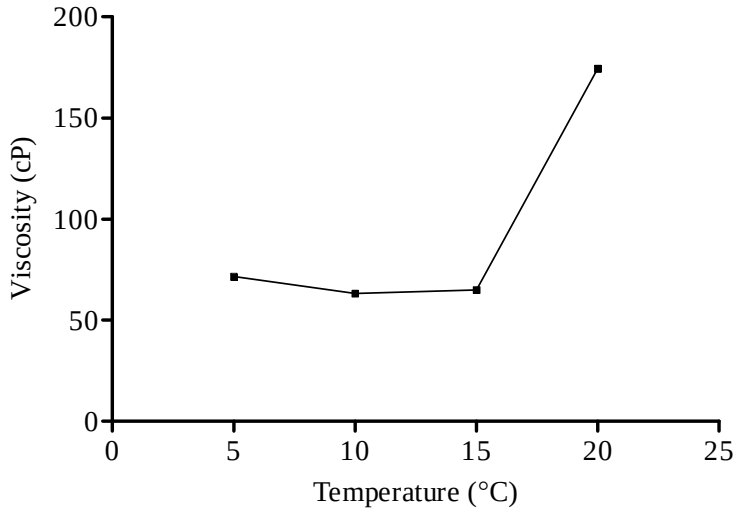


Figure 4.17 *Relationship between the initial temperature increase and the viscosity of a 20 % w/w solution of PF-127*

Lenaerts *et al.* [493] noted a similar phenomenon to that observed for the 20% w/w solution of PF-127 that is illustrated in Figure 4.17. The initial decrease in viscosity is most likely due to a rise in the activation energy of the system due to the increase in the temperature and was noted for solutions of PF-127 in the concentration range of 18 – 25% w/w where no gelation occurred at these temperatures. The slight decrease in viscosity as the temperature is increased has an

important implication for the clinical use of dosage forms manufactured from PF-127 polymers. Following removal of the ampoule/vial from a refrigerator, the dosage form can be allowed to warm slightly, thereby allowing a less viscous liquid to form and be administered conveniently.

As the temperature is increased from 5 °C to approximately 25 °C, there is a small rise in viscosity for each solution tested, followed by a dramatic and rapid increase in the bulk viscosity of the system, depending on the concentration of PF-127 as shown in Figure 4.18. The rapid rise in viscosity occurs concurrently with visual observations of gelation.

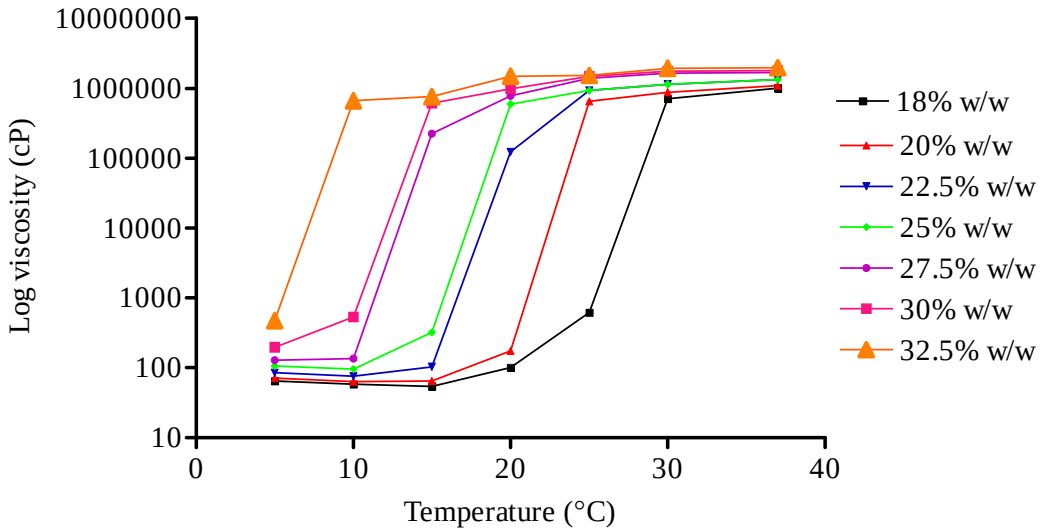


Figure 4.18 *Effect of increasing the temperature on the viscosity of different concentrations of PF-127 in solution*

It is clear on visual inspection of the curves shown in Figure 4.18 that the temperature at which the gelation occurs is dependent on the concentration of polymer in solution and in general occurs at lower temperatures for solutions of higher concentration of PF-127. These observations are in agreement with previously published work [493,494,497,498].

There is a rapid increase in the viscosity at T_m1 for the PF-127 systems studied and the increase in the viscosity is primarily due to the presence of micelles at the high concentrations of poloxamer. As the temperature is increased, the micelles undergo swelling and increase in size. Conformational changes that occur as the temperature is increased are due to de-solvatation of water from the micelles resulting in polymeric units and the different micelles being exposed to each other with a resultant increase in the bulk viscosity of the system as the temperature is increased. It is thought that pseudo-cross-linkages occur between the PF-127 units of different micelles that subsequently cause an increase in the viscosity [493].

4.5.7 Gravimetric Analysis

4.5.7.1 Introduction

Pluronic® copolymers undergo slow dissolution or erosion when exposed to aqueous media, a phenomenon that has been studied by several researchers [460,499-502]. Furthermore, it has been shown [500-502] that there is a strong correlation between the rate of erosion and the rate of drug release from poloxamer gels with resultant r^2 values of > 0.99 obtained when modelling the relationship between the rates of gel dissolution and drug release.

It has also been shown that the dissolution rate of the gel is dependent on the concentration of the gel former present in the dosage form and formulations with lower concentrations of PF-127 resulting in a faster rate and extent of drug release [500,501]. This is expected since as PF-127 concentrations increase there is an increase in the viscosity of the gels (§ 4.5.6) with a corresponding decrease in the rate of drug release [500-502].

4.5.7.2 Gravimetric Analysis

The rate of gel dissolution of PF-127 formulations of three (3) different concentrations was determined using the method described in § 4.4.2.9 and are summarised in Figure 4.19 and Table 4.4, which summarises the linearity curves for the dissolution of PF-127 under the specified conditions. The linearity curve for 20% w/w PF-127 gel was determined from t = 0 min to t = 300 min after which 100% of the gel had dissolved.

Table 4.4 *Dissolution curve parameters summary (y = mx + c) for PF-127 gels*

Concentration of PF-127 (% w/w)	Slope	Intercept	R^2
20	0.3747	2.4191	0.9934
25	0.2055	2,2575	0.9975
30	0.1459	2.5931	0.9978

As expected, the rate and extent of dissolution of the gel decreased with an increase in PF-127 concentration in the formulation. The rate of erosion was found to be zero order (r^2 > 0.99) for all three (3)-gel formulations studied. Therefore, the rate of release of a drug included in such formulations would be expected to be zero order if dissolution of the polymer is the only mechanism by which drug is liberated from the polymer matrix. The mechanism of release will be discussed further in Chapter 5, *vide infra*, where mathematical models were used to assess the mechanism of drug release from these systems.

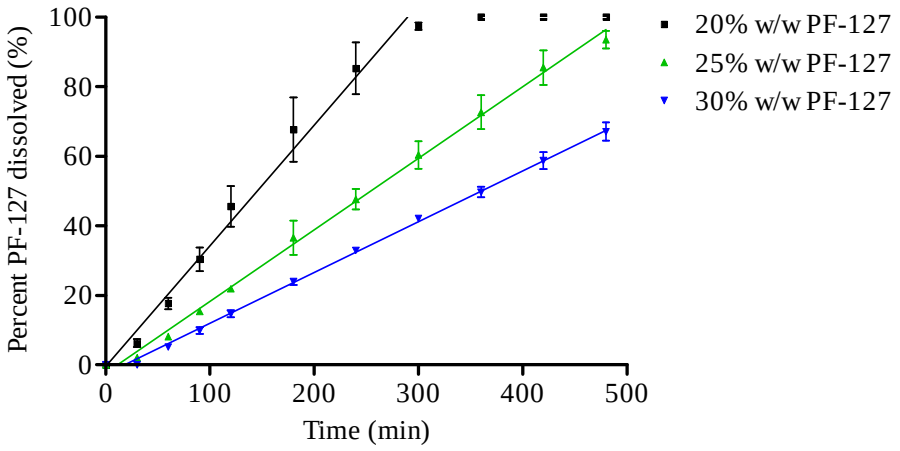


Figure 4.19 *The influence of PF-127 concentration on percent gel dissolution in an aqueous medium*

4.6 CONCLUSION

Preformulation studies of a primary excipient allow for appropriate formulation improvements and decisions to be made when developing dosage forms. This information generated in these studies provides a formulation scientist with invaluable information prior to optimisation of the dosage form, preventing a waste of valuable resources and time during early stage development projects.

The data generated from spectral studies of PF-127, alone and in combination with OT gave preliminary information about the possibility of an interaction occurring between OT and the vehicle of choice that may have had an impact on the integrity and the performance of the final dosage form. From preliminary studies using UV and IR, it was concluded that the vehicle was suitable for use with OT because of the likelihood of minimal interaction.

The use of an analytical method for PF-127 in solution was developed and validated by assessing linearity, accuracy, and precision parameters. This method will be invaluable in the formulation process and may be used to reveal information about the potential mechanism of drug release from the gel. The analytical method can be applied to the determination of the amount of gel that has dissolved at different time points during *in vitro* release testing and can therefore be used in conjunction with mathematical models and drug release rates in elucidating the mechanism of release of OT from PF-127 formulations [460].

The determination of the CMC of PF-127 was vital in the development of a dissolution method for the drug to develop an *in vitro* release test method in which micelle formation was avoided. The information is critical in assessing dosage forms where poorly soluble drug candidates and/or excipients such as preservatives may be solubilised thereby reducing the efficiency of the test method. The use of PF-127 as micelles as carriers for drugs has been evaluated by Kabanov *et al.* [443,444] and therefore the ability of PF-127 to solubilise API and other excipients is a possibility that must be considered during formulation and *in vitro* assessment of PF-127 containing dosage forms.

The thermodynamic properties of PF-127 were also characterised since the delivery system was intended to be a thermoresponsive system that exists as a flowable viscous liquid at low temperatures and forming a stiff gel at body temperature and a potential depot for sustained drug release. Knowledge of the transition temperatures is important in establishing storage conditions for the dosage form and for subsequent handling of that dosage form prior to clinical use. For example, the transition temperature for a formulation to be usable must preferably be above approximately 15 °C, to allow the formulation to remain in a syringeable form following removal of the product from the refrigerator. The formulation of products with solutions above approximately 25% w/w PF-127 is therefore limited because of the increased viscosity above this temperature. In addition, the low transition temperatures for higher concentrations (> 25% w/w) of PF-127 means that on slight warming (> 10 °C) they form stiff gels and can not be administered using a syringe and a needle. Furthermore, the second transition temperatures, T_m2 for the concentration range 18 – 25% w/w PF-127 solutions were determined and the thermo-reversible nature of the copolymer was confirmed. This information may be important in the formulation of the parenteral dosage form, indicating that heating the gel solutions, e.g. in sterilisation processes will not interfere with the integrity of the dosage form, and therefore this method can be used for sterilisation. The investigation of the effect of additives on the transition temperatures is also important as this allows the formulation scientist to consider the potential impact of these additives on product performance during formulation development. The potential additives that were investigated *viz.*, sodium chloride, acetate buffer at pH = 4 and chlorbutol were found to decrease the transition temperatures, but not to any great extent although it is imperative that formulation optimisation must be performed in the presence of the additives.

The assessment of the rheological properties of such systems is central in the development of sustained release dosage form as such properties are likely to impact on the release of API from

the dosage form following administration of the product. In addition, the behaviour of the viscosity as a function of temperature is of critical importance when establishing clinical use parameters of such systems. For example, it is necessary to answer questions such as to what extent does the viscosity change on warming to room temperature and can the dosage form still be administered using a syringe. Solutions of PF-127 containing lower concentrations of gel former, *viz*., 18 – 25% w/w when warmed from 5 °C to approximately 20 °C show a slight decrease in the viscosity and this is a potentially useful property for the use of PF-127 as a gel for sustained release as the reduced viscosity would allow the administration of the formulation with a needle. Higher viscosities for higher concentrations of PF-127 (> 25% w/w) solutions limit the clinical application of these gels compared to their more dilute counterparts. Although higher viscosity solutions of PF-127 have the potential of a more sustained release rate of API, the syringeability is limited. Another important consideration with respect to the viscosity is the clinical administration of the drug via a needle and the size of the syringe and needle assembly that would be necessary. Further evaluation that is recommended is an investigation of the flow properties of the drug and monitoring how the viscosity is affected by changes in the shear rate and the temperature.

Gravimetric analysis was used to assess the impact of dissolution of the gel in an aqueous medium and was considered appropriate since the rate of polymer dissolution is likely to influence the rate and extent of drug release from such gels. Gravimetric analysis yields key information to the formulation scientists and allows strategies to be put in place to increase or reduce the rate of dissolution in order to design a formulation with a specific and desired rate of drug release. Strategies could involve the inclusion of additional polymers that can retard the rate of dissolution of the gel without influencing significantly on the viscosity or chemical modification of PF-127 [503,504], which has been performed in an attempt to increase gel viscosity.

PF-127 was found to be a potentially useful vehicle for the formulation of a sustained release dosage form of OT, since it has no obvious potential for interaction with the drug. Furthermore, PF-127 gels have the potential to produce thermoresponsive delivery systems that undergo viscosity increases on warming, thereby proving suitable for the production of a depot injection for the prolonged release of OT release. Furthermore, the dosage form can dissolve in aqueous medium facilitating release of OT from the depot.

CHAPTER 5

THE DEVELOPMENT OF AN *IN VITRO* RELEASE TEST FOR THE ASSESSMENT OF OXYTOCIN RELEASE FROM PLURONIC® F127 GELS

5.1 INTRODUCTION

The value of *in vitro* drug dissolution and release testing in the pharmaceutical industry cannot be overlooked as information gained from such testing is used for quality control purposes, batch uniformity assessments, and homogeneity testing of dosage forms within a batch. Prediction of *in vivo* behaviour of dosage forms and assessments of the impact of formulation changes or method of manufacture on overall dosage form performance *in vivo* may be inferred from dissolution profiles [505]. In addition, *in vitro* release profiles generated during testing reveal information about the structure of disperse systems and behaviour of formulations at a molecular level, as well as possible interactions between a drug molecule and polymer matrices in which the molecule is contained. The mechanism of drug release may also be inferred from *in vitro* release profiles by the application of mathematical models to those profiles [506].

The intrinsic variability of *in vitro* release testing warrants careful development of methods in order for a test to reflect the true drug release characteristics from a drug delivery system. The type of drug and apparatus that are used must be carefully considered as these factors can influence the rate and extent of drug release obtained during *in vitro* release testing [507]. The selection of an *in vitro* release method and dissolution medium must consider the potential for an *in vitro in vivo* correlation of the method of choice, if possible [508].

There are no accepted compendial guidelines for *in vitro* testing to assess drug release for controlled and sustained release parenteral formulations, such as those made from PF-127. The USP [10] provides guidance for dissolution testing of oral and transdermal dosage forms, but no guidelines for assessing the *in vitro* release of an active ingredient from controlled release (CR) parenteral preparations are reported.

The objective of these studies was to develop an appropriate discriminatory *in vitro* test for assessing formulation factors that may affect drug release during early formulation development studies for an OT formulation prepared from PF-127 as the gel-forming matrix. An appropriate release assessment method should be able to discriminate between the performance of different

formulation variants [496,509] and this was the primary criterion used for selection of an appropriate method during the *in vitro* release test method development process. Mathematical models and statistical tests were used to evaluate the discriminatory behaviour of different release test methods that were investigated in these studies and were also applied to drug release data generated using the different release test methods to assess the impact of the test method on possible mechanisms of OT release from the dosage form.

5.2 METHODS FOR ASSESSING *IN VITRO* RELEASE OF PARENTERAL PRODUCTS

5.2.1 Introduction

The selection of an appropriate apparatus for *in vitro* release testing of CR parenteral systems, including microparticulate, nanoparticulate, hydrogel and liposomal dosage forms, has been the subject of several conferences and publications [496,508-511]. Important considerations for the evaluation of CR parenteral systems include the use of appropriate apparatus, dissolution media, sampling methods and total percent release attainable from an *in vitro* release test method [496]. Guidelines for the evaluation of novel delivery systems such as orally disintegrating and chewable tablets have been reported although recommendations for parenteral formulations have not yet been established. However, the use of compendial and modified flow-thorough cell apparatus has been applied with some success [509]. The most commonly used methods that have been used for *in vitro* release methods for CR parenteral systems can be classified into three broad classes, *viz.*, sample and separate, continuous flow and dialysis methods [511].

5.2.2 USP Dissolution Apparatus

Compendial apparatus recommended in the USP [10] for *in vitro* release testing have specific attributes that may result in each apparatus not being entirely suitable for *in vitro* release testing of CR parenteral preparations. USP Apparatus 1 (basket) and 2 (paddle) were designed for testing immediate and modified-release oral formulations and USP Apparatus 3 (reciprocating cylinder) and 4 (flow through cell) are appropriate for testing of extended release oral preparations. USP Apparatus 5 (paddle over disc), 6 (cylinder) and 7 (reciprocating holder) are recommended for *in vitro* testing of transdermal dosage forms. It has been reported that USP Apparatus 1 and 2 may not be entirely suitable for CR parenteral products but Apparatus 3 and 4 may be useful with appropriate modifications [496].

The use of the USP paddle apparatus is recommended for testing drug release from tablets, at a stirring speed of 50 rpm, while the USP basket apparatus is set at a speed of 100 rpm for pharmaceutical dosage forms that tend to float in dissolution media including capsules [512]. The USP basket and paddle apparatus contain a cylindrical vessel with a hemispherical bottom, which may be covered, a motor with a metallic drive shaft and a cylindrical basket or paddle, respectively. The vessel is placed in a water bath maintained at 37 $\pm$ 0.5 °C. There is no source of agitation, motion, or vibration except that provided by the rotating stirring element connected to a cylindrical basket or paddle. A dosage form is then placed into the basket when using the USP Apparatus 1 or submerged at the bottom of the vessel when USP Apparatus 2 is utilised, prior to the commencement of the dissolution test [10]. Sampling of dissolution medium may be performed at predetermined intervals with or without replacement of the dissolution medium.

One of the main disadvantages of using USP Apparatus 1 or 2 for the assessment of drug release from parenteral formulations is that a large volume of dissolution medium is used in the test procedure. Large volumes of test medium may not be relevant to small volume parenteral preparations for intramuscular and/or subcutaneous administration, as they are not exposed to such large volumes of plasma, *in vivo* [508]. Furthermore, the presence of a stationary dissolution medium limits the suitability of the basket and paddle apparatus for testing such products, as pharmaceutical preparations for parenteral use are exposed to dynamic body fluid compositions and volumes *in vivo*. Another disadvantage of the paddle apparatus is containment of the samples to be tested in the dissolution vessel, although modifications to *in vitro* release apparatus may be attempted to overcome the shortcomings of the apparatus for parenteral preparations. These include the use of a sinker to submerge a dosage form and the use of dialysis tubing for sample containment. Disadvantages in the use of sinker and dialysis tubing modifications are that resultant uneven dissolution of drug from the dosage forms being tested may produce unreliable and poorly reproducible results in the former, or aggregation of microspheres and prevention of gel dissolution and violation of sink conditions in the latter cases [508].

The USP Apparatus 3 or reciprocating cylinder apparatus is made up of rows and columns of outer flat bottom glass vessels, a set of inner glass reciprocating cylinders with inert fittings and screens that fit the tops and bottoms of the cylinders. A motor and drive assembly is attached to the cylinders, which results in reciprocation of the cylinders vertically inside outer glass vessels containing the dissolution test medium. The outer vessels are immersed in a water bath that is maintained at 37 $\pm$ 0.5 °C and only the reciprocating action of the cylinders provides significant

agitation to the system. An evaporation cap is used for the duration of an *in vitro* release test to minimise dissolution fluid loss over extended test periods. Dosage forms to be tested are placed inside the reciprocating cylinders prior to the commencement of a dissolution test and during a test, the cylinders move through several rows of dissolution media in the outer vessels at predetermined intervals [10]. The amount of drug released from a dosage form during an *in vitro* release test is determined by analysing the contents of the dissolution vessels. Modifications of the apparatus may make it more relevant for the testing of parenteral CR preparations and may be achieved by using small samples vials and small vessels, with or without agitation [508].

The USP Apparatus 4 or flow-through apparatus is made up of a reservoir with a pump, a flow-through cell that can be made of different sizes and a water bath, which is used to maintain the temperature of the reservoir at 37 $\pm$ 0.5 °C. The pump forces dissolution medium upward through a flow cell at different flow rates *viz.*, 4, 8 and 16 ml/min. A filter located at the top of the flow cell prevents the escape of undissolved drug particles and glass beads at the bottom of the cell protect the fluid entry probe. The tablet/dosage form to be tested is placed in the vessel prior to the commencement of the dissolution test [10]. The main advantages of the flow-through apparatus are the flexibility in the volume, sample cell and flow rate, suggesting that this apparatus may be the most suitable for the assessment of *in vitro* release from parenteral CR formulations [508].

There is consensus that the use of USP Apparatus 5, 6 and 7 do not offer any advantages for the *in vitro* release assessment of parenteral CR preparations [508]. Further research is necessary in order to design an appropriate *in vitro* release test apparatus that considers the geometry, hydrodynamics, dissolution media, and acceptance criteria that are suitable for the assessment of *in vitro* drug release from CR parenteral dosage forms [496].

5.2.3 Sample and Separate Methods

Sample and separate methods are the most extensively used techniques for assessing *in vitro* drug release from parenteral CR systems [513-519] and are extensively used for testing of microparticulate dosage forms, but have not found much use in the assessment of gel systems such as those manufactured using PF-127. Drug loaded microparticles are introduced into a media containing vessel and drug release is assessed over a specified time. The differences in

the reported techniques are variation in the size of the container, presence, or absence of agitation and sampling methods that are used in monitoring *in vitro* drug release.

Tubes or vials may be used to contain the dissolution medium when small volumes of dissolution media are used or alternatively large bottles or Erlenmeyer flasks can be used for *in vitro* release tests that require larger volumes of fluid. Agitation may be constant, intermittent or absent and possible sources of agitation include the use of magnetic stirrers, rotating shakers or incubating water baths. Filtering or centrifuging of aliquots prior to quantitation may be necessary depending on the type of dosage form being tested and sink conditions can be maintained by replacing the medium after sampling [511]. Sample and separate methods are useful for dosage forms where the dispersed phase can be separated from the dissolution medium without influencing the overall *in vitro* release profile generated [506]. The main advantages of this test method are that it is relatively easy to set up, quick and accurate, although the aggregation of microparticles may present an analytical challenge [511]. The utility of sample and separate methods is also limited by the size and size distribution of the microparticles in a formulation, which may be difficult to filter or centrifuge if their particle size is too small [506].

5.2.4 Continuous Flow Methods

Continuous flow methods describe modifications to the USP flow-through apparatus and other similar techniques where dissolution medium is circulated through a column/cell that contains a drug-loaded CR parenteral dosage form and the *in vitro* release characteristics of a drug are assessed over time. Variations in the medium, apparatus set-up, pumps and flow rates make each method specific for a dosage form [511]. Continuous flow methods that have been reported are varied and include placing a sample in a filtration cell and withdrawing samples at specified intervals from the cell and replacing fresh medium into the cell [520]. Modifications to this method include the use of jacketed column or water-bath to maintain the temperature at 37 °C [511]. An example of a custom made continuous flow system [521] is depicted in Figure 5.1 and that of a commercial continuous flow system is shown in Figure 5.2 [522].

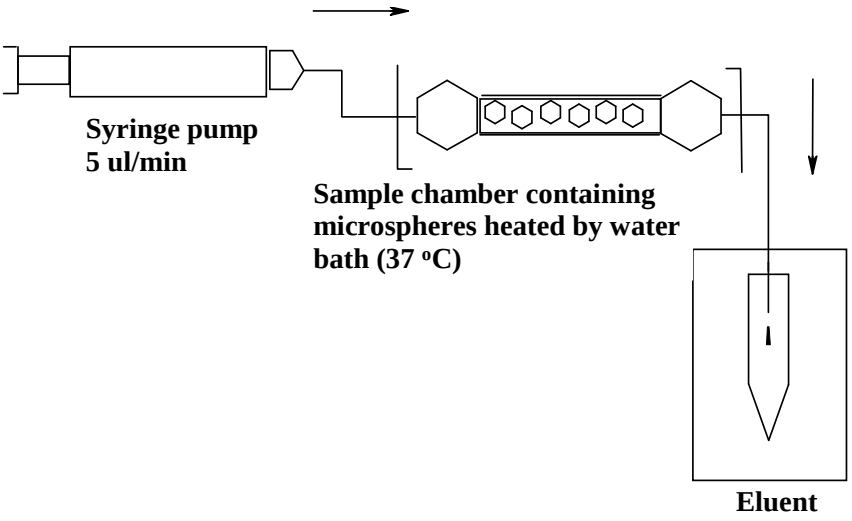


Figure 5.1 *Apparatus for a continuous flow assessment method using a syringe pump (adapted from [521])*

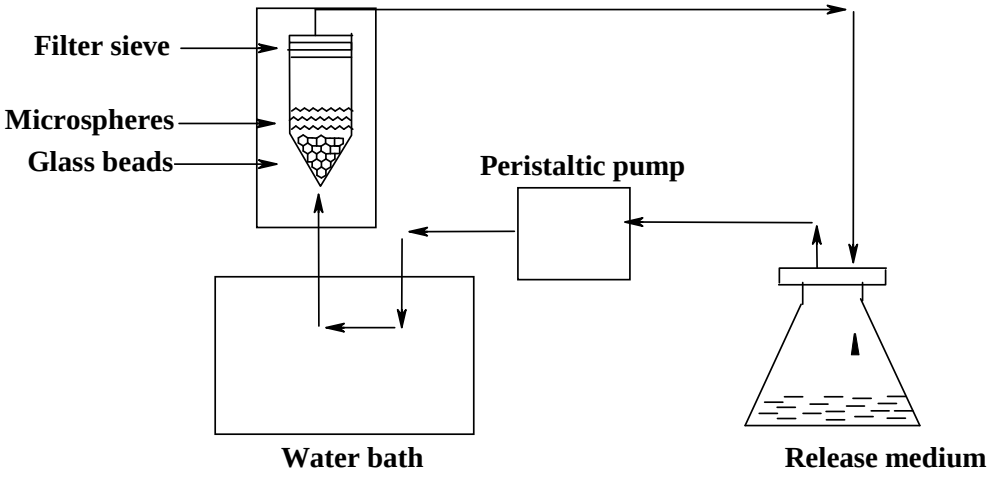


Figure 5.2 *Commercial apparatus for continuous flow assessment (adapted from [522])*

Peristaltic pumps and syringes or HPLC pumps may be used to ensure a constant flow rate of the dissolution medium is maintained and modifications to the flow rate may be required to optimise *in vitro* release profiles for specific dosage forms. The use of low flow rates may result in incomplete release of a drug due to slow hydration and dissolution of a polymeric delivery system because polymer hydration may affect the rate of erosion of the polymer thereby affecting the overall rate of release rate of a drug from the delivery system [511].

The primary advantage of continuous flow methods for assessing *in vitro* release from CR parenteral formulations is that these systems attempt to simulate the *in vivo* environment by

providing a small dynamic volume of media that passes through a stationary depot of drug. In addition, these methods allow continuous sampling of the dissolution medium [511] and if the receptor medium is removed and replaced rapidly, the rate of release of a drug is proportional to the instantaneous release of the drug from the formulation, since there is no accumulation of drug in the receptor medium [506]. Limitations of continuous flow methods include variations in flow rate because of filters clogging when microparticulate systems are assessed. Using low flow rates may result in low release rates of drug, which may not be indicative of the *in vivo* performance characteristics of a delivery system. In addition, rapid replacement of buffer in the sampling procedure is quite difficult to achieve and may affect the resultant *in vitro* release profiles that are obtained [506,511].

5.2.5 Dialysis Methods

In dialysis methods, the physical separation of a drug loaded CR system from the dissolution medium is achieved by use of a dialysis membrane. Dialysis techniques have been used extensively for assessment of *in vitro* release characteristics from different dosage forms including parenteral depot dosage forms of an oily nature, suppositories, liposomes, nanoparticles, implants, and micelles [511]. Dialysis methods have also been used extensively for *in vitro* drug release characterisation of CR parenteral dosage forms [523-527].

Dialysis techniques usually include placing a formulation in a dialysis bag, which is then placed in a vessel that contains the dissolution medium. The diffusion of drug through the membrane may be enhanced by the use of agitation and this may be achieved by use of a horizontal shaker or USP Apparatus 2. The assessment of quantity of drug released is performed by sampling the bulk media over time and analysing the samples with an appropriate analytical technique [511].

Several modifications can be made to dialysis methods for the assessment of *in vitro* drug release from dosage forms and such variations include the use of a tube with a dialysing membrane at one end as shown in Figure 5.3. The tube and membrane were placed in an appropriate dissolution medium thereby allowing for the diffusion of drug through the dialysis tube into the medium that was sampled at appropriate intervals [528].

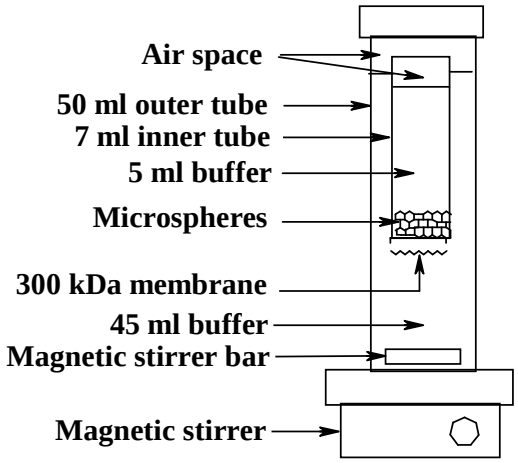


Figure 5.3 *Dialysis method with a membrane on one side (adapted from [528])*

The selection of membranes with specific molecular weight cut-offs (MWCO) may be arbitrary, but the MWCO must be sufficiently high to not limit diffusion of a drug entity from a dosage form into the dissolution medium [511]. The volume of medium in the dialysis bag must be at least 6 – 10-fold less than the volume of the bulk dissolution medium to ensure that sink conditions are maintained and that the driving force for drug transport-diffusion is unimpeded [511].

The advantages of using dialysis methods for the assessment of CR parenteral delivery systems include ease of sampling and medium replacement, as there is no interference of the dissolution medium by delivery systems. In addition, dialysis methods mimic *in vivo* conditions for subcutaneous and intramuscular administration, where a preparation is immobilised and permits only slow diffusion of a drug into the dissolution medium. However, the main disadvantage of dialysis systems is that sink conditions are not readily maintained, thereby limiting the utility and applicability of this type of method. The surface area of dialysis tube exposed to the dissolution medium affects the *in vitro* release profiles that are obtained since when a small surface area is exposed, the time taken for equilibration between the internal and external phases is increased with a subsequent decrease in the rate of release. Long equilibration times may limit the accurate analysis of drug release when a burst effect is present. Agitation may be used to minimise the effect of an unstirred water layer outside the membrane thereby reducing the time taken for equilibration, promoting of rapid release of drug from CR parenteral dosage forms [511].

5.2.6 Methods Used for *In Vitro* Release Assessment of Hydrogels

Hydrogels have been extensively explored as potential delivery systems for protein and peptide delivery systems, as was previously discussed in Chapter 3, *vide infra* and different methods have been used to assess the rate and extent of *in vitro* drug release from such systems.

For methods that do not use agitation, hydrogels containing drug are placed in a relatively large volume of a dissolution medium and aliquots are removed for analysis at predetermined sample times. The sample medium may be replaced with an equivalent volume maintaining sink conditions in the test system [529-534]. A variation in this method is one in which the hydrogel formulation is exposed to a small volume of dissolution medium on a surface of the formulation and the fluid is removed and replaced by fresh buffer at specific sample times [449,535].

Methods that use agitation have also been used for assessing the *in vitro* release of drug from hydrogel systems. By way of example, drug loaded hydrogel pellets were placed in a phosphate buffer in a shaking water bath that was maintained at 37 °C and agitation was achieved using a reciprocating motion at 100 rpm. The dissolution medium was sampled periodically with replacement of fresh buffer [536]. Ruel-Garièpy *et al*. [537] placed a drug containing hydrogel in circular moulds and the hydrogel was allowed to set at 37 °C for 12 hours, after which the gels were removed from the moulds and placed in histology cassettes. These were then suspended in 500 ml of phosphate buffered saline (PBS) at pH 7.4 in which the surfactant, sodium dodecyl sulphate was used to increase the *in vitro* release rate. A shaking incubator set at 100 rpm maintained at 37 °C was used and samples were drawn periodically [537]. Alternatively, the entire dissolution medium could be removed at sampling intervals and replaced with fresh medium [538-541] or different rates of agitation may be used e.g. 150 rpm [541,542], 100 rpm [537], 70 rpm [538], and 50 rpm [541].

Kim *et al*. [543] used a dialysis tube for *in vitro* testing. A known amount of drug containing hydrogel was placed in a dialysis bag with 1 ml of PBS, the bag was positioned in 10 ml PBS maintained at 37 °C and stirred at 100 rpm, and the entire medium was removed and replaced at specific sample times.

5.2.7 *In Vitro* Testing of Drug Release from PF-127 Systems

Several methods have been reported for the assessment of *in vitro* drug release from PF-127 systems that were intended for parenteral use, membrane-less diffusion systems and/or modifications thereof have been utilised [450,460,499-501,544-546]. In summary, a drug containing PF-127 formulation is placed in a small vessel, which is then placed in a larger vessel, such as a beaker and the dissolution medium is gently poured directly above the delivery system and is in direct contact with the gel layer. The drug released is then analysed from samples of medium following removal of the receptor medium at predetermined time intervals. The system is usually maintained at 37 °C for the duration of the experiment, and gentle stirring can provide agitation in these systems.

Anderson *et al*. [499] used a membrane-less diffusion system in which a petri dish fitted with a metal holding plate was placed in an inverted position in a beaker containing dissolution medium. Figure 5.4 depicts the apparatus that was placed in a beaker with the gel exposed to the dissolution medium.

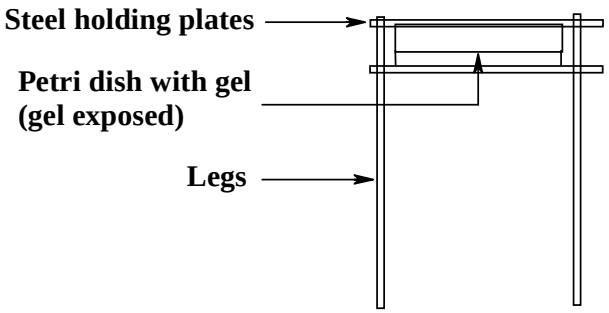


Figure 5.4 *Schematic diagram of the gel holder used by Anderson et al. (adapted from [499])*

Membrane diffusion systems have also been used for the assessment of PF-127 formulations [452,547,548]. In these systems, the gel layer is separated from the dissolution or receptor media by means of a non-rate limiting membrane and the receptor fluid is sampled for analysis at specified times. The test system is usually maintained at 37 °C for the duration of the *in vitro* release test.

A dialysis system has also been used for the assessment of *in vitro* drug release from PF-127 formulations [451], where a drug containing cold PF-127 solution was placed in a cellulose dialysis tube that provided a cylindrical gel compartment. The gel was allowed to stiffen prior to commencement of the *in vitro* test and the preparation was then placed into a receptor medium

and maintained at 37 °C with agitation by a magnetic stirrer bar. The amount of drug release was assessed following sampling of the receptor phase at predetermined times.

Paalova *et al*. [547] used a two compartment *in vitro* system depicted in Figure 5.5 to study drug release from PF-127 and other cellulose polymer gels. The donor compartment of the system was separated from the receptor fluid by a non-rate limiting membrane. The method was found to have a strong *in vitro in vivo* correlation since the amount of drug released and the pharmacological effect that was observed *in vivo* were directly related.

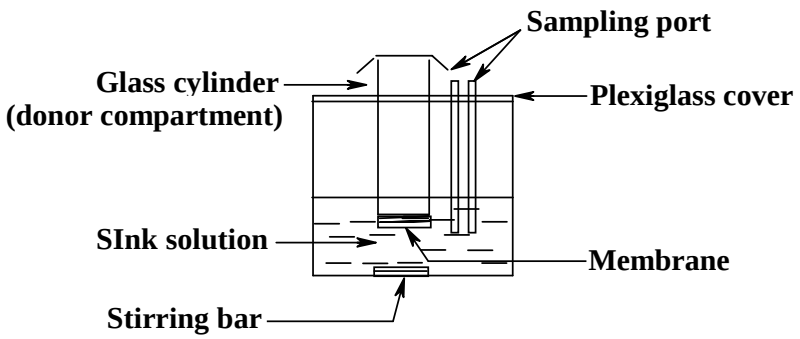


Figure 5.5 *A two-compartment dialysis method used to assess in vitro release from Pluronic® gels, (adapted from Paalova et al. [547])*

Moore *et al*. [502] used USP Apparatus 2 to determine drug release from PF-127 gels. The gels were placed in containers with a diameter of 60 mm and a height of 15 mm and drug release was assessed at 37 °C. The speed of rotation of the paddle was varied between 20 and 80 rpm to determine the effect on release rates and to develop a mathematical model to evaluate the release of drugs from dissolving PF-127 gel systems.

5.3 *IN VITRO* RELEASE TEST METHOD DEVELOPMENT

New technologies and advances in the development of CR delivery systems have mandated the development of specific *in vitro* release test methods. Furthermore, modification of current compendial methods or the development of new procedures may be necessary in order to suit the specific needs for product assessment. An in-depth understanding of drug release mechanisms and the hydrodynamics of *in vitro* dissolution test apparatus on is necessary in order to develop discriminatory *in vitro* release methods that produce reliable and reproducible data that is also biorelevant [549].

Biorelevance is an important consideration in the development of *in vitro* dissolution methods for CR parenteral products and where applicable, *in vitro* release tests should be developed for specific clinical outcomes. In order to achieve *in vivo* relevance, consideration of the physiological site of administration including body temperature, muscle pH, buffer capacity, and dosage form considerations such as volume and osmolarity of a product must be considered. Another important consideration is the duration over which the *in vitro* release test is conducted and sampling times and intervals in order to, where possible, mimic *in vivo* release characteristics and generate relevant drug release profiles [497,509,510]. For CR parenteral products, a multipoint drug release profile is recommended for the appropriate assessment of dosage form performance [509].

A recommended approach for *in vitro* release test method development has been described [496,508] as follows:

i. Identification of appropriate dissolution medium/media and the conditions for ensuring reproducible release rates,
ii. Preparation of different formulation compositions that are likely to result in changes in the performance of a dosage form,
iii. Testing of different formulations both *in vitro* and *in vivo* and,
iv. Modification of the *in vitro* test method to allow for discrimination between the different formulations that have different *in vivo* release profiles.

The extent of drug release is a further consideration and for *in vitro* release assessment of CR parenteral delivery systems, a desirable outcome is that at least 80% be released as this is considered to be economical and safe, reducing costs and maintaining a low toxicity profile respectively [508]. *In vitro* release methods that are developed must also be able to identify burst release of the API and in cases where burst release occurs, the duration and extent of the burst release must be characterised as this may affect the toxicity of a dosage form and produce unwanted effects *in vivo* [508].

5.4 COMPARISON OF *IN VITRO* RELEASE PROFILES

5.4.1 Introduction

The comparison of *in vitro* release profiles can be achieved by the use of mathematical methods of analysis. Mathematical analysis for assessment of *in vitro* release profiles can be divided into

3 types *viz*, model-dependent (curve fitting), model-independent methods and statistical analysis [550].

5.4.2 Statistical Methods

Statistical methods have been used for the evaluation and comparison of dissolution profiles following *in vitro* dissolution testing [551,552] and for development and optimisation of *in vitro* dissolution tests [553-555]. Statistical methods can be used to compare different formulations tested under the same experimental conditions as well as for the comparison of dissolution tests for the same formulation tested under different test conditions [550,553,554].

Analysis of variance methods can be distinguished into univariate or one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA). ANOVA tests may be used to assess the difference between means of two dissolution or drug release data sets in single time point dissolution tests and MANOVA analysis is useful for the evaluation of multiple time point dissolution tests [550]. It has been reported that ANOVA analysis is more informative and is easier to interpret compared to MANOVA analysis, which requires data transformation on repeated measures and makes interpretation of results difficult [552].

For the comparison of dissolution of *in vitro* release profiles a repeated measures design may be used, where the percent drug dissolved is the dependent variable and time is the repeated factor [552]. Univariate ANOVA analysis can be applied at each time point when comparing dissolution profiles to determine where differences, if any, exist between the dissolution profiles being compared [552]. Different *post hoc* tests can be used to determine the exact points of difference between dissolution profiles including the Least Significant Difference Test, Tukey's Multiple Range Test, Scheffé Method, Newman-Keuls Test and Dunnett's Test [556].

ANOVA based methods of analysis reveal information about differences in the shape and levels of different dissolution data sets that are being compared [552], although they have been criticised for being too discriminatory, showing statistical differences in dissolution profiles that may not necessarily reflect pharmaceutical differences [551].

5.4.3 Model Independent Methods

5.4.3.1 Introduction

Model-independent methods make no assumptions about the shape of the curves being evaluated and are used to compare drug release profiles from different data sets. Model-independent methods can be divided into ratio tests and pair-wise procedures.

5.4.3.2 Ratio Test Procedures

In ratio test procedures, the ratio between parameters derived from dissolution data from a test and reference product are compared at the same time point. The test procedures vary in complexity for the analysis of selected parameters. Ratio test procedures can include the evaluation of the ratio of percent released/dissolved ($t_{x\%}$) at a certain time point, area under the curve (AUC) or ratio of the mean dissolution time (MDT) [550,551]. The relationship that may be used to calculate the MDT, which is the sum of the individual times during which a specific fraction of the total dose in a dosage form is released is depicted in Equation 5.1 [550].

$$MDT = \frac{\sum_{j=1}^{n} t_j \Delta M_j}{\sum_{j=1}^{n} \Delta M_j} \qquad \textbf{\textit{Equation 5.1}}$$

Where,

j = sample number

n = number of dissolution sample times

t_j = time at midpoint between t_j and t_{j-1}

ΔM = additional amount of drug dissolved between t_j and t_{j-1}

5.4.3.3 Pair-wise Procedures

Examples of pair-wise test procedures include the Rescigno index [557] and the difference (f_1) and similarity (f_2) factors [558].

The bioequivalence index derived by Rescigno [557] was proposed as useful for the comparison of drug plasma levels as a function of time for a test and reference product. The same index may be applied to the comparison of *in vitro* dissolution profiles obtained when comparing drug release from a test and reference product. The expression used to calculate the Rescigno index for the comparison of dissolution profiles is depicted in Equation 5.2 [550,551].

$$\xi_i = \left(\frac{\int_0^\infty \left| d_R(t) - d_T(t) \right|^i dt}{\int_0^\infty \left| d_R(t) + d_T(t) \right|^i dt} \right)^{1/i}$$ ***Equation 5.2***

Where,

$d_R(t)$ = amount/percent of drug dissolved for reference product at time t

$d_T(t)$ = amount/percent of drug dissolved for test product at time t

i = any positive integer

t = time

The Rescigno index is always a fraction between zero (0) and one (1). The index is zero (0) when the test and reference and products are identical and in cases where no drug is released from either the test or reference formulation, the index is equivalent to one (1) [557].

Moore and Flanner [558] proposed the use of difference and similarity factors, f_1 and f_2 respectively, for the comparison of dissolution profiles and these factors are known as fit factors. The difference factor, f_1 measures the percent error between dissolution profiles of a test and reference product at all time points. The percent error is zero (0) when the *in vitro* release profiles of the test and reference product are identical and this value increases proportionally as dissolution profiles become dissimilar [558]. The similarity factor, f_2 is a logarithmic transformation of the sum-squared error of the differences between a test and reference product over all time points. The factor falls between 0 and 100 and a value of 100 indicates that the dissolution profiles of a test and reference product are identical. At least three (3) or four (4) dissolution time points are needed to calculate the similarity factor, f_2 [558]. The difference and similarity factors can be calculated from Equations 5.3 and 5.4, respectively.

$$f_1 = \left\{ \frac{\sum_{t=1}^{n} \left| R_t - T_t \right|}{\sum_{t=1}^{n} R_t} \right\} \times 100$$ ***Equation 5.3***

$$f_2 = 50 \log \left\{ \left[1 + \frac{1}{n} \sum_{t=1}^{n} w_t \left(R_t - T_t \right)^2 \right]^{-0.5} \times 100 \right\}$$ ***Equation 5.4***

Where,

n = number of dissolution time points

R_t = percent of the reference product dissolved at time t

T_t = percent of the test product dissolved at time t

w_t = an optional weight factor, usually set at 1

In general, if the f_1 value is lower than 15 and the f_2 value falls between 50 and 100, then the two dissolution profiles being compared can be considered to be similar [558]. The Centre for Drug Evaluation and Research (FDA) [512,559] has adopted the use of the similarity factor, f_2 as a valid criterion for the comparison of *in vitro* dissolution profiles between test and reference products. The guidance to industry [512,559] recommends that the similarity factor be used to compare *in vitro* dissolution profiles when at least 12 individual dosage units are used.

Recommendations for the use of the f_2 similarity factor include that the number of sample points included in the calculation should be limited to no more than one after more than 85% of the label claim of the product has been released, since the sensitivity of the test decreases after this point and fails to discriminate between measurements [560].

The main advantages of the use of the f_1 and f_2 difference and similarity factors are that they are easy to compute and each gives a single number that indicates the degree of closeness between the two *in vitro* dissolution profiles being compared [552]. However, limitations in the use of the similarity factor include failure to take into account the shape of the curve and unequal spacing between time points on *in vitro* release profiles. Furthermore, it is impossible to evaluate false positive and false negative values when the similarity factor is used and the similarity factor is often too liberal in concluding similarity between two dissolution profiles [550].

5.4.3.4 Other Model-Independent Methods

A newer model-independent approach has been proposed by Gohel and Panchal [561] for the comparison of dissolution profiles of a test and reference product and this model uses a similarity factor, S_d for the comparison. The Gohel similarity factor can be calculated using Equation 5.5. [561].

$$S_d = \frac{\sum_{t=1}^{n-1} \left| \log\left(\frac{AUC_{Rt}}{AUC_{Tt}} \right) \right|}{n-1}$$ ***Equation 5.5***

Where,

n = number of data points collected during an *in vitro* release test

AUC_{Rt} = area under the curve of the reference product at time t

AUC_{Tt} = area under the curve of the test product at time t

Two dissolution profiles are considered similar when the S_d value is zero (0) or close to zero (0). The advantages of using the similarity factor for the comparison of dissolution profiles are that the method is simple and flexible as the data for dissolution profile comparison may be expressed as either amount or percent release/dissolved [561]. The calculation of the S_d value may be used to infer a percent difference between the formulations being tested and such differences have been reported and are summarised in Table 5.1 [561].

Table 5.1 *Standard data for Gohel similarity factor, S_d*

Percent difference	Sd
0	0.0000
5	0.0212
10	0.0414
15	0.0607
20	0.0792
25	0.0969
30	0.1139

The dissolution efficiency (DE) of a pharmaceutical dosage form is defined as the area under the dissolution curve up to a specific time, *t*, expressed as a percentage of the area of a rectangle representing 100% release following dissolution at the same time, *t* [562]. DE can be calculated using Equation 5.6.

$$D.E. = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100\%$$ ***Equation 5.6***

Where,

y = percent of drug released at time t

t = time

5.4.4 Model-Dependent Methods

5.4.4.1 Introduction

Model-dependent methods make assumptions about the shape of the curve, fitting data using equations in which parameters defining the shape of the curve are optimised [563]. Commonly used model-dependent methods that have been used to characterise dissolution curves include zero order, first order, Higuchi, Hixson-Crowell, and the Korsmeyer-Peppas models [550]. The models can be used to evaluate the mechanism of drug release from delivery systems and are useful in understanding formulation factors that alter the *in vitro* release of an API from a dosage form, thereby optimising formulation development [564]. Furthermore, drug release from hydrogel systems can be characterised by the primary mechanism of release, which may be diffusion-controlled, chemically-controlled, and/or swelling controlled systems [423].

5.4.4.2 Zero Order Model

The zero order model can be used to model drug release from dosage forms that do not disaggregate and that release the same amount of drug per unit time to obtain a predictable and sustained release of a medicinal agent [550]. The zero order model can be expressed using Equation 5.7.

$$Q_t = Q_0 + K_0 t \qquad \textbf{\textit{Equation 5.7}}$$

Where,

Q_t = amount of drug released at time t

Q_0 = initial amount of drug present in solution

K_0 = zero order release constant

t = time

Diffusion controlled systems consist of a polymeric membrane which encapsulates an API within a core and the membrane separates the API from the external environment [423]. Drug release from encapsulated systems often follows a zero order model and is independent of time for planar, cylindrical and spherical geometries. The rate of release from a spherical diffusion controlled system may be determined by use of Equations 5.8 and 5.9.

$$\frac{dM_t}{dt} = \frac{4\pi D_{ip} K}{(r_e - r_i)/(r_e r_i)}(c_{i2} - c_{i1}) \qquad \textbf{Equation 5.8}$$

$$M_t = \frac{4\pi D_{ip} K (c_{i2} - c_{i1})}{(r_e - r_i)/(r_e r_i)} \qquad \textbf{Equation 5.9}$$

Where,

M_t = amount of drug released at time t

$\frac{dM_t}{dt}$ = rate of change of the amount of drug released per unit time

D_{ip} = diffusion coefficient of species i

K = drug partition coefficient

r_e = external radius of sphere

r_i = internal radius of sphere

c_{i1} = drug concentration of species i inside polymer matrix

c_{i2} = drug concentration of species i outside polymer matrix

In reservoir diffusion systems as described, drug release is controlled by the geometry of the system [423].

5.4.4.3 First Order Model

The application of a first order model to describe the *in vitro* release/dissolution kinetics of API was reported by Gibaldi and Feldman [565]. The first order release model may be expressed mathematically using Equation 5.10.

$$\ln Q_t = \ln Q_0 + K_1 t \qquad \textbf{Equation 5.10}$$

Where,

Q_t = amount of drug release in time t

Q_0 = initial amount of drug present in solution

K_1 = first order release constant

t = time

Water-soluble drugs that are formulated into porous matrices are released according to first order kinetic principles [550,566] and that the rate of release is proportional to the amount of

drug remaining to be released from a matrix. The rate of release therefore declines with time as the amount of drug remaining in the dosage form diminishes over time.

5.4.4.4 Higuchi Model

The models for drug release developed by Higuchi [567,568] describe the mechanism of release of water-soluble and water-insoluble drugs that were homogeneously incorporated into non-eroding semi-solid and/or solid matrices. A simplified version of the Higuchi model is depicted in Equation 5.11.

$$Q_t = K_H t^{1/2} \qquad \textbf{Equation 5.11}$$

Where,

Q_t = amount of drug released at time t

K_H = Higuchi dissolution constant

t = time

The Higuchi model describes drug release based on Fick's First Law of diffusion and release is considered to be diffusion controlled if the release of drug is square root time dependent [550].

5.4.4.5 Hixson-Crowell Model

The Hixson-Crowell model describes drug release from systems that undergo a change in diameter and surface area with proceeding drug release. The model is applied to dosage forms where dissolution occurs in planes that are parallel to the surface of the dosage form and where the dimensions of the dosage form diminish in a proportional manner over time [550]. The Hixson-Crowell model can be described using Equation 5.12.

$$Q_0^{1/3} - Q_t^{1/3} = K_s t \qquad \textbf{Equation 5.12}$$

Where,

Q_0 = initial amount of drug in dosage form

Q_t = amount of drug remaining in dosage form at time t

K_s = constant incorporating the surface/volume ratio

t = time

5.4.4.6 Korsmeyer-Peppas Model

Korsmeyer *et al*. [569] proposed a semi-empirical comprehensive model known as the power law, to describe mechanism of release from different dosage forms and the power law may be described mathematically as shown in Equation 5.13 and Equation 5.14, which depicts the logarithmic transformation of the relationship.

$$\frac{M_t}{M_\infty} = kt^n \qquad \textbf{\textit{Equation 5.13}}$$

$$\log\left(\frac{M_t}{M_\infty}\right) = n\log t + \log k \qquad \textbf{\textit{Equation 5.14}}$$

Where,

$\frac{M_t}{M_\infty}$ = fraction of released drug

k = constant incorporating geometric characteristics of dosage form

n = release exponent indicating mechanism of release

t = time

Drug release from hydrophilic matrices is controlled by the inward flux of solvent into the interior of the delivery system, which results in swelling of a polymer. In the initial stages of swelling of a hydrogel, two distinct phases are observed, *viz*., an inner glassy phase and outer swollen rubbery phase, from which drug molecules are able to diffuse [423]. The phenomenon of macromolecular relaxation occurs at the glassy-rubbery interface and the extent to which this affects the release of drug from the delivery system can be inferred from the exponent, *n*.

The numerical value of the exponent, *n*, describes the mechanism of drug release from a dosage form [570]. For a slab, when $n = 0.5$ the mechanism of drug release in the system is governed by Fickian diffusion and is termed Case I transport. When *n* lies between 0.5 and 1.0 then mass transfer of drug from the matrix is reported to follow a non-Fickian model that corresponds to both drug diffusion and polymer relaxation. When the exponent $n \geq 1.0$ the mechanism of drug release is considered to be swelling controlled or Case II transport. The interpretation of *n* also depends on the shape of the dosage form tested, and the limits for slab, cylinder, and sphere geometries are summarised in Table 5.2.

Table 5.2 *Interpretation of release mechanism from polymeric films using the power law [550,570].*

Geometry	**Case I Fickian diffusion**	**Intermediate/ Anomalous**	**Case II Swelling controlled**
Slab	$n = 0.50$	$0.50 < n < 1.0$	$n = 1.0$
Cylinder	$n = 0.45$	$0.45 < n < 0.89$	$n = 0.89$
Sphere	$n = 0.43$	$0.43 < n < 0.85$	$n = 0.85$

The Korsmeyer-Peppas power law is only valid for the first 60% of drug released from a dosage form, and must therefore be used accordingly to avoid misinterpretation of the mechanism of drug release.

5.4.5 Mathematical Treatment of *In Vitro* Release Data

The different test methods that were used for the assessment of OT release from PF-127 gels were evaluated for potential discriminatory behaviour using ANOVA analysis and model independent methods. In addition, the similarity of dissolution profiles for OT release from PF-127 dosage forms containing different concentration of gel former was evaluated. Univariate ANOVA analysis was performed using GraphPad Prism software Version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) to assess whether differences between each time point of *in vitro* dissolution profiles existed for each of the formulations tested. Tukey's Multiple Range test was used as a *post hoc* test to assess which of the formulations were different for at the individual time points evaluated during *in vitro* dissolution tests.

Model independent approaches that were used to assess the similarity and/or difference between *in vitro* dissolution profiles were the f_1 and f_2 difference and similarity factor and the Gohel similarity factor, S_d, approaches. Formulations were considered different if $f_1 > 15$, $f_2 < 50$ and an S_d value equivalent to a percent difference of at least 25% was considered to be similar. The model independent methods were used in an attempt to determine whether dissolution profiles and methods were discriminatory. In this study model independent approaches were used to evaluate the differences in release profiles that were generated for different concentrations of PF-127 in a formulation evaluated using a specific *in vitro* test method. The difference and similarity factors cannot be applied to the evaluation of the same formulation under different experimental conditions and fit factors have been reported to be of use during the development of an appropriate *in vitro* dissolution/release test [555,571].

Model dependent methods were used to assess differences, if any, on the mechanism of release of OT from PF-127 gels when different *in vitro* dissolution/release tests were used. The power law was used as the primary model to assess the mechanism of OT release from dosage forms. The zero order, first order, Higuchi and Hixson-Crowell models were also used to determine the mechanism of drug release from the gels produced in these studies.

The determination of goodness of fit of data to the models was performed by assessing the adjusted coefficient of determination ($R^2_{adjusted}$). Other methods that may be used are the coefficient of determination (R^2), correlation coefficient (R), sum of squares of residuals (SSR), mean square error (MSE) and the Akaike's Information Criterion (AIC) [550]. The limitation of R^2, despite its easy computation is that the value of R^2 tends to increase as more model parameters are added, irrespective of the significance of the parameter added to a specific model [550]. The use of $R^2_{adjusted}$ however is more meaningful to the interpretation of mathematical models as this value may increase or decrease when new parameters are added to a model.This indicates the impact of the added parameter on the performance of the model when used to assess the mechanism of drug release from a dosage form or formulation [550]. The $R^2_{adjusted}$ may be calculated using Equation 5.15.

$$R^2_{adjusted} = 1 - \frac{n-1}{n-p}(1-R^2) \qquad \textit{Equation 5.15}$$

Where,

n = number of dissolution data points

p = number of parameters in the model

R^2 = coefficient of determination

To determine the most likely mechanism of release of OT from PF-127 gels based on the mathematical models described above a value of $R^2_{adjusted} > 0.950$ was considered acceptable.

5.5 EXPERIMENTAL

5.5.1 Dissolution Test Conditions

OT containing PF-127 gels of concentration 20%, 25% and 30% w/w were prepared using the cold method as described in § 4.4.2.1 by addition of an appropriate amount of gel former to a previously cooled aqueous solution of OT (200 IU/ml). The solutions were assayed by HPLC

before use and the gels were set in moulds of dimensions 23.6 X 11.0 X 8.0 mm (L X W X D) and such that only one (1) surface of the gel was exposed to the dissolution medium. An appropriate amount of gel was weighed out such that each dosage unit contained approximately 300 IU of OT and the gels were set in a convection oven (Weiss Gallenkamp, Loughborough, United Kingdom) at 37 °C prior to *in vitro* release testing. All *in vitro* release tests were performed with three dosage units (n = 3).

The dissolution medium used in the initial stages of formulation development for all three formulations in each of the different test methods that were investigated was a 0.1 M phosphate buffer (pH = 7.2). The buffer was prepared by weighing 68 g of potassium dihydrogen phosphate and dissolving the salt in approximately 4 *l* of HPLC grade water in a beaker and adjusting the pH to the required value using sodium hydroxide pellets. The volume finally made up to 5 *l* in an A-grade volumetric flask using HPLC water.

5.5.2 USP Apparatus 1 and 2

In vitro dissolution/release testing using 500 ml of dissolution medium that was prepared as described in § 5.5.1, maintained at 37 $\pm$ 0.5 °C for the basket (USP Apparatus 1) and paddle (USP Apparatus 2) apparatus was used. The dissolution tests were conducted using a fully automated Hanson Research SR 8 PLUS dissolution tester fitted with an Autoplus™ Multifill™ and Maximizer Syringe Fraction Collector (Hanson Research Cooperation, Chatsworth, CA, USA). The baskets (40-mesh) and the paddles were rotated at 25 rpm for the duration of the experiments. The gel retained in the moulds were placed in either the basket or placed at the bottom of a dissolution vessel when using USP Apparatus 1 or 2, respectively. The dissolution vessels were covered to minimise evaporation. Samples of 1.5 ml were withdrawn for analysis at 30, 60, 90, 120, 180, 240, 360, and 480 min and an equivalent amount (1.5 ml) of fresh dissolution medium was replaced automatically. The amount of drug released was quantitated using a validated HPLC method (Chapter 2). Dissolution profiles were generated by plotting the percent of OT released versus time and the resultant dissolution profiles were assessed by statistical methods and mathematical models as described in § 5.4.5.

5.5.3 USP Apparatus 3

A VanKel® Bio-Dis® dissolution tester (VanKel® industries, New Jersey, USA) was used for *in vitro* release assessment. A model VK 750D digitally controlled water circulation/heater

(VanKel® industries, New Jersey, USA) was used to maintain the temperature at 37 ± 0.5 °C. A screen mesh of pore size of 177 µm and the dip speed of 5 dpm were used to retain the dosage form and provide agitation, respectively. The dosage form was moved through the different vessels at 30, 60, 120, 240, 360, and 480 min. The amount of drug released was determined using a validated HPLC method (Chapter 2) and appropriate the dissolution profiles that were generated were assessed by statistical methods and mathematical models as described in § 5.4.5.

5.5.4 Dialysis Tubing Method

Dialysis tubing cellulose membrane, 25 mm flat width (Sigma Aldrich, St Louis, MO, USA) was hydrated prior to use after which one end of the tube was tied and an appropriate amount of OT containing PF-127 gel was weighed out and placed in the dialysis tubing, such that there was approximately 300 IU in each dosage unit. The dialysis tube was then carefully tied at the other end. The gel was allowed to set in a convection oven (Weiss Gallenkamp, Loughborough, United Kingdom) at 37 °C prior to commencing the *in vitro* release test. USP Apparatus 2 set at 50 rpm, with a dissolution medium comprised of a 0.1 M phosphate buffer that was prepared as previously described in § 5.5.1, was used to assess drug release and samples were harvested at 60, 120, 180, 240, 360, and 480 min. The amount of drug released was determined using a validated HPLC method (Chapter 2). Dissolution profiles were generated and assessed by statistical methods and mathematical models as described in § 5.4.5.

5.5.5 Membrane-less Diffusion System

Appropriate amounts of gel (equivalent to 300 IU) (20%, 25% and 30%w/w) were weighed and placed in 5 ml test tubes. The gels were allowed to set in a water bath (Grant Instruments (Cambridge) Ltd, Cambridge, UK) maintained at 37 ˚C. 500 µl of dissolution medium 0.1 M phosphate buffer at pH 7.2 was prepared as described previously in § 5.5.1 and was carefully placed on the surface of the gel and the tubes were placed in the water bath for the duration of the study. At predetermined times of 30, 60, 90, 120, 180, 240, 360, and 480 min, the receptor fluid was completely removed and replaced with fresh receptor fluid. The sample that had been removed was diluted and analysed using a validated HPLC method (Chapter 2) and the resultant dissolution profiles were assessed by statistical methods and mathematical models as described in § 5.4.5.

5.5.6 Optimisation of *In Vitro* Release Test Method

The *in vitro* dissolution test method that was selected as the most appropriate for the assessment of OT gels was then tested for further discriminating power by comparing drug release profiles obtained following testing a 25% w/w PF-127 formulation that had been manufactured to contain 25, 50, 100 and 200 IU of OT per dosage unit.

The effect of dynamic pH was investigated to mimic *in vivo* conditions, where the pH of the muscle undergoes subtle changes as labour progresses. The formulation was exposed to a pH of 6.85 for the first 30 min of the test and thereafter a pH of 7.2 was maintained for the duration of the *in vitro* dissolution test using USP Apparatus 3.

5.6 RESULTS AND DISCUSSION

5.6.1 The Discriminating Power of the Different *In Vitro* Release Test Methods

5.6.1.1 In Vitro Release Profiles of OT from the different test methods

The *in vitro* release profiles of OT from PF-127 gels using the different test methods described in § 5.5 are shown in Figures 5.6 – 5.10.

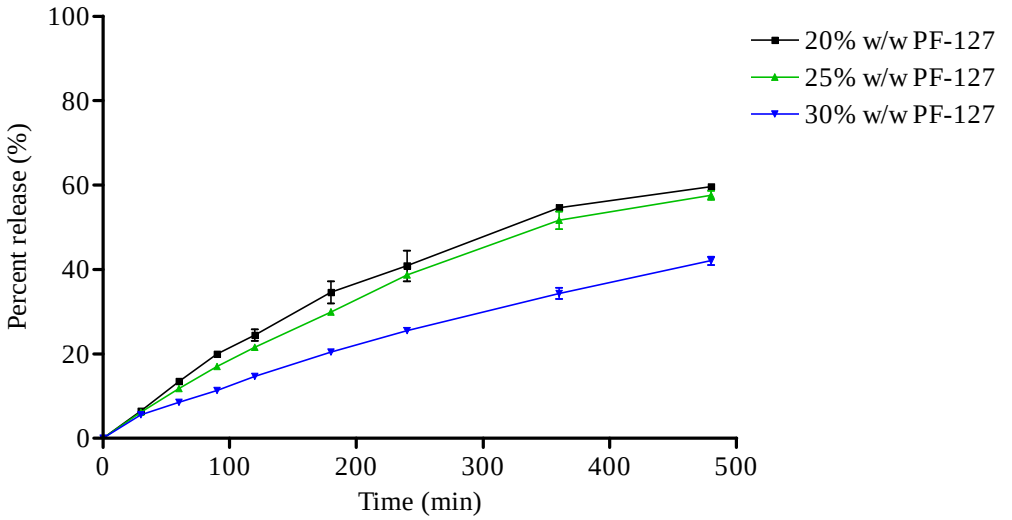


Figure 5.6 *The extent of OT release from PF-127 gels using USP Apparatus 1(n = 3) for assessing in vitro release*

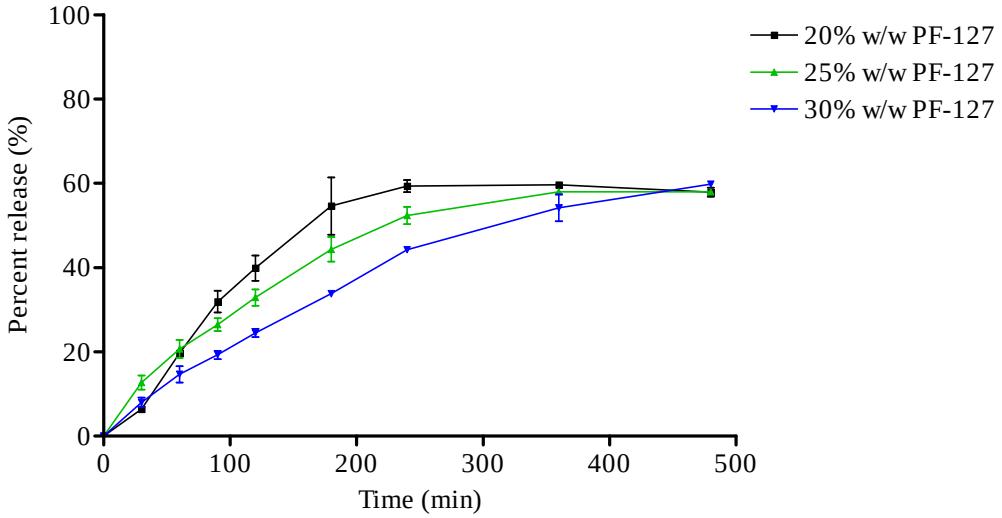


Figure 5.7 *The extent of OT release from PF-127 gels using USP Apparatus 2 (n = 3) for assessing in vitro release*

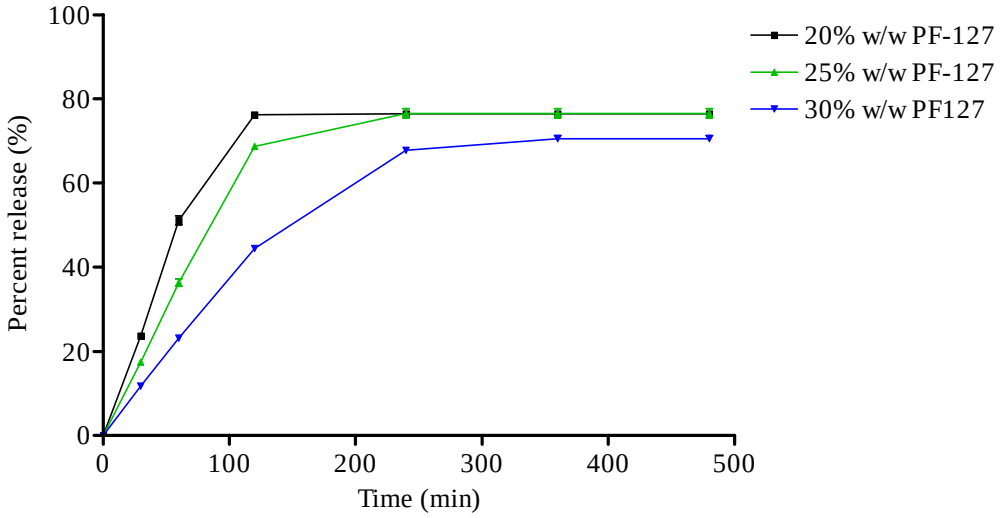


Figure 5.8 *The extent of OT release from PF-127 gels using USP Apparatus 3 (n = 3) for assessing in vitro release*

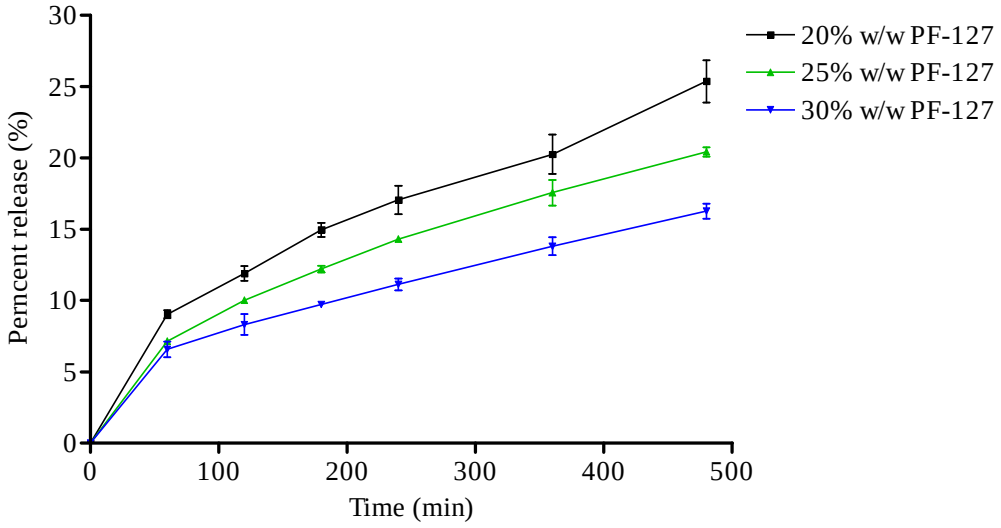


Figure 5.9 *The extent of OT release from PF-127 gels using the dialysis method (n = 3) for assessing in vitro release*

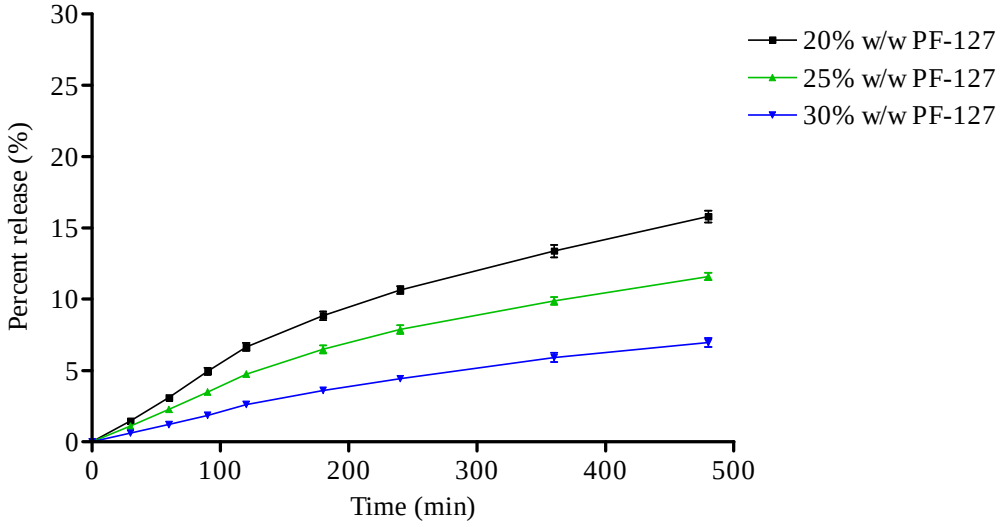


Figure 5.10 *The extent of OT release from PF-127 gels using the membrane-less diffusion method (n = 3) for assessing in vitro release*

As expected, the rate of OT release from the different formulations tested was dependent on the concentration of PF-127 that was used in the formulation with the lower concentrations of gel former providing the fastest rate of release of the OT from the dosage forms. The trend was observed for all *in vitro* dissolution apparatus tested. Formulations with a low gel content have a lower viscosity in comparison to formulations with higher gel content (See § 4.5.6) and undergo faster gel dissolution (See § 4.5.7). These effects ultimately result in a faster rate of OT liberation and other studies have shown that the rate of release of drug from PF-127 systems is controlled by dissolution of the gel [500,502].

5.6.1.2 Analysis of Variance

ANOVA analysis was used to compare the *in vitro* release profiles of OT from the different formulations when different *in vitro* release methods were used to assess which of the tests were discriminatory. The primary advantage of using ANOVA analysis for this purpose is that the statistical test allows for the detection of differences between *in vitro* release profiles at individual time points. Furthermore, the ANOVA analysis allows for careful monitoring of the profiles to determine if there are any changes in the discriminatory pattern of the test system as *in vitro* release testing proceeds. The results of the ANOVA analysis performed comparing *in vitro* release profiles for the different formulations with different PF-127 content are summarised in Tables 5.3 - 5.7.

Table 5.3 *ANOVA analysis summary for in vitro release profiles generated using USP Apparatus 1*

Time (min)	Comparison	Mean difference	95% Confidence interval to mean difference		P value	Summary
			Lower limit	Upper limit		
30	20% vs. 25%	0.3021	-0.5800	1.184	P > 0.05	Not significant
	20% vs. 30%	0.9569	0.07475	1.839	P < 0.05	Significant
	25% vs. 30%	0.6548	-0.2274	1.537	P > 0.05	Not significant
60	20% vs. 25%	1.665	0.2874	3.043	P < 0.05	Significant
	20% vs. 30%	4.939	3.561	6.317	P < 0.01	Significant
	25% vs. 30%	3.274	1.896	4.652	P < 0.01	Significant
90	20% vs. 25%	2.961	1.734	4.189	P < 0.01	Significant
	20% vs. 30%	8.594	7.367	4.189	P < 0.001	Significant
	25% vs. 30%	8.594	4.406	6.860	P < 0.001	Significant
120	20% vs. 25%	2.903	-1.890	7.696	P > 0.05	Not significant
	20% vs. 30%	9.846	5.053	14.64	P < 0.01	Significant
	25% vs. 30%	6.943	2.150	11.74	P < 0.05	Significant
180	20% vs. 25%	4.612	-4.439	13.66	P > 0.05	Not significant
	20% vs. 30%	14.15	5.098	23.20	P < 0.05	Significant
	25% vs. 30%	9.537	0.4866	18.59	P < 0.05	Significant
240	20% vs. 25%	2.146	-10.40	14.69	P > 0.05	Not significant
	20% vs. 30%	15.37	2.822	27.91	P < 0.05	Significant
	25% vs. 30%	13.22	0.6752	25.77	P < 0.05	Significant
360	20% vs. 25%	2.983	-5.357	11.32	P > 0.05	Not significant
	20% vs. 30%	20.31	11.97	28.65	P < 0.01	Significant
	25% vs. 30%	17.33	8.987	25.67	P < 0.01	Significant
480	20% vs. 25%	2.058	-3.278	7.394	P > 0.05	Not significant
	20% vs. 30%	17.57	12.24	22.91	P < 0.01	Significant
	25% vs. 30%	15.52	10.18	20.85	P < 0.01	Significant

Table 5.4 *ANOVA analysis summary for in vitro release profiles generated using USP Apparatus 2*

Time (min)	Comparison	Mean difference	95% Confidence interval to mean difference		P value	Summary
			Lower limit	Upper limit		
30	20% vs. 25%	-6.316	-13.41	0.7769	P > 0.05	Not significant
	20% vs. 30%	-1.612	-8.705	5.482	P > 0.05	Not significant
	25% vs. 30%	4.705	-2.389	11.80	P > 0.05	Not significant
60	20% vs. 25%	-0.9969	-11.05	9.052	P > 0.05	Not significant
	20% vs. 30%	5.052	-4.997	15.10	P > 0.05	Not significant
	25% vs. 30%	6.049	-4.000	16.10	P > 0.05	Not significant
90	20% vs. 25%	5.433	-5.343	16.21	P > 0.05	Not significant
	20% vs. 30%	12.62	1.849	23.40	P < 0.05	Significant
	25% vs. 30%	7.192	-3.584	17.97	P > 0.05	Not significant
120	20% vs. 25%	7.014	-5.647	19.68	P > 0.05	Not significant
	20% vs. 30%	15.42	2.758	28.08	P < 0.05	Significant
	25% vs. 30%	8.404	-4.256	21.07	P > 0.05	Not significant
180	20% vs. 25%	10.27	-14.96	35.50	P > 0.05	Not significant
	20% vs. 30%	20.82	-4.411	46.05	P > 0.05	Not significant
	25% vs. 30%	10.55	-14.68	35.78	P > 0.05	Not significant
240	20% vs. 25%	6.988	-1.476	15.45	P > 0.05	Not significant
	20% vs. 30%	15.15	6.688	23.62	P < 0.05	Significant
	25% vs. 30%	8.164	-0.2997	16.63	P > 0.05	Not significant
360	20% vs. 25%	1.631	-9.472	12.74	P > 0.05	Not significant
	20% vs. 30%	5.443	-5.661	16.55	P > 0.05	Not significant
	25% vs. 30%	3.812	-7.292	14.92	P > 0.05	Not significant
480	20% vs. 25%	-0.1106	-4.397	4.176	P > 0.05	Not significant
	20% vs. 30%	-1.956	-6.242	2.331	P > 0.05	Not significant
	25% vs. 30%	-1.845	-6.132	2.441	P > 0.05	Not significant

Table 5.5 *ANOVA analysis summary for in vitro release profiles generated using USP Apparatus 3*

Time (min)	Comparison	Mean difference	95% Confidence interval to mean difference		P value	Summary
			Lower limit	Upper limit		
30	20% vs. 25%	6.236	4.620	7.852	P < 0.01	Significant
	20% vs. 30%	11.93	10.32	13.55	P < 0.001	Significant
	25% vs. 30%	5.699	4.083	7.314	P < 0.01	Significant
60	20% vs. 25%	14.80	9.749	19.85	P < 0.01	Significant
	20% vs. 30%	27.93	22.88	32.97	P < 0.001	Significant
	25% vs. 30%	13.13	8.080	18.18	P < 0.01	Significant
120	20% vs. 25%	7.502	5.617	9.387	P < 0.001	Significant
	20% vs. 30%	31.77	29.88	33.65	P < 0.001	Significant
	25% vs. 30%	24.26	22.38	26.15	P < 0.001	Significant
240	20% vs. 25%	-0.09111	-4.318	4.136	P > 0.05	Not significant
	20% vs. 30%	8.688	4.461	12.91	P < 0.01	Significant
	25% vs. 30%	8.779	4.552	13.01	P < 0.01	Significant
360	20% vs. 25%	-0.09111	-4.798	4.615	P > 0.05	Not significant
	20% vs. 30%	5.892	1.186	10.60	P < 0.05	Significant
	25% vs. 30%	5.984	1.277	10.69	P < 0.05	Significant
480	20% vs. 25%	-0.09111	-4.798	4.615	P > 0.05	Not significant
	20% vs. 30%	5.892	1.186	10.60	P < 0.05	Significant
	25% vs. 30%	5.984	1.277	10.69	P < 0.05	Significant

Table 5.6 *ANOVA analysis summary for in vitro release profiles generated using the dialysis method*

Time (min)	Comparison	Mean difference	95% Confidence interval to mean difference		P value	Summary
			Lower limit	Upper limit		
60	20% vs. 25%	1.893	-0.2980	4.085	P > 0.05	Not significant
	20% vs. 30%	2.464	0.2730	4.656	P < 0.05	Significant
	25% vs. 30%	0.5710	-1.620	2.762	P > 0.05	Not significant
120	20% vs. 25%	1.872	-1.215	4.958	P > 0.05	Not significant
	20% vs. 30%	3.586	0.4989	6.673	P < 0.05	Significant
	25% vs. 30%	1.714	-1.373	4.801	P > 0.05	Not significant
180	20% vs. 25%	2.755	0.8885	4.621	P < 0.05	Significant
	20% vs. 30%	5.244	3.378	7.111	P < 0.01	Significant
	25% vs. 30%	2.489	0.6228	4.356	P < 0.05	Significant
240	20% vs. 25%	2.731	-0.9598	6.422	P > 0.05	Not significant
	20% vs. 30%	5.921	2.230	9.612	P < 0.05	Significant
	25% vs. 30%	3.190	-0.5006	6.881	P > 0.05	Not significant
360	20% vs. 25%	2.695	-3.338	8.728	P > 0.05	Not significant
	20% vs. 30%	6.457	0.4245	12.49	P < 0.05	Significant
	25% vs. 30%	3.762	-2.271	9.795	P > 0.05	Not significant
480	20% vs. 25%	4.949	-0.4982	10.40	P > 0.05	Not significant
	20% vs. 30%	9.107	3.660	14.55	P < 0.05	Significant
	25% vs. 30%	4.158	-1.289	9.605	P > 0.05	Not significant

Table 5.7 *ANOVA analysis summary for in vitro release profiles generated using the membrane-less diffusion method*

Time (min)	Comparison	Mean difference	95% Confidence interval to mean difference		P value	Summary
			Lower limit	Upper limit		
30	20% vs. 25%	0.3581	-0. 1599	0.8762	P > 0.05	Not significant
	20% vs. 30%	0.8357	0.3177	1.354	P < 0.01	Significant
	25% vs. 30%	0.4776	-0.04045	0.9957	P > 0.05	Not significant
60	20% vs. 25%	0.8112	0.2285	1.394	P < 0.05	Significant
	20% vs. 30%	1.884	1.302	2.467	P < 0.001	Significant
	25% vs. 30%	1.073	0.4904	1.656	P < 0.01	Significant
90	20% vs. 25%	1.446	0.6220	2.269	P < 0.01	Significant
	20% vs. 30%	3.085	2.261	3.909	P < 0.001	Significant
	25% vs. 30%	1.639	0.8155	2.463	P < 0.01	Significant
120	20% vs. 25%	1.922	1.066	2.778	P < 0.01	Significant
	20% vs. 30%	4.049	3.192	4.905	P < 0.001	Significant
	25% vs. 30%	2.127	1.271	2.983	P < 0.001	Significant
180	20% vs. 25%	2.348	1.315	3.381	P < 0.01	Significant
	20% vs. 30%	5.238	4.205	6.272	P < 0.001	Significant
	25% vs. 30%	2.890	1.857	3.924	P < 0.001	Significant
240	20% vs. 25%	2.783	1.711	3.854	P < 0.001	Significant
	20% vs. 30%	6.233	5.162	7.305	P < 0.001	Significant
	25% vs. 30%	3.451	2.379	4.522	P < 0.001	Significant
360	20% vs. 25%	3.500	1.969	5.030	P < 0.01	Significant
	20% vs. 30%	7.464	5.933	8.994	P < 0.001	Significant
	25% vs. 30%	3.964	2.434	5.495	P < 0.001	Significant
480	20% vs. 25%	4.211	2.738	5.684	P < 0.001	Significant
	20% vs. 30%	8.839	7.366	10.31	P < 0.001	Significant
	25% vs. 30%	4.627	3.154	6.100	P < 0.001	Significant

ANOVA analysis of data generated using USP Apparatus 1 and summarised in Table 5.3 reveal that for the initial 30 min of the dissolution test, there was no discrimination between the 25% w/w PF-127 formulation with both the 20% and 30% w/w PF-127 formulations, although discrimination was observed between the extremes of gel concentration that were used. However, between 60 and 120 min into the *in vitro* release test, it can be observed from the summarised ANOVA results Table 5.3 and on visual inspection of the graph, that the three curves corresponding to the different concentrations of PF-127 are separated from each other. In the later stages dissolution testing using USP Apparatus 1, the ANOVA results indicate that there were no significant differences between the *in vitro* release profiles that were obtained from the 20% and 25% w/w PF-127 formulations, although the 30% w/w PF-127 formulation can be discriminated from both the lower and intermediate PF-127 formulations.

The ANOVA analysis data presented in Table 5.4 reveal that the use of USP Apparatus 2 does not allow for discrimination between the formulations tested and that no significant differences exist between the dissolution profiles. Visual inspection of Figure 5.7 clearly shows that the early and late segments of the *in vitro* release profiles are very close together. However, according to the ANOVA analysis, the *in vitro* release profiles show differences between the 20% and the 30% w/w PF-127 formulations at times points in between the early and late sample points and that there was no discrimination between the 25% w/w PF-127 formulation and the 20% ww and 30% w/w formulations. The lack of discrimination using the USP apparatus 2 may also be observed from visual inspection of Figure 5.7, as the dissolution profile for the 25% w/w PF-127 formulation appears to fall midway between the two formulations in which low and high concentrations of PF-127 were used.

A summary of the ANOVA results for comparison of the *in vitro* release profiles generated using USP Apparatus 3 is reported in Table 5.5 and the data indicate that significant differences exist between the percent OT dissolved at different time points in the early stages of test procedure, specifically between 0 min and 240 min. Thereafter, a plateau is reached for the 20% and 25% w/w PF-127 formulations and no significant differences were observed between the OT release profiles. Thereafter, discrimination between the 20% and 25% w/w concentrations of PF-127 is not achieved as these systems reached a plateau at the same time and level.

The dialysis method for *in vitro* release testing of OT from PF-127 gels indicates that there are no significant differences between the 25% and both the 20% and 30% w/w PF-127 formulations for the majority of the time points tested and the data are summarised in Table 5.6.

The lack of discrimination can also be observed by visual inspection of Figure 5.9 as the 25% w/w PF -127 formulation *in vitro* release profile for OT falls approximately midway between the two (2) formulations in which 20% w/w and 30% w/w PF-127 were used. However, discrimination was achieved between the 20% and 30% w/w PF-127 formulations for all time points using this dissolution or *in vitro* release method.

The use of membrane-less diffusion indicates that the *in vitro* release profiles are different for the majority of all time points tested and that significant differences between the means were observed according to the ANOVA analysis performed and summarised in Table 5.7. Visual inspection of the dissolution profiles generated and shown in Figure 5.10 confirm these observations as the *in vitro* release profiles for the different formulations are clearly well distinguished from each other.

5.6.1.3 Model Independent Methods

The f_1 and f_2 difference and similarity factors and the Gohel similarity factor, S_d were used to compare the *in vitro* release profiles generated for OT using the different *in vitro* release test methods, from PF-127 gels of different concentration. For the purposes of assessing the discriminatory behaviour of the *in vitro* release tests evaluated, the 25% w/w formulation was used as the reference product and the results of these analyses are summarised in Table 5.8.

Table 5.8 *f_1, f_2 and S_d values for the different formulations, using the 25% w/w formulation as the reference and shaded areas indicates discriminatory behaviour*

***In vitro* release test**	**20% w/w PF-127**			**30% w/w PF-127**		
	f_1	f_2	S_d	f_1	f_2	S_d
USP apparatus 1	9.9	72.8	0.0452	31.1	49.1	0.129
USP apparatus 2	13.7	72.6	0.0944	15.7	66.6	0.147
USP apparatus 3	23.3	49.4	0.0899	26.1	41.9	0.155
Dialysis tube	21.9	75.7	0.0883	19.4	81.6	0.0677
Membrane-less diffusion	36.6	81.4	0.134	43.2	78.9	0.258

On consideration of the difference factor, f_1 , both USP Apparatus 1 and 2 were not able to discriminate between the dissolution profiles generated for the 20% and 25% w/w formulation as the f_1 factors that were calculated were < 15 for these comparisons. The similarity factor, f_2, for the comparison of the 20% and 25% w/w formulations indicate that the *in vitro* release profiles are similar and therefore it may be concluded that both the USP Apparatus 1 and 2 are unable to produce data in which discrimination between different formulations is achieved. The Gohel S_d values that were calculated for the comparison of the 20% and 25% w/w PF-127 containing formulations using USP Apparatus 1 and 2 indicate that differences that are detected are approximately 10% and between 20% and 25%, respectively, indicating similarity in the *in*

vitro release profiles that were generated. However, the use of f_1 for the comparison of the 25% and the 30% w/w formulation indicated that discrimination is achieved ($f_1 > 15$) for both USP Apparatus 1 and 2. However this is not the case on consideration of f_2 for the profiles generated using USP Apparatus 2 ($f_2 = 66.6$) which indicates similarity between the *in vitro* release profiles but the test indicates that for the f_2 calculated for profiles generated using USP Apparatus 1 ($f_2 = 49.1$), discrimination is achieved. The S_d values that were obtained when the 25% and 30% w/w PF-127 OT containing gels were compared indicate percent differences that are greater than 30% for both USP Apparatus 1 and 2 indicating that the profiles are different.

Data generated using USP Apparatus 3 show that the use of this apparatus is able to discriminate between different formulations since $f_1 > 15$ and $f_2 < 50$ in all cases, indicating that differences were detected when the 25% w/w PF-127 formulation was compared to the 20% and 30% w/w gel formulations. Furthermore, the S_d factors that were calculated for the comparison of the 25% w/w PF-127 gel concentration with both the 20% and 30% w/w PF-127 produced S_d values with equivalent percent differences greater than 30%, indicating differences in the formulations do exist.

The use of a dialysis bag with the paddle apparatus and the membrane-less diffusion system did not allow for discrimination between all formulations tested as f_2 values that were calculated from the relevant *in vitro* release profiles were greater than 50, although values of greater than 15 were obtained for some of the f_1 factors calculated. The use of S_d for the comparison of dialysis membrane test method indicated that there was a percent difference of between 20% and 25% when the *in vitro* release profiles of OT of the 20% and 25% w/w PF-127 gels were compared. An S_d value equivalent to approximately 20% difference was obtained when *in vitro* release profiles of the 25% w/w PF-127 was compared to that obtained when a 30% w/w gel was used. These results indicate that according to the criterion to determine similarity using Gohel's S_d factor, the dialysis *in vitro* release test method is unable to discriminate between formulation variants. However, the values for S_d, indicate that membrane-less diffusion system studied resulted in differences of greater than 30% when the 25% w/w PF-127 formulation was compared to the 20% and 30% w/w formulation suggesting a potentially discriminatory test procedure may exist.

5.6.1.4 Conclusion

The three tests that were used to assess the discriminatory power of the dissolution methods produce some conflicting results although there is also some consensus between the tests and the results are summarised in Tables 5.9 and 5.10.

Table 5.9 *Summary of comparison of the mathematical models used to compare the 20% vs. 25%*

	ANOVA	f_1	f_2	S_d
USP apparatus 1	**X**	**X**	**X**	**X**
USP apparatus 2	**X**	**X**	**X**	**X**
USP apparatus 3	✓	✓	✓	**X**
Dialysis tube	**X**	✓	**X**	**X**
Membrane-less diffusion	✓	✓	**X**	✓

✓ indicates discriminatory behaviour and **X** indicates failure to discriminate

Table 5.10 *Summary of comparison of the mathematical models used to compare the 25% vs. 30%*

	ANOVA	f_1	f_2	S_d
USP apparatus 1	✓	✓	✓	✓
USP apparatus 2	**X**	✓	**X**	✓
USP apparatus 3	✓	✓	✓	✓
Dialysis tube	**X**	✓	**X**	**X**
Membrane-less diffusion	✓	✓	**X**	✓

✓ indicates discriminatory behaviour and **X** indicates failure to discriminate

A comparison of the different mathematical tools used to assess the similarity/difference of the dissolution profiles generated using different apparatus indicate that there is some consensus between the data obtained for comparison of the *in vitro* release test methods. Consensus in the results can be observed in Tables 5.9 and 5.10, which show that the different methods for comparison yield the same conclusions for USP Apparatus 1 and 2 in Table 5.9 and for USP Apparatus 1 and 3 in Table 5.10.

The different sensitivities of the methods to assess discrimination between dissolution profiles is evident on comparison of the f_1 and f_2 fit factors when they are used for the dialysis tube and the membrane-less diffusion methods, which yield conflicting results. Specifically, the f_1 test indicates a difference between the *in vitro* release profiles whereas the f_2 factor indicates that the *in vitro* release profiles are similar. This is due to the sensitivity of the method when comparing dosage forms with a relatively low percent release from the dosage forms and results in insensitivity of the methods that was not observed when a large amount of OT was released using compendial apparatus. Although the use of ANOVA analysis has been deemed too discriminatory for comparison of *in vitro* release profiles between a test and reference product, these studies indicate that there is a general consensus between data generated using ANOVA and that generated using the other comparison techniques that were applied. Although the use of

the f_2 fit factor for the membrane-less diffusion system indicated that there were no differences between the formulation variants, the use of ANOVA and S_d which are based on the differences between means and AUC respectively indicate that there are differences. The f_2 factor is based on the differences between the percent released which is relatively low and the data are close together and therefore the tool fails to distinguish between the dissolution profiles for the different formulations tested, despite the mean amount released and the AUC being evaluated as different.

The selection of the appropriate dissolution method for use from the different methods evaluated was based on the ease of use of the method and the ability of the method to discriminate between formulations. In addition to the fact that the use of USP Apparatus 3 permits discrimination between formulations, the system is automated and therefore convenient to use. Furthermore, the use of USP Apparatus 3 permits further optimisation of an *in vitro* dissolution test method by allowing for changes in pH and other *in vivo* conditions to be mimicked as drug is released. In addition, the system allows for the assessment of whether such changes will affect the release kinetics of OT from the gel.

5.6.2 The Impact of the Type of Apparatus on the *In Vitro* Release

5.6.2.1 OT release rate

The different compendial apparatus result in different hydrodynamic conditions *in vitro* and as a result drug release profiles show fundamental differences that can be understood by considering the type of dosage form (swelling, eroding, non-eroding *etc*) and the hydrodynamic effects present when using the different apparatus [554,571].

The *in vitro* release profiles generated for OT release from a 25% w/w PF-127 gel using the different dissolution methods were compared and are shown in Figure 5.11.

The rates (IU/min) of release of OT determined from samples tested in all apparatus for the 25% w/w formulation were compared, using the total amount of OT that was released at the end of the test period or the time taken to reach a plateau and the results are summarised in Table 5.11.

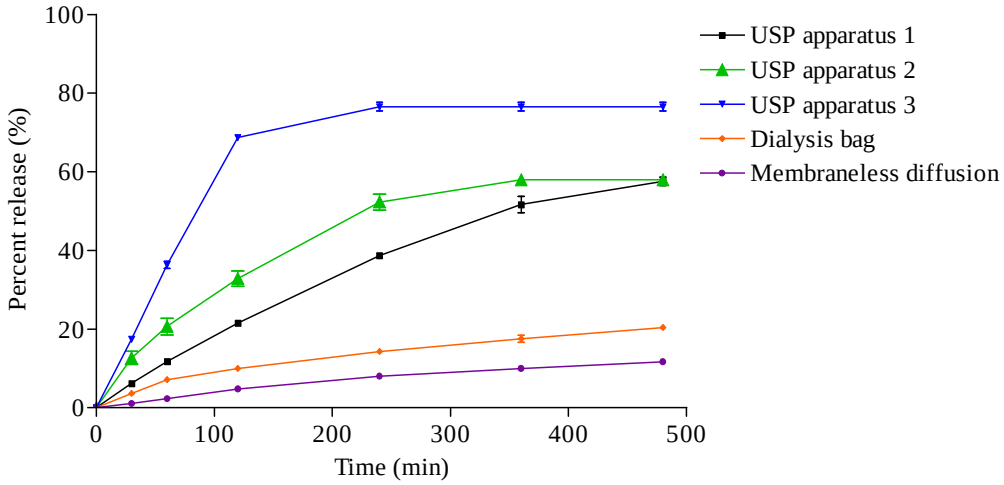


Figure 5.11 *Comparison of the rate and extent of OT release from a 25% w/w PF-127 gel using different dissolution test methods*

Table 5.11 *Rate of release of OT from the 25% w/w formulation for the different test methods*

***In vitro* release test**	**Rate of OT release (IU/min)**
USP Apparatus 1	0.361
USP Apparatus 2	0.489
USP Apparatus 3	0.964
Dialysis tube method	0.129
Membrane-less diffusion method	0.077

A comparison of the rate of release of OT from the 25% w/w PF-127 gel using the three compendial apparatus reveals that the overall release rate and pattern of release is such that USP 3 > USP 2 > USP 1 which may observed when comparing the profiles in Figure 5.11 and the data summarised in Table 5.11. This is in agreement with observations from another study comparing the three compendial methods that noted the same pattern of release [571]. The rate of OT liberation from the paddle apparatus was faster than that from the basket apparatus because of the greater efficiency of the paddle to create agitation in a dissolution test compared to a basket rotating at the same speed of 50 rpm [553]. The faster rate of release observed when using the paddle apparatus has been proposed to be due to the difference in the hydrodynamic properties of the medium with the paddle resulting in increased agitation of the medium [507] and therefore a faster erosion/dissolution of the matrix in the system, resulting in faster drug release.

The finding that the basket apparatus results in slower release rates is not conclusive but is found to depend on the nature of the dosage form and the drug candidate [507]. It has been reported that the dissolution rate of cetirizine and pseudoephedrine from a dosage form tested

using a basket and paddle apparatus did not differ significantly for the two apparatus [555]. The importance of selecting an appropriate dissolution test apparatus considering formulation factors and drug properties has been emphasised by Fernandes *et al*. [554], who showed that for one of the formulations of lamivudine tested, the basket apparatus resulted in faster dissolution of the drug. However, for some other formulations the dissolution rate of lamivudine was independent of the medium, the apparatus used and the time over which the test was conducted.

The hydrodynamics of a test system describe the flow of fluid within the dissolution vessel and considerations about how fluid flow is likely to impact on *in vitro* release of a drug must be taken into account. The velocity of the fluid within a basket apparatus and at the bottom of the vessel when using the paddle apparatus has been compared using computational fluid dynamics [572]. The velocity of fluid for the basket ranges between $0.5 – 57.4 \times 10^{-3}$ ms^{-1} at 50 rpm and the velocity at the bottom of the vessel at the same speed ranges between $0.003 – 3.5 \times 10^{-3}$ ms^{-1}. At 50 rpm, the velocity at the bottom of the vessel of the paddle apparatus ranges between $0.8 – 79.6 \times 10^{-3}$ ms^{-1}. The comparison of the velocities indicates that there is a possibility that the rates of dissolution for a dosage form may be similar as the velocity ranges that are attainable in both Apparatus overlap. For a non-disintegrating dosage form, where diffusion controls drug release the similar velocities within the basket apparatus and at the bottom of the paddle apparatus means that comparable dissolution rates can be achieved [572]. However, for a delivery system such as those manufactured using PF-127, which undergoes dissolution [500,502], slightly higher *in vitro* release rates can be expected from the paddle apparatus compared to the basket apparatus. This occurs due to the higher velocity of the dissolution medium in the USP paddle apparatus vessel, resulting in faster dissolution of the gel and hence faster drug release.

Dissolution profiles generated using USP Apparatus 3 show the fastest rates of release as compared to the other four methods evaluated and these findings are in agreement with other observations for an eroding matrix system [571]. The oscillating movement of the inner cylinder within the outer cylinder results in full exposure of all surfaces of a dosage form to the dissolution medium in which it is reciprocating, resulting in a higher degree of erosion [571]. In addition, the oscillating movements of the cylinder cause more vigorous hydrodynamics compared to the other two systems, intensifying the erosion of a matrix and increase in the rate of *in vitro* release compared to the basket and paddle apparatus [571].

The dialysis method of dissolution rate testing reveals a slower rate of release as compared to that generated using the three compendial apparatus. The gel was separated from the aqueous dissolution medium by a physical barrier that prevents the dissolution of the gel matrix and hence OT molecules must therefore diffuse from the interior of the gel into the dissolution medium in order for release to occur. Drug release that occurs as result of diffusion from a matrix system is slower as compared to systems in which dissolution of the matrix may play an important role in drug release rates. The release of drug candidates from PF-127 matrices has been found to be diffusion controlled when a membrane is used to separate the gel from the dissolution medium [448,573] and such release has been shown to follow Higuchi release kinetics [567,568]. It was noted for the dialysis test system that the rate of release was faster in the initial stages of the dissolution test as the gradient of the release profile is steeper but this decreases as the *in vitro* release test proceeds over time. The reduction in release rate may be attributed to changes in the concentration gradient that controls diffusion and where during the initial stages of the test there is a steep concentration gradient and faster diffusion of the drug from the dialysis bag into the dissolution medium. As more drug is released into the dissolution medium, so the release rate slows down due to a change in the concentration gradient. In addition, as the drug diffuses out of the gel the distance over which OT diffuses from the inner regions of the dialysis bag is increased during the later stages of the test when compared to the initial stages of release and therefore the release rate is slower as the *in vitro* release test proceeds. The extent of release for the dialysis system is also low which can be attributed to the attainment of equilibrium for OT in the dissolution media and the gel. As a result, there is no more OT diffusing out of the gel and this may be because in this system, there is the physical separation of the gel from the dissolution medium which prevents dissolution of the gel, which is important in the release of drug from the PF-127 matrices [500,502].

The membrane-less diffusion method revealed the slowest rate of release (0.077 IU/min) of OT from the gels. In the membrane-less diffusion method the test gel is in direct contact with the dissolution medium at only interface of the gel and the dissolution medium and since there is no agitation of the dissolution medium during the *in vitro* release test, dissolution of the gel is slower compared to the other *in vitro* release test methods. This results in slower liberation of OT as dissolution of the gel matrix has been determined to control the rate of release of drugs from PF-127 matrices [500,502].

5.6.2.2 Application of the Korsmeyer-Peppas Model

Drug release from polymeric systems can be a complex process that is primarily dependent on the nature of a drug and the physico-chemical characteristics of a hydrogel and the interaction of the system with an aqueous dissolution medium. Drug release from polymeric systems can be generally attributed to occur by one or a combination of three basic mechanisms, *viz.*, diffusion controlled, swelling controlled, and erosion CR [564].

Diffusion controlled polymeric systems describe release mechanisms that are limited by the rate of diffusion of a drug through the polymer in which the drug is contained and include reservoir systems and matrix systems, in which the drug is surrounded by a polymeric membrane or may be either be dissolved or dispersed within the polymer, respectively [564]. However, polymeric systems are often swelling-controlled and show non-Fickian or anomalous diffusional behaviour and this is reported to be due to the interaction between water molecules and polymeric chains, which often rearrange to accommodate water molecules [564]. Extremes of anomalous diffusion behaviour have been termed Case I and Case II transport [574], where Case I transport refers to the condition in which solvent mobility is lower than the segmental relaxation rate of the polymeric chains and this describes diffusion controlled systems. Conversely, Case II transport describes mechanisms in which solvent mobility is higher than the relaxation rate of the polymer, which are swelling controlled systems with anomalous diffusional characteristics. Often, however release of a drug from a polymeric matrix often exhibits attributes intermediary to Case I and Case II transport [564].

The Korsmeyer-Peppas power law is one of the most comprehensive mathematical models that may be used to infer the mechanism of drug release from a polymeric dosage form, such as those prepared from PF-127 gels. The power law was applied to *in vitro* release profiles of OT from a 25% w/w PF-127 gel concentration that were analysed using the different *in vitro* dissolution methods described in § 5.5. A summary of Korsmeyer-Peppas parameters that were obtained following modelling of the *in vitro* release profiles generated using the different *in vitro* release tests to the power law are summarised in Table 5.12.

Table 5.12 *Summary of Korsmeyer-Peppas best-fit parameters.*

***In vitro* release test**	**$M_t/M_{\infty\,(max)}$**	***N***	**k (%/min^n)**	**$R^2_{adjusted}$**
USP Apparatus 1	0.5783	0.8190	0.004127	0.9937
USP Apparatus 2	0.5296	0.6855	0.012503	0.9992
USP Apparatus 3	0.6102	0.9904	0.006139	0.9983
Dialysis membrane	0.2067	0.5055	0.009066	0.9996
Membrane-less diffusion	0.1224	0.8472	0.000759	0.9809

The geometry of the dosage forms was assumed to be that of a slab as only one flat surface was exposed to the dissolution medium during *in vitro* release testing performed using compendial and membrane-less diffusion systems and a cylindrical geometry was assumed for the dialysis membrane system.

The predominant mechanism of release of OT from PF-127 gels following modelling of data generated using all test apparatus was determined to occur by anomalous transport. Therefore drug release is characterised by both diffusion and swelling control as *n* values are between 0.5 and 1 for the slab geometries and for the cylinder assumed for the dialysis membrane an *n* value of > 0.45 was obtained. However, the main differences in the mechanisms of release as indicated by the numerical value of the exponent is the extent to which the transport processes are governed by swelling and diffusion in the systems. The different hydrodynamics described in § 5.6.2.1 are likely factors that influence the type of release that is observed from these polymeric systems.

For the assumed slab geometries, USP Apparatus 2 has the lowest numerical value of the exponent, *n* indicating that diffusion in an important contributing factor when this *in vitro* release test is used to generate *in vitro* release profiles. Mixed transport mechanisms are observed when USP Apparatus 1 and 3 and the membrane-less diffusion method were used. The results that were obtained from the Korsmeyer-Peppas model are in close agreement with the work of Anderson *et al*. [499], who derived a mathematical model to describe the mechanism of drug release form poloxamer gels. Anderson *et al*. reported that whilst dissolution of the gel was important for the liberation of drug from the gels, diffusion of a drug through the polymeric system also contributed to the mechanism of release. The dissolution of the gel in poloxamer systems may be considered to be due to the interaction of polymeric micelles, described in § 4.5.5 for gel systems, with water molecules causing dissociation of micellar aggregates from the gel surface into the bulk dissolution medium.

The use of a dialysis bag for dissolution profile generation is a slightly different system to those described for the compendial apparatus and for the membrane-less diffusion system. The impact of the release test on the mechanism of release differs due to the assumption that a cylindrical geometry is necessary and that there is physical separation of the gel phase from the dissolution media. The results that were obtained from the Korsmeyer-Peppas model indicate that the exponent that was obtained had an *n* of 0.5055, which is Case I transport for cylindrical geometry, indicating that diffusion is an important mechanism of release of OT from the

dialysis system. Nonetheless, the impact of swelling CR cannot be neglected as dialysis tubing is permeable to water molecules and this allows interaction of polymeric micellar aggregates with water, causing relaxation of the micelles, although these are contained within the dialysis bag.

The Korsmeyer-Peppas model is an attractive mathematical model for use for understanding the mechanism of drug release from polymeric systems. The exponent is relatively easy to compute using appropriate mathematical tools and is easy to interpret, assuming that correct assumptions are made about the geometry of the dosage form being tested. However, limitations to the use of the power law are that release from CR devices must be carefully applied as certain assumptions to the power law may not be completely applicable to CR devices being test. These include assumption about constant diffusivities of a drug with a polymer, constant dimensions of a device during an *in vitro* release test, one-dimensional diffusional behaviour, and perfect sink conditions in an *in vitro* release test [564].

5.6.2.3 Other Mathematical Models

Zero and first order models, Higuchi and Hixson-Crowell models were also used to further elucidate the mechanism of release of OT from the dosage forms manufactured using PF-127 gels from profiles generated using different *in vitro* release test methods. The suitability of each model to describe the mechanism of OT liberation was based on the calculation of the $R^2_{adjusted}$ for the formulations. The model parameters and the values of $R^2_{adjusted}$ are summarised in Table 5.13.

Table 5.13 *Summary of other mathematical model parameters*

***In vitro* release test**	**Zero Order**		**First Order**		**Higuchi**		**Hixson-Crowell**	
	K_0	$R^2_{adjusted}$	K_1	$R^2_{adjusted}$	K_H	$R^2_{adjusted}$	K_s	$R^2_{adjusted}$
USP Apparatus 1	0.3688	0.9663	0.0044	0.8108	8.7029	0.9656	-0.0061	0.8833
USP Apparatus 2	0.6369	0.9690	0.0063	0.9103	10.46	0.9779	-0.0093	0.9466
USP Apparatus 3	1.7342	0.9990	0.0146	0.9476	18.424	0.9143	-0.0234	0.9729
Dialysis membrane	0.1165	0.9088	0.0023	0.9200	2.8244	0.9998	-0.0027	0.9476
Membrane-less diffusion	0.0768	0.9554	0.0044	0.7622	1.8273	0.9707	-0.0036	0.8465

The application of the zero order model may be considered as suitable for describing the mechanism of release of OT from PF-127 gels as the $R^2_{adjusted}$ values that were obtained were greater than 0.950 for all systems except for the dialysis membrane method. Drug release from PF-127 gels has been determined to be controlled by dissolution of the gel in an aqueous medium and as described in § 4.5.7, where gel dissolution is a zero order process. The

correlation that exists between gel dissolution and drug release has been noted in earlier studies [500-502] and therefore the mechanism of release of a drug is zero order. Due to the absence of dissolution when using the dialysis membrane method a similar mechanism of release as described for the other systems is not observed. Generally, zero order models are useful in describing mechanisms of drug release if the dosage forms being tested do not disaggregate during the course of an *in vitro* release test. However, PF-127 undergoes dissolution in aqueous media as was observed in § 4.5.7 and therefore the zero order model may not be entirely suitable to describe the mechanism of OT release from PF-127 gels.

On inspection of the $R^2_{adjusted}$ values that were obtained following modelling of OT release data with a first order model indicated that the mechanism of release was not first order and was unable to describe OT liberation from PF-127 gels. In general, water-soluble drugs that are incorporated into porous matrices are released according to first order kinetics and the PF-127 gel matrix is made up of micellar aggregates that do not form a porous structure to enable liberation of OT by this mechanism.

The Higuchi model may be useful in explaining the *in vitro* release mechanism of OT when the USP Apparatuses 1 and 2, the dialysis bag and the membrane-less diffusion methods are used to assess OT dissolution rates. The Higuchi model explains the role of diffusion in liberation of drug from a matrix and these results are in agreement with those presented in Table 5.12, where anomalous transport mechanisms were observed for the four (4) *in vitro* release tests that exhibited Higuchi kinetics, indicating the role of diffusion in the release of OT from these systems. However, the *in vitro* release profile for OT generated when using USP Apparatus 3 produced a value for *n* very close to one (1), which indicated very little diffusion in the system and on application of Higuchi kinetics, diffusion was not determined to be an important mechanism of OT release from PF-127 dosage forms when tested using this apparatus.

The Hixson-Crowell model was not particularly useful for the description of OT liberation from PF-127 systems as the $R^2_{adjusted}$ values that were obtained were generally less than 0.950 except for data generated using USP Apparatus 3. The Hixson-Crowell model describes drug release from systems that experience a change in diameter and surface area of particles with proceeding drug release. When USP Apparatus 3 is used vigorous hydrodynamics are produced in the system compared to other *in vitro* release tests and may therefore cause more significant changes in the shape of the dosage form compared to the other *in vitro* release test methods that were assessed.

5.6.3 The Impact of Drug Loading on the *In Vitro* Release of OT

The amount of OT that was initially used in the comparison of the dissolution profiles is in excess of the amount of OT that would normally be used for therapeutic purposes. Dosages of 5 – 20 IU are normally used in clinical practice [9,14], but due to the large sample volume required when using the USP Apparatus 1 and 2, a larger dose was used to facilitate analysis of samples using the HPLC method described in Chapter 2. However, the dissolution fluid volume for USP Apparatus 3 is smaller than that for USP Apparatus 1 and 2 and therefore the formulations could be optimised with regard to dose strength to produce dosage forms that were clinically relevant.

The impact of drug loading was studied for 200 IU, 100 IU, 50 IU and 25 IU dosage strengths in a 25% w/w PF-127 formulation and the resultant profiles are shown in Figure 5.12.

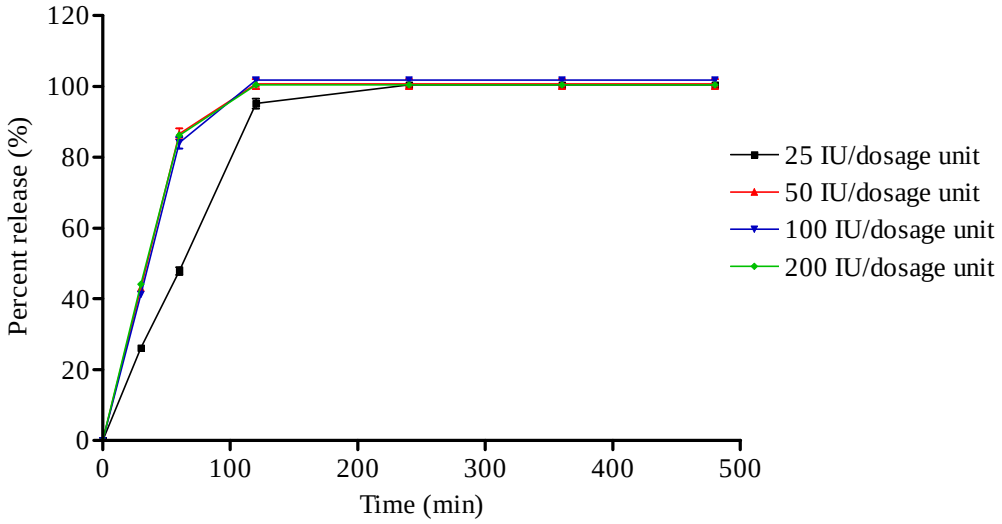


Figure 5.12 *Impact of drug loading on the in vitro release profile of OT from the USP apparatus 3*

Table 5.14 summarises the percent OT released at 120 min and the calculated rate of release as a percent/minute for each of the dosage strengths tested.

Table 5.14 *The rate of release of OT for the different dosage strengths*

Dosage strength (IU)	Percent release at 120 min (%)	Percent release /min (%/min)
25	95.31	0.794
50	100.68	0.839
100	101.79	0.848
200	100.47	0.837

It was observed that the rate of the release of OT was similar for the higher dosage strengths, but this is slightly lower for the lowest dosage strength. These observations indicate that diffusion of the drug through the matrix may also play a role in the mechanism of drug release as described in § 5.6.2.2 and § 5.6.2.3. Although the dissolution rate of the gel is the same for the PF-127 gels in the formulations and is likely to be the most important factor controlling the release of the drug, the role of diffusion can not be neglected.

The ANOVA analysis results for the comparison of the different dosage strengths of OT that were tested using USP apparatus 3 are summarised in Table 5.15.

The ANOVA results that were generated indicate that the extent of OT release is dependent on the amount of OT that is used in the formulation. It was noted that significant differences between the individual time points for the formulations were observed for example between the 25 IU/dosage form and the other concentrations that were investigated, although there was no discrimination between the higher strength dosage forms. The significant differences between the 25 IU/dosage unit and the higher dosage strengths may be a result of the differences in diffusion rates of OT from the dosage form. Although diffusion was concluded not to be a primary mechanism for OT release when USP Apparatus 3 is used for *in vitro* release testing (See § 5.6.2.2), the role of diffusion is still a contributing factor at higher concentrations. This is likely to be due to steeper concentration gradients existing when higher concentrations of OT are used, resulting in faster release of OT from these dosage forms. The difference in concentration may not be as apparent as the amount of OT per dosage unit increases, but this is evident when very low amounts of drug are used.

Table 5.15 *ANOVA analysis summary for in vitro release profiles generated for different dosage strengths of OT.*

Time (min)	Comparison	Mean difference	95% Confidence interval to mean difference		P value	Summary
			Lower Limit	Upper Limit		
30	25 IU vs. 50 IU	-16.91	-19.40	-14.41	P < 0.001	Significant
	25 IU vs. 100 IU	-15.09	-17.58	-12.60	P < 0.001	Significant
	25 IU vs. 200 IU	-17.92	-20.41	-15.43	P < 0.001	Significant
	50 IU vs. 100 IU	1.818	-0.6733	4.310	P > 0.05	Not significant
	50 IU vs. 200 IU	-1.015	-3.506	1.477	P > 0.05	Not significant
	100 IU vs. 200 IU	-2.833	-5.325	-0.3415	P < 0.05	Not significant
60	25 IU vs. 50 IU	-38.74	-45.83	-31.64	P < 0.001	Significant
	25 IU vs. 100 IU	-36.20	-45.41	-29.10	P < 0.001	Significant
	25 IU vs. 200 IU	-38.31	-43.30	-31.21	P < 0.001	Significant
	50 IU vs. 100 IU	2.539	-4.557	7.523	P > 0.05	Not significant
	50 IU vs. 200 IU	0.4274	-6.669	9.635	P > 0.05	Not significant
	100 IU vs. 200 IU	-2.112	-9.208	4.984	P > 0.05	Not significant
120	25 IU vs. 50 IU	-5.509	-11.34	0.3187	P > 0.05	Not significant
	25 IU vs. 100 IU	-6.622	-12.45	-0.7941	P > 0.05	Not significant
	25 IU vs. 200 IU	-5.297	-11.12	0.5310	P > 0.05	Not significant
	50 IU vs. 100 IU	-1.113	-6.940	4.715	P > 0.05	Not significant
	50 IU vs. 200 IU	0.2122	-5.615	6.040	P > 0.05	Not significant
	100 IU vs. 200 IU	1.325	-4.502	7.153	P > 0.05	Not significant
240	25 IU vs. 50 IU	-0.2007	-4.605	4.204	P > 0.05	Not significant
	25 IU vs. 100 IU	-1.314	-5.718	3.091	P > 0.05	Not significant
	25 IU vs. 200 IU	0.01160	-4.393	4.416	P > 0.05	Not significant
	50 IU vs. 100 IU	-1.113	-5.517	3.291	P > 0.05	Not significant
	50 IU vs. 200 IU	0.2122	-4.192	4.617	P > 0.05	Not significant
	100 IU vs. 200 IU	1.325	-3.079	5.729	P > 0.05	Not significant
360	25 IU vs. 50 IU	-0.2007	-4.605	4.204	P > 0.05	Not significant
	25 IU vs. 100 IU	-1.314	-5.718	3.091	P > 0.05	Not significant
	25 IU vs. 200 IU	0.01160	-4.393	4.416	P > 0.05	Not significant
	50 IU vs. 100 IU	-1.113	-5.517	3.291	P > 0.05	Not significant
	50 IU vs. 200 IU	0.2122	-4.192	4.617	P > 0.05	Not significant
	100 IU vs. 200 IU	1.325	-3.079	5.729	P > 0.05	Not significant

5.6.4 The Impact of a Dynamic pH on OT Release

During labour the reduction in blood flow in uterine muscles occurs which is known to impact metabolic activity in the uterus. Effects that occur *in vivo* include stimulation of anaerobic metabolism, alteration of adenosine-5'-triphosphate (ATP) and a decrease in the pH [575]. Larcombe-McDouall *et al.* [575] investigated the impact of occlusion (reducing blood flow) in the rat uterus on certain metabolites and pH. From their studies, the pH at rest was determined to be 7.23 $\pm$ 0.01 and with occlusion, there was a decrease in the pH that was related to the extent of occlusion and the pH ranged from 7.20 to 6.88 with maximal occlusion [575]. However, the recovery of pH following occlusion was very rapid, returning to normal levels within 10 min of allowing the return of blood flow to the muscle tissues. These results have important implications with regard to studying the effect of pH on the release kinetics of OT from the PF-127 dosage forms. If the dosage form is to be administered after labour muscle pH would be expected to be lower than that of resting muscle but the body recovers rapidly and therefore muscle pH would return to normal after a relatively short period. The impact of pH on the release of the OT was studied and these data are shown in Figure 5.13.

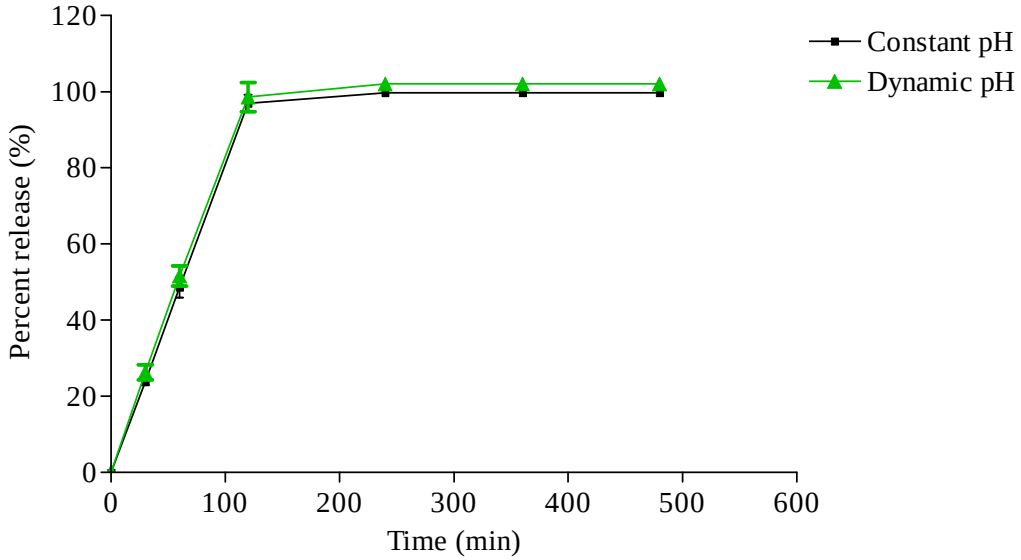


Figure 5.13 *The impact of dynamic pH on the rate and extent of OT release.*

It is evident that pH does not have any impact on the rate and extent of release of OT from this dosage form. The release rates are very similar and computation of the f_1 and f_2 difference and similarity factors (f_1 = 3.5 and f_2 = 81.8) reflects this observation. The impact of pH on the release of API from PF-127 systems has been studied [501] and the similar results were

observed. PF-127 is a non-ionic block copolymer [427] and is therefore unlikely to be affected when exposed to media of different pH and therefore pH should not impact the rate and extent of drug release from these systems. However, OT is an ionisable peptide with an isoelectric point at pH = 7.7 and the increase in pH towards this value means that a greater proportion of OT molecules exist in the unionised state. However, the change in the proportion of unionised/ionised OT molecules is unlikely to impact on the rate of OT liberation from the dosage form because the interaction with the non-ionic poloxamer is unlikely to be altered to any significant extent.

5.7 CONCLUSION

The development of a discriminatory *in vitro* dissolution test is necessary in early formulation studies in order to develop an effective and safe dosage form. A novel *in vitro* release test for the assessment of parenteral dosage form of OT extemporaneously prepared using PF-127 as the gel former was developed.

Mathematical models are important in assessing the mechanism of drug release from a dosage form. The use of models has the primary advantage of facilitating the optimisation of drug release characteristics from a delivery system and for dosage form performance evaluation and the impact of excipients on the mechanism of drug release. However, for the purposes of this study model dependent mathematical models were used to enhance the understanding of the impact of the apparatus and *in vitro* release test methodology on the liberation of OT from a matrix formulation. This is important in the development of *in vitro* release test methods in cases where the mechanism of drug release may be known or in cases where a specific mechanism of release is desirable *in vivo*. In such cases, an *in vitro* release test may be optimised based on the specific mechanistic requirements for drug release. The optimisation of an *in vitro* release test method is essential in assessing whether an *in vitro in vivo* correlation exists for a specific *in vitro* release method and establishing a correlation is a logical next step in the development and optimisation of *in vitro* release tests as described in § 5.3.

Following an evaluation of five (5) different test methods using the f_1 and f_2 difference and similarity factors, the Gohel similarity factor, S_d and ANOVA analysis, USP Apparatus 3 was found to be the most discriminatory method and therefore the most suitable tool for further optimisation. Furthermore, the extent of drug release from this test method was higher in comparison to other *in vitro* release test methods that were assessed (See § 5.6.2.1 and § 5.6.2.2) and therefore the method was found to be suitable to assess the release of OT from

test formulations that were developed for optimisation. The smaller volume that is required for when using USP Apparatus 3 as compared to USP Apparatus 1 and 2, (180 ml compared to 500 ml) also allowed for the formulation to be further optimised with respect to drug loading, permitting lower amounts of drug to be used in accordance with the relevant doses used in clinical practice. In addition, an assessment of the impact that drug loading on the release of the drug from the dosage form was undertaken. The impact of variation in pH on drug delivery can also be easily assessed using USP Apparatus 3. Although the pH was found not to impact on the rate and extent of drug release from PF-127 gels, it was decided that the dynamic pH be used as the desired *in vitro* release method. This is because excipients and additives that can be used to modify release of OT from the formulation as well as to optimise a delivery system of OT may be affected by pH and therefore impact on the mechanism of release. The optimal release test developed for the assessment of OT from parenteral dosage forms prepared from PF-127 gels is summarised in Table 5.16.

Table 5.16 *Optimal in vitro release test conditions for assessing OT release from PF-127 gels*

Aspect	**Specifications**
In vitro release test apparatus	USP apparatus 3
Dissolution medium	0.1 M phosphate buffer, pH = 6.85 for 30 min, then 0.1 M phosphate buffer pH = 7.2 for 7 hours 30 min
Sampling intervals	30, 60, 120, 240, 360 and 480 min
Speed	5 dips/minute
Temperature	37 $\pm$ 0.5 °C

CHAPTER 6

CONCLUSION

OT is an endogenous peptide with a diverse pharmacological profile *in vivo*, although the primary use of OT is for the induction of labour and promotion of lactation. Therapeutically OT is used for labour induction, treatment, and prevention of post partum haemorrhage and for the promotion of lactation. For the purposes of preventing post partum haemorrhage, OT is often administered as an intramuscular dose, which has a short duration of action. Therefore, the use of a long acting parenteral preparation for treatment and prevention of post partum haemorrhage may be necessary and appropriate to provide sustained tone to the uterus, thereby preventing excessive bleeding.

A sensitive, selective, accurate, and precise stability indicating HPLC method for the quantitation of OT in pharmaceutical dosage was developed and validated according to ICH guidelines. The analytical method was found to be specific and therefore selective for OT in the presence of degradation products and the preservative, chlorbutol. The method was further optimised for the quantitation of *in vitro* release rate of OT from extemporaneously prepared PF-127 dosage forms. The HPLC method was found to be linear over a concentration range of 0 – 0.50 IU/ml and precise with % RSD values that were < 5% RSD. The method was also found to be accurate with a % Bias that was determined to be < 5%.

Current trends in protein and peptide delivery systems were reported and an alternative route or dosage form for sustained OT delivery was proposed. Various alternatives including covalent modification of OT by attachment of fatty acids (lipidisation) or PEG (PEGylation), nasal and transdermal delivery were reviewed and considered. Parenteral delivery of OT using liposomes, hydrogels and particulate systems were evaluated as viable means of OT delivery. However the selection of an appropriate delivery system require an appropriate pharmacokinetic profile of OT, ease of manufacture and scale up of the dosage form and the biocompatibility and toxicity of delivery system components. Thermo-sensitive hydrogel systems which exist as flowing viscous liquids at low temperatures, but that stiffen on warming to body temperature thereby forming a potential depot were considered as a viable and appropriate means to deliver OT. Pluronic® copolymers are commercially available polyethylene glycol polypropylene glycol block copolymers that behave in this manner and have been investigated for use for the sustained delivery of drug molecules. Furthermore, Pluronic® block copolymers have been approved by the FDA for inclusion in parenteral

formulations, therefore the toxicity profile and biocompatibility of these molecules is well established.

Investigation into the types of Pluronic® block copolymers revealed that PF-127 was an appropriate and potentially useful polymer for the sustained release of OT. PF-127 was characterised in order to determine the properties of the copolymer that may be important in developing and optimising a pharmaceutical dosage form as well as to assess whether the release of a molecule may be predicted from the behaviour of PF-127 with changes in concentration of PF-127 in a formulation and temperature. UV and IR absorption spectra of OT and PF-127 combinations revealed that a potential interaction between OT and PF-127 was unlikely. The CMC of PF-127 was determined to be 0.5% w/v in aqueous solution. An analytical method for quantifying PF-127 in solution was developed and validated with respect to linearity, accuracy and precision, where the method yielded % RSD values < 2% in the range 0.01 – 0.4% w/v. The onset of gelation was found to be dependent on the concentration of gel former in the solution, and higher concentrations underwent gelation at lower temperatures compared to lower concentrations of PF-127. For example, a 30% w/w solution of PF-127 formed a stiff gel at only 12 °C and a 20% w/w solution formed a gel at 23 °C. The viscosity of PF-127 solutions was found to be dependent on the temperature and concentration of gel former that was used, where an increase in viscosity was generally observed with increases in both temperature and concentration of PF-127. Furthermore, there was a dramatic change in viscosity of the solution that occurred simultaneously with the onset of gelation. Gravimetric analysis of PF-127 dissolution at 37 °C revealed that dissolution of PF-127 is a zero order process that depends on the concentration of gel that is in a formulation, where higher concentrations of gel dissolve at slower rates compared to lower concentrations.

The lack of compendial or official guidelines for the development of *in vitro* release test methods for controlled release parenteral preparations is a challenge for the development of such dosage forms. Five different *in vitro* test systems were evaluated as possible tools for the assessment of OT release from PF-127 gel systems prepared using the cold method. The amount of PF-127 that was used to prepare dosage forms of OT that contained 300 IU of active were 20%, 25% and 30% w/w. The release rate of OT from these formulations was evaluated using USP Apparatus 1, 2 and 3, a dialysis bag in USP Apparatus 2 and a membrane-less diffusion system. The different test methods were assessed for their discriminatory capacity using ANOVA analysis, the f_1 and f_2, difference and similarity factors, and the Gohel (S_d) similarity factor as model independent methods using the 25% w/w formulation as the reference product. The results indicated that the USP Apparatus 1 and 2

failed to discriminate adequately between the 20% and 25% w/w formulations, although discrimination was observed between the 25% and 30% w/w formulations. USP Apparatus 3 showed adequate discrimination between the different formulation compositions that were tested. The results indicate that, in general the dialysis bag method showed poor discrimination between the formulations and although the f_1 and f_2 tests indicated that the membrane-less diffusion system was unable to discriminate between formulations, ANOVA analysis and the S_d factor showed that discrimination was achieved. USP Apparatus 3 was found to be the most appropriate apparatus for the assessment of PF-127 OT formulations.

The Korsmeyer-Peppas power law was the primary model dependent method that was used to elucidate the mechanism of OT release from the different formulations tested using the different *in vitro* release tests. It was observed that the release of OT from the dosage forms generally showed anomalous transport mechanisms suggesting that drug release is a consequence of both Fickian diffusion and swelling controlled transport mechanisms. However, the extent to which these mechanisms contribute to drug release differ according to the apparatus used to generate dissolution profiles and it was noted that the dialysis bag method displayed release kinetics that were predominantly diffusion controlled whereas the USP Apparatus 3 method revealed that swelling controlled transport mechanisms were prevalent. The application of zero order, first order, Higuchi, and Hixson-Crowell models to assess the kinetics of drug release indicated that the first order and Hixson-Crowell models were not generally applicable to describing the mechanism of OT release from PF-127 gels using the apparatus described. However, the application of zero order and the Higuchi models indicated the importance of zero order dissolution of the PF-127 gel as a release controlling mechanism and diffusion as a control element in liberating drug from the gel matrix, respectively.

In vitro release testing using USP Apparatus 3 was conducted to optimise the amount of OT in each dosage form and the impact of pH of the release media on OT release was assessed. It was observed that dosage forms that contain 25 IU can be tested using the USP Apparatus 3 and that although pH changes may occur in the muscle during labour, these changes did not influence the liberation of OT from OF-127 dosage forms.

The studies conducted and reported in this thesis represent the early stages in the development and optimisation of an OT parenteral dosage form. Further studies that would be necessary for progression of the project would be the *in vivo* optimisation of drug release and establishing whether an *in vitro in vivo* correlation exists for this system. An established *in vitro in vivo* correlation would ensure the clinical applicability of the *in vitro* release test and allow

strategic decisions to be made during product development that would allow quality control tests to predict *in vivo* behaviour of the product. The dosage form that is developed must therefore be administered to test animals in order to achieve a realistic rate of OT delivery *in vivo* and then further optimisation of the *in vitro* release test method.

In order to produce an optimal delivery system for OT, the desired rate of OT release must be evaluated and compared to intravenous infusion rates that are used to administer OT in a clinical setting for the prevention of post partum haemorrhage. The desired rate of OT release from an optimised dosage form for a period of 8 hours was calculated and a dose of 9.6 – 19.2 IU is administered when OT is infused at a rate of 20 – 40 mU/min [9]. This would ensure delivery of pharmaceutically relevant doses of OT to a patient from a parenteral sustained release delivery system.

The project therefore looks at some important aspects that are vital in the development of pharmaceutical dosage forms. Literature review of not only current trends in drug delivery, but also of drug properties are highlighted as important in the pharmaceutical development stages in order to ensure that practical solutions to drug delivery challenges are addressed. An accurate, precise and sensitive analytical method is a key element in successful dosage form design as this permits the optimisation of delivery systems. Preformulation studies to assess drug and matrix properties, including stability, excipient behaviour with changing environmental conditions and compatibility are important, especially if no such data has been published. *In vitro* release tests are important in the development of dosage forms in order to ensure that optimal drug delivery devices are produced.

APPENDIX I
BATCH RECORD REPORTS

Rhodes University
Faculty of Pharmacy, Department of Pharmaceutics
Grahamstown 6140

BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	300 IU Oxytocin acetate in 20 %w/w Pluronic® F127
Date of Manufacture	11 August 2006

Formula:

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	20 %w/w	20 g	RM000175
200 IU/ml Oxytocin solution	80 %w/w	80 ml	RM000174

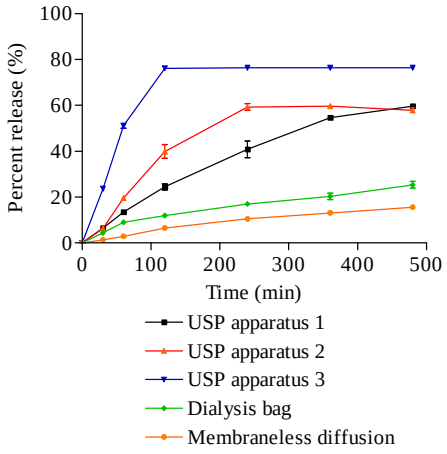
Methodology

20 g of Pluronic® F127 was weighed and dispersed in 80 ml of a 200 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	154.12 IU/g	
Weight used (300 IU)	1.97 ± 0.02 g	
Gel transition temperature	21 °C	
Viscosity	5 °C	73.33 ± 2.08
	25 °C	648 000 ± 2200
	37 °C	1 068 000 ± 30 500

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

Rhodes University
Faculty of Pharmacy, Department of Pharmaceutics
Grahamstown 6140

BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	300 IU Oxytocin acetate in 25 %w/w Pluronic® F127
Date of Manufacture	11 August 2006

Formula:

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	25 %w/w	25 g	RM000175
200 IU/ml Oxytocin solution	75 %w/w	75ml	RM000174

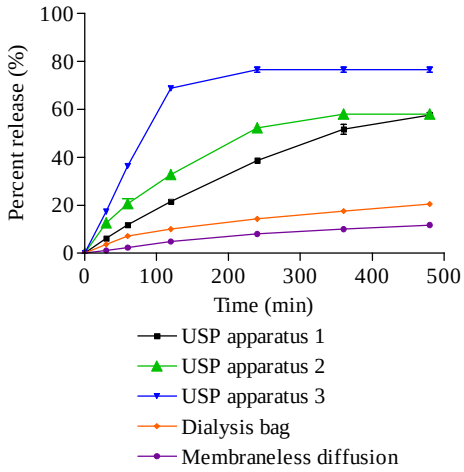
Methodology

25 g of Pluronic® F127 was weighed and dispersed in 75 ml of a 200 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	147.12 IU/g	
Weight used (300 IU)	2.08 ± 0.05 g	
Gel transition temperature	15 °C	
Viscosity	5 °C	106.33 ± 3.51
	25 °C	1 270 000 ± 15 000
	37 °C	1 480 000 ± 20 000

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

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BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	300 IU Oxytocin acetate in 30 %w/w Pluronic® F127
Date of Manufacture	13 August 2006

Formula

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	30 %w/w	30 g	RM000175
200 IU/ml Oxytocin solution	70 %w/w	70 ml	RM000174

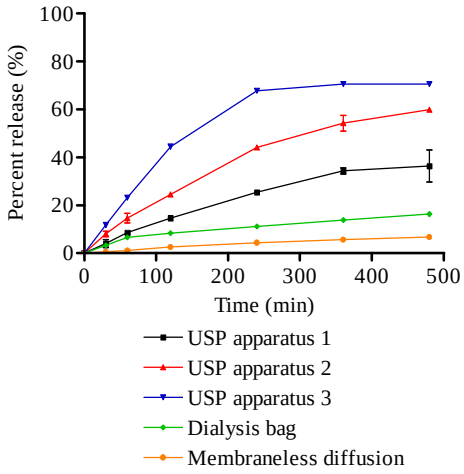
Methodology

30 g of Pluronic® F127 was weighed and dispersed in 70 ml of a 200 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	137.57 IU/g	
Weight used (300 IU)	2.20 ± 0.03 g	
Gel transition temperature	11 °C	
Viscosity	5 °C	198.21 ± 2.00
	25 °C	1 500 000 ± 20 000
	37 °C	1 800 000 ± 19 000

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

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BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	200 IU Oxytocin acetate in 25 %w/w Pluronic® F127
Date of Manufacture	21 August 2006

Formula

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	25 %w/w	3.33 g	RM000175
150 IU/ml Oxytocin solution	75 %w/w	10.00 ml	RM000174

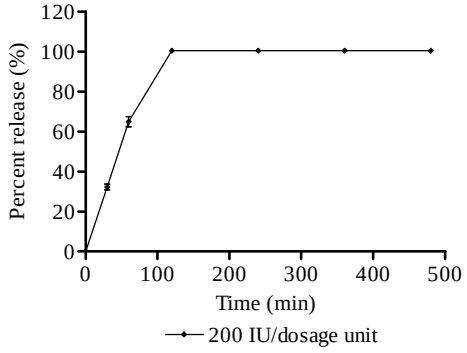
Methodology

3.33 g of Pluronic® F127 was weighed and dispersed in 10 ml of a 150 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	105.66 IU/g
Weight used (200 IU)	1.89 ± 0.03 g

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

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BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	100 IU Oxytocin acetate in 25 %w/w Pluronic® F127
Date of Manufacture	21 August 2006

Formula

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	25 %w/w	3.33 g	RM000175
100 IU/ml Oxytocin solution	75 %w/w	10.00 ml	RM000174

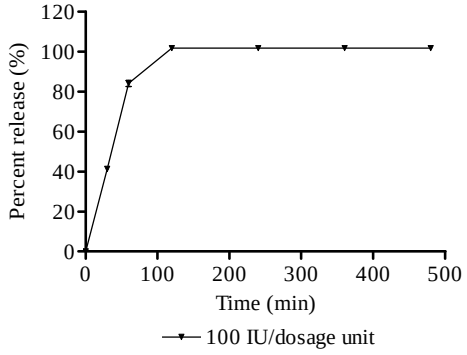
Methodology

3.33 g of Pluronic® F127 was weighed and dispersed in 10 ml of a 200 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	65.78 IU/g
Weight used (100 IU)	1.52 ± 0.02 g

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

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BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	50 IU Oxytocin acetate in 25 %w/w Pluronic® F127
Date of Manufacture	21 August 2006

Formula

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	25 %w/w	3.33 g	RM000175
50 IU/ml Oxytocin solution	75 %w/w	10.00 ml	RM000174

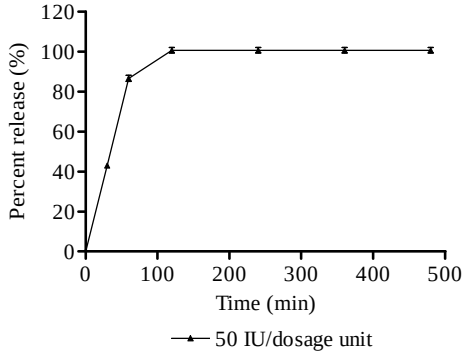
Methodology

3.33 g of Pluronic® F127 was weighed and dispersed in 10 ml of a 200 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	35.02 IU/g
Weight used (50 IU)	1.43 $\pm$ 0.02 g

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

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BATCH RECORD SUMMARY

Formulator	Faith Chaibva
Product	25 IU Oxytocin acetate in 25 %w/w Pluronic® F127
Date of Manufacture	24 August 2006

Formula

Name	Original formula	Working formula	Rhodes Batch Number
Pluronic® F127	25 %w/w	3.33 g	RM000175
20 IU/ml Oxytocin solution	75 %w/w	10.00 ml	RM000174

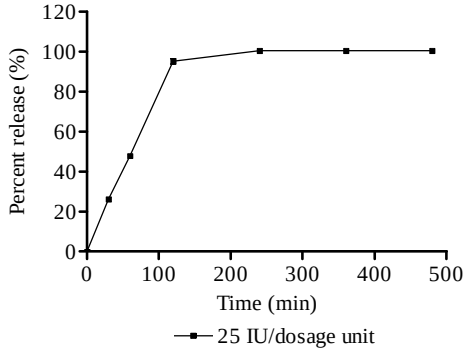
Methodology

3.33 g of Pluronic® F127 was weighed and dispersed in 10 ml of a 200 IU/ml solution of OT using a magnetic stirrer over a period of 2 – 3 min. This was placed in the refrigerator for at least 24 hours or until complete dissolution had occurred and a clear viscous solution obtained.

Tests performed

Assay	19.51 IU/g
Weight used (25 IU)	1.28 ± 0.03 g

IN VITRO RELEASE AND COMMENTS



- A clear gel viscous gel was produced
- The gel that was placed in casts set forming a stiff gel after placing the gel in a convection oven at 37 °C
- The surface of the gel was smooth and had no bubbles

APPENDIX II

ANOVA SUMMARY TABLES

Table A1 *ANOVA summary statistics of the in vitro release of OT from USP apparatus 1 comparing the means of percent OT released at different time points for 20%, 25% and 30% w/w PF-127 formulations*

Time (min)	**R squared**	**F**	**P value**	**Are means significantly different? ($p < 0.05$)**
30	0.8774	10.74	0.0429	Yes
60	0.9873	116.2	0.0014	Yes
90	0.9966	442.0	0.0002	Yes
120	0.9629	38.92	0.0071	Yes
180	0.9367	22.20	0.0159	Yes
240	0.9111	15.37	0.0265	Yes
360	0.9758	60.40	0.0038	Yes
480	0.9869	113.2	0.0015	Yes

Table A2 *ANOVA summary statistics of the in vitro release of OT from USP apparatus 2 comparing the means of percent OT released at different time points for 20%, 25% and 30% w/w PF-127 formulations*

Time (min)	**R squared**	**F**	**P value**	**Are means significantly different? ($p < 0.05$)**
30	0.8329	7.477	0.0683	No
60	0.7081	3.638	0.1577	No
90	0.8894	12.06	0.0368	Yes
120	0.8964	12.99	0.0333	Yes
180	0.7985	5.946	0.0904	No
240	0.9492	28.04	0.0114	Yes
360	0.5957	2.210	0.2570	No
480	0.6047	2.295	0.2485	No

Table A3 *ANOVA summary statistics of the in vitro release of OT from USP apparatus 3 comparing the means of percent OT released at different time points for 20%, 25% and 30% w/w PF-127 formulations*

Time (min)	**R squared**	**F**	**P value**	**Are means significantly different? ($p < 0.05$)**
30	0.9969	476.8	0.0002	Yes
60	0.9944	267.5	0.0004	Yes
120	0.9994	2710	P<0.0001	Yes
240	0.9707	49.71	0.0050	Yes
360	0.9251	18.54	0.0205	Yes
480	0.9251	18.54	0.0205	Yes

Table A4 *ANOVA summary statistics of the in vitro release of OT from dialysis tubing in USP apparatus 2 comparing the means of percent OT released at different time points for 20%, 25% and 30% w/w PF-127 formulations*

Time (min)	**R squared**	**F**	**P value**	**Are means significantly different? ($p < 0.05$)**
60	0.8897	12.10	0.0366	Yes
120	0.8871	11.79	0.0379	Yes
180	0.9787	68.99	0.0031	Yes
240	0.9375	22.52	0.0156	Yes
360	0.8706	10.10	0.0465	Yes
480	0.9422	24.47	0.0139	Yes

Table A5 *ANOVA summary statistics of the in vitro release of OT from the membrane-less diffusion system comparing the means of percent OT released at different time points for 20%, 25% and 30% w/w PF-127 formulations*

Time (min)	**R squared**	**F**	**P value**	**Are means significantly different? ($p < 0.05$)**
30	0.8043	12.33	0.0075	Yes
60	0.9429	49.53	0.0002	Yes
90	0.9566	66.10	P<0.0001	Yes
120	0.9723	105.3	P<0.0001	Yes
180	0.9759	121.4	P<0.0001	Yes
240	0.9816	159.9	P<0.0001	Yes
360	0.9739	112.1	P<0.0001	Yes
480	0.9826	169.6	P<0.0001	Yes

Table A6 *ANOVA summary statistics of the in vitro release of OT from USP apparatus 3 using different amounts of OT per dosage unit, viz., 25, 50, 100 and 200 IU per dosage unit*

Time (min)	**R squared**	**F**	**P value**	**Are means significantly different? ($p < 0.05$)**
30	0.9965	376.8	P<0.0001	Yes
60	0.9944	235.3	P<0.0001	Yes
120	0.8653	8.563	0.0325	Yes
240	0.3391	0.6842	0.6068	No
360	0.3391	0.6842	0.6068	No
480	0.3391	0.6842	0.6068	No

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