Analytical Methods for the Quantitative Determination of Oxytocin

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Abstract

Oxytocin is a clinically important nonapeptide that is used for the induction and/or augmentation of labor and is normally administered as a slow intravenous infusion diluted with normal saline or Ringer's lactate solution. Oxytocin is also indicated for use in the prevention and treatment of post partum hemorrhage and may be administered via either the intramuscular or intravenous routes in order to increase uterine tone and/or reduce bleeding. The analysis of oxytocin in different media has evolved over the past 30 years with the result that more sophisticated, selective and sensitive techniques are used for the determination of the compound. A variety of techniques have been applied to the determination of oxytocin in different matrices ranging from simple paper chromatography to hyphenated liquid chromatographic such as liquid chromatography coupled with mass-spectrometry. Additionally enzyme linked immuno-sorbent assays (ELISA) and radio immuno-assays (RIA) are used for the determination of low concentrations of oxytocin in biological matrices. This manuscript provides a systematic survey of the analytical methods that have been reported for isolation and quantitation of oxytocin in different matrices.

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Introduction

Oxytocin (Figure 1) is a nonapeptide that is synthesized in the cell bodies of the paraventricular and supraoptic nuclei of the hypothalamus [1-3].

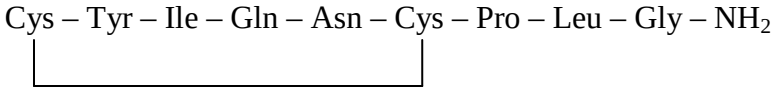

Figure 1. Amino acid sequence of oxytocin

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 [4]. The chemical structure of OT is depicted in Figure 2 [4].

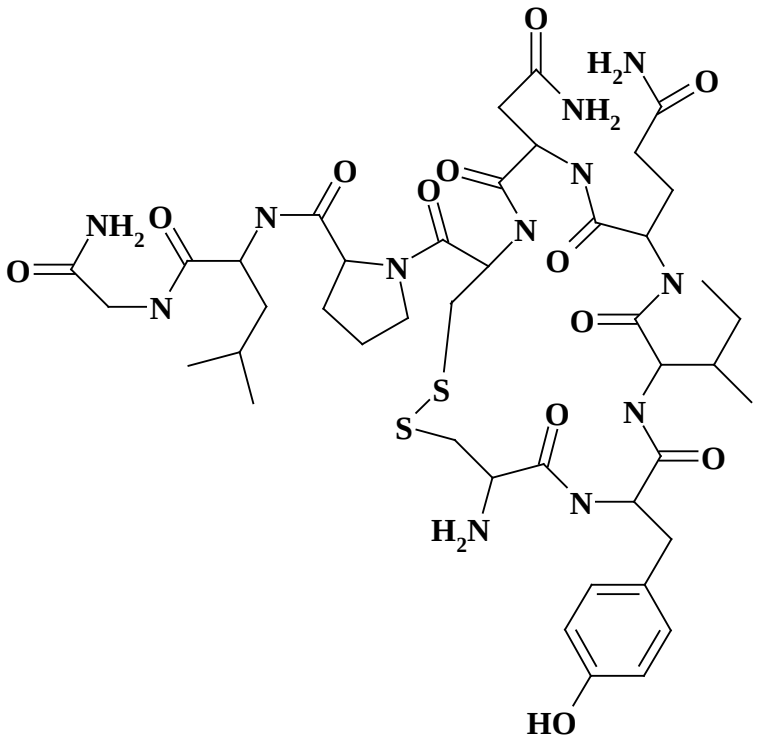

Figure 2 The chemical structure of OT ($C_{43}H_{66}N_{12}O_{12}S_2$) MW = 1007.23 [4]

Following synthesis in the hypothalamus, oxytocin is attached to the carrier protein, neurohypophysin and is subsequently transported via axonal processes to the posterior lobe of the pituitary gland, where it is stored until required for use [1, 2].

Oxytocin activity in mammals include both uterogenic and galactogenic effects that result in uterine contractions and milk letdown respectively [4-7]. The extensive expression of oxytocin receptors in organs other than the uterus and mammary glands suggests that oxytocin has diverse pharmacological effects. Indeed it has been shown that oxytocin plays a role in the stimulation of prostaglandin production in the endometrium, T-cell function, bone and muscle formation, secretion of prolactin, luteolysis among many other diverse biological functions [8, 9].

Analytical methods for the determination of oxytocin need to be sensitive and precise for several reasons. Specifically, commercially available pharmaceutical preparations of oxytocin contain 5 and 10 IU/ml [10], which with reference to the 4th International Standard [11] is equivalent to only 8.4 and 16.8 µg/ml, respectively. In addition oxytocin solutions are often diluted with large volumes of intravenous fluids such as normal saline, Ringer's lactate and Ringer's lactate/dextrose solutions resulting in the delivery of low doses of the drug over extended periods of time. [7]. Furthermore, oxytocin is rapidly cleared from the systemic circulation via liver metabolism and kidney excretion [12] and therefore plasma levels of circulating oxytocin are likely to be low, necessitating the use of highly sensitive analytical techniques for its determination in biological matrices. Sensitivity may be achieved by use of selective and efficient extraction procedures to isolate oxytocin from different matrices or by use of analytical methods with highly sensitive detection systems or that are selective for oxytocin.

Several methods reporting the analysis of oxytocin from different matrices have been published and this review provides an overview of these methods and their diverse application. A summary of the different analytical methods that have been reported and an overview of the subsequent progress made in the determination and quantitation of oxytocin is included.

Bioassay

The use of biological assay methods are the earliest techniques reported for the determination of oxytocic activity [13-15]. These techniques involved the measurement of the depression of blood pressure in a chicken or the assessment of uterine contractions in comparison to those produced from a known standard [4, 16]. As recently as the early 1990's, official compendia

such as the 1990 United States Pharmacopeia [17] and the 1993 British Pharmacopoeia [18] recommended the use of such assays to quantitate the activity of oxytocin.

Bioassays however have a number of limitations including a lack of precision and selectivity and hence they have limited applicability for use as quality control procedures and their use for the determination of low concentrations of analyte is problematic [19]. Furthermore, the high cost of analysis, total analysis time, limited sensitivity and poor reproducibility of bioassays further restrict their use [20]. In addition bioassay methods cannot provide adequate information about the presence or absence and/or levels of related substances or degradation products in a sample [20] further limiting their possible use for routine analysis and therefore other techniques are required for the quantitation of oxytocin.

Partition and Thin Layer Chromatography

The use of classic partition chromatography with a Sephadex® G-25 separation system and a mobile phase consisting of butanol, acetic acid, pyridine and water was successfully used to separate oxytocin from its diastereoisomers [21].

Thin-layer chromatography (TLC) has been successfully used for the purposes of identifying oxytocin during synthetic procedures [22, 23] and TLC was found to be adequate for the identification of oxytocin during solid phase and solution based synthetic procedures [24-26]. In general the retention factor (R_f) of a known standard in a particular mobile phase system was used to compare TLC plates developed following synthesis of oxytocin. The mobile phase compositions used included the use of butanol-acetic acid-water (4:1:1 (v/v/v)) [22, 23] or methanol-chloroform-acetic acid-water (38:62:2:2(v/v/v/v)) with a Pauly and/or chlorine-o-toluidine color reagent for oxytocin identification [27].

The separation of oxytocin from other nonapeptides, including [8-lysine]-vasopressin, [8-ornithine]-vasopressin, [2-phenylalanine,8-lysine]-vasopressin and [des-1-amino]-oxytocin was achieved using commercially available silica gel plates with a mobile phase consisting of methanol-chloroform-acetic acid-water (30/70/1/6 (v/v/v/v)) [28]. TLC was also used for the separation of oxytocin and related nonapeptides with a mobile phase comprised of chloroform-isopropanol-water (2:8:1 (v/v/v)) or acetone-ethyl acetate-methanol-water

(3:2:1:1 (v/v/v/v)) and included a derivatization reaction with fluorescamine for the identification process [29].

An advantage of TLC when used for the purposes of identification of oxytocin during synthetic procedures is that the technique permits a rapid qualitative assessment of the compounds and analytes of interest [30]. However the technique is not appropriate for the accurate quantitation of the exact amount of oxytocin present in reaction mixtures [30]. Therefore more accurate and selective methods of analysis using techniques such as high performance liquid chromatography (HPLC) for the determination of oxytocin in different media are required.

Electrophoretic Separations

Electrophoretic methods of separation have also been applied to the identification of oxytocin during synthesis [23, 27]. Cellulose-coated plates in an electrolyte solution of pyridine-acetic acid-water (1:10:90) at pH 4.6 with a gradient of approximately 23 V/cm for 45 – 60 minutes was found to be suitable for the identification of oxytocin produced during such procedures [23]. In addition a thin-layer electrophoretic method developed using an electrolyte solution comprised of 0.1 N pyridine-acetic acid buffer at pH 5.6 and an applied voltage of 400 V over a 2 hour period was also found to be suitable for the identification of oxytocin during synthetic procedures [27].

Sutcliffe and Corran [31] compared the selectivity of capillary zone electrophoresis (CZE), micellar capillary zone electrophoresis (MCZE) and HPLC and established that successful separations were possible using MCZE and HPLC whereas complete separation was not achieved using CZE. However MCZE did not offer an advantage over HPLC with respect to speed of analysis and therefore the method was not recommended as an alternative to HPLC for the analysis and identification of oxytocin.

Reversed Phase High Performance Liquid Chromatography

The advent of HPLC and the subsequent development of speciality chemically modified stationary phases have facilitated the use of this technique for the routine analysis of peptides during synthesis and for the purposes of quality control. Furthermore the selectivity of HPLC makes it possible to acquire accurate quantitative data for peptides of interest [30]. It is clear

that HPLC is considered the method of choice for the analysis of oxytocin as the majority of published and compendial methods for the determination of oxytocin us the technique due to its relative simplicity, wide applicability and sensitivity. Several studies [32-37] have shown that it is possible to establish a good quality correlation between results generated using HPLC analysis with those obtained using bioassays in which the contraction of the rat uterus or drop in blood pressure of chickens were monitored. Furthermore the standard error of estimates for HPLC methods (< 1%) is considerably better than those observed when using bioassay techniques (approximately 7%) [35]. A summary of the HPLC methods that have been reported for the determination of oxytocin in different matrices is shown in Table 1.

Table 1. HPLC methods for the analysis of oxytocin

Column	Mobile phase	Elution	Flow rate (ml/minute)	Detection	Retention time (minutes)	Ref
L1 packing, 5 µm, 4.6 mm x 120 mm	A: 0.1 M monobasic sodium phosphate buffer B: 50% acetonitrile in water Linear gradient from 70% mobile phase A to 50% mobile phase B over 20 minutes	Gradient	1.5	UV 220 nm	~ 10	[5]
ODS, 5 µm, 4.6 mm x 125 mm	A: 15.6 g/l sodium dihydrogen phosphate B: 50% acetonitrile in water Linear gradient from 70% mobile phase A to 40% over 30 minutes, and then switch to 70% over 0.1 minute then 70% mobile phase A for 15 minutes	Gradient	1.0	UV 220 nm	~7.5	[6]
LiChrosorb® RP-8 (E. Merck), 5 µm, 3.2 mm i.d. x 150 mm	18% acetonitrile in 0.15 M phosphate buffer, pH = 3.0	Isocratic	0.7	UV 215 nm	~ 7.5	[30]
Merck® RP 8, 10 µm, 25 cm x 3 mm i.d.	20% v/v acetonitrile in phosphate buffer, pH = 7	Isocratic	3.0	UV 215 nm	~ 2	[16]
Nucleosil® C_8 5 µm, 15 cm x 0.4 mm i.d.	20% v/v acetonitrile in phosphate buffer, pH = 7	Isocratic	2.0	UV 210 nm	~ 5	[16]
Spherisorb® S5 ODS, 5 µm, 7.5 cm x 3 mm i.d.	17.5% v/v acetonitrile in borate buffer, pH = 10	Isocratic	1.0	UV 220 nm	~ 2.5	[16]
Nucleosil® C_{18}, 10 µm, 25 cm x 4 mm i.d.	20% v/v acetonitrile in 267 mM phosphate buffer, pH = 7	Isocratic	4.0	UV 210 nm	~ 4	[38]
Nucleosil® C_8, 5 µm, 15 cm x 4 mm i.d.	20% v/v acetonitrile in 0.67 M phosphate buffer, pH = 7	Isocratic	1.8	UV 220 nm	~ 7	[38]
Merck® RP 8, 10 µm, 25 cm x 3 mm i.d.	20% v/v acetonitrile in 0.67 M phosphate buffer, pH = 7	Isocratic	3.0	UV 215 nm	~ 2.5	[38]
RP-C18, 12.5 cm x 4.6 mm i.d. e.g. Shandon Hypersil®	A: 50% acetonitrile B: 0.1 M sodium dihydrogephosphate Gradient: 30% A to 60% A in 30 minutes	Gradient	1.0	UV 220 nm	~ 9	[35]
LiChrosopher® 60 RP-select, 5 µm	18% v/v acetonitrile in phosphate buffer pH = 2.1	Isocratic	1.0	UV 220 nm	10	[32]
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 acetonitrile	Gradient	1.5	UV 220 nm	10	[39]
C_{18} micro bond-a-clone 10 µm column (Phenomenex®)	20% acetonitrile in a 0.1 M potassium dihydrogen phosphate buffer at pH 7.0.	Isocratic	1.6 – 1.8	UV 210 nm	7.4	[40]

Phenomenex® Hypersil C_{18} 5 µm, 4.6 mm x 150 mm	20% acetonitrile in 0.08 M phosphate buffer, pH = 5.0	Isocratic	1.5	UV 220 nm	5.0	[41]
Agilent Zorbax® SB-C18, 5 µm, 2.1 mm x 150 mm	50% v/v acetonitrile in 0.05% v/v formic acid	Isocratic	0.25	Mass spectrometry	4.51	[42]

One of the earliest applications of HPLC to monitor the synthesis of oxytocin was achieved using a LiChrosorb® RP-8 5 µm, 3.2 mm i.d. x 150 mm stationary phase and a mobile phase comprised of sodium phosphate buffer and acetonitrile [30]. This method was selective since it was possible to separate oxytocin from its immediate precursor, i.e. a reduced nonapeptideamide which contains reduced cysteine groups as compared to oxytocin which contains oxidized residues that form a disulphide bridge [30]. Furthermore the method was found to be suitable for the quantitative determination of oxytocin and its synthetic intermediates with a limit of detection in the nanogram range.

Reversed-phase HPLC methods that have been used for the separation of oxytocin and its diastereoisomers have selected various stationary phases including µBondapak® C_{18} columns with mobile phase compositions of 10% v/v tetrahydrofuran in ammonium acetate buffer or 18% v/v acetonitrile in ammonium acetate buffer [21]. The separation of oxytocin from 2-D-tyrosineoxytocin, 4-D-glutaminyloxytocin and 2-D-tyrosine-4-D-glutaminyloxytocin has also been achieved using reversed-phase HPLC in which both gradient and isocratic separations were successful [33]. By use of a gradient system the resolution of oxytocin from its diastereoisomers was better than that achieved using an isocratic separation method. Isocratic separation was achieved using a LiChrosorb® RP 8 (5 or 10 µm) column with a mobile phase consisting of 12% v/v acetonitrile in a phosphate buffer of pH 3 or 7 whereas the gradient separation was achieved using an octadecylsilanized silica gel (5 µm) stationary phase with phosphate buffer (pH 2.3) initially and a buffer-acetonitrile (1:1) gradient to separate oxytocin and similar nonapeptides over the analysis time [33]. However, the isocratic method was found to be suitable for monitoring the quality and stability of synthetic oxytocin [33, 34].

The determination of oxytocin and other nonapeptides in liquid dosage forms such as intravenous solutions and concentrated oxytocin products has also been achieved using reversed-phase HPLC [38]. Binary mixtures of 20% v/v acetonitrile in phosphate buffer were used as the mobile phase for the quantitation of oxytocin in the aforementioned products. The limit of detection for oxytocin was reported to be 0.88 and 1.17 ng/µl at wavelengths of 210 and 215 nm, respectively. The methods were found to be reproducibile for the analysis of oxytocin in ampoules and concentrates with relative standard deviation values of between 1 and 1.5%, respectively [38].

The separation of oxytocin from other peptide hormones using gradient elution HPLC with a Hypersil® ODS (5 mm x 100 mm) stationary phase and 0.1 M phosphate buffer at pH 2.1 and acetonitrile as the primary and secondary solvents, respectively, has been reported [43]. The method applied a gradient of 0 – 60% v/v acetonitrile over a 50 minute period with oxytocin eluting after approximately 19.5 minutes. The eluant was monitored by UV detection at a wavelength of 225 nm [43].

Several other authors have recommended the use of HPLC for the determination of oxytocin in pharmaceutical preparations [32, 36, 39, 41, 44].

Dudkiewicz et al., [32] used a 5 µm LiChrosopher® 60 RP-select stationary phase with a mobile phase of 18% v/v acetonitrile in phosphate buffer at pH = 2.1 at a flow rate of 1.0 ml/minute and a detection wavelength 220 nm to determine the concentration of oxytocin in pharmaceutical dosage forms. A similar method reported by Ohta et al., [36] made use of a 5 µm, 4.6 mm i.d. x 250 mm Zorbax® TMS column maintained at 40 °C and a mobile phase comprised of 18% v/v acetonitrile with a 50 mM phosphate buffer at pH 5.0 and a flow rate of 1.0 ml/minute for the determination of oxytocin in pharmaceutical preparations.

The determination of oxytocin in parenteral formulations was achieved using a validated stability indicating assay that used reversed-phase gradient chromatography [39]. The stationary phases used in these studies were 5 µm, 120mm X 4.6 mm i.d. Alltech Hypersil® ODS or 150 mm X 4.6 mm i.d. Beckman Ultrasphere® ODS with mobile phase A consisting of 100 mM monobasic sodium phosphate of varying pH between 3.1 and 4.5 and mobile phase B consisting of 50% v/v acetonitrile in water. The flow rate was set at 1.5 ml/minute and detection was achieved at 220 nm. The resultant retention time for oxytocin was approximately 10.2 minutes and chlorobutanol, the preservative used in the formulations eluted at approximately 21.1 minutes. Stress studies were conducted by exposing Oxytocin Injection USP and synthetic oxytocin containing 10 units/ml to thermal, acidic, basic, oxidative and fluorescent light conditions. In all cases oxytocin was well resolved from any degradation products and the percent degradation was calculated from the peak area response of samples relative to a calibration curve [39].

An analytical method for the assay a combination formulation of oxytocin and ergometrine has been reported and a Nucleosil® C_{18} column was used as the stationary phase [44]. The mobile phase used to achieve the separation was comprised of 35% v/v acetonitrile in a buffer containing 0.05% sodium tetradecyl sulfate and 0.83 mM phosphoric acid buffer adjusted to pH 5 with triethylamine. The flow rate for the analysis was 2.5 ml/minute and the analytes of interest were detected using UV detection with a retention time for oxytocin of approximately 8 minutes.

A simple isocratic stability indicating HPLC method for the determination of oxytocin in ampoules was developed by Chaibva and Walker [41]. Stress studies conducted during validation revealed that the method was stability indicating as oxytocin was found to be well separated from any degradation products. Separation was achieved on a 5 µm, 4.6 mm i.d. X 150 mm Phenomenex® C_{18} Hypersil®, stationary phase using a mobile phase consisting of 20% v/v acetonitrile in an 80 mM phosphate buffer at a pH of 5. The limits of quantitation and detection were reported to be 0.4 IU/ml and 0.1 IU/ml respectively with a maximum of 4.84% RSD indicating that the method was precise.

The stability of oxytocin in fluids commonly used for intravenous adminsitration was evaluated using a C_{18} bond-a-clone 10 stationary phase with ethylphychoxybenzoate as an internal standard [40]. Two vials of oxytocin (10 IU/ml) were injected into 1000 ml of an intravenous solution after which samples were periodically removed from the solutions for analysis. Samples were concentrated prior to analysis that was conducted using a mobile phase of 20% v/v acetonitrile in a 10 mM potassium dihydrogen phosphate buffer at pH 7.0 at a flow rate of 1.6 to 1.8 ml/minute with UV detection at 210 nm. The retention time of oxytocin under these conditions was reported to be approximately 7.4 minutes [40]. Solid phase extraction has also been used for concentrating oxytocin from dilute Ringer's Lactate solutions [45]. A Supelco® C_8 (5 µm, 150 mm x 4.6 mm) reversed-phase column attached to a pellicular guard column with a mobile phase consisting of 20% v/v acetonitrile in a 50 mM potassium dihydrogen orthophosphate buffer at pH 7 at a flow rate of 1.25 ml/minute with detection at 220 nm was used for sample analysis. Under these conditions oxytocin and internal standard were eluted at approximately 8.5 minutes and 17 minutes respectively. The lower limit of quantitation was found to be 0.0075 IU/ml.

Bridges et al, [46] reported the use of a simple isocratic HPLC technique for the determination of neurohypophyseal hormones including oxytocin with UV detection. The method was reported to be highly sensitive and levels of oxytocin as low as 200 fmol were analyzed.

UV Detection

The ultraviolet (UV) spectrum of oxytocin reveals that oxytocin has a λ_{max} absorbance at approximately 275 nm with an additional peak at approximately 280 nm with an additional region of increased absorbance occurring at between 200 – 240 nm [4]. Therefore it is not surprising that the majority of analytical methods summarized in Table 1 reveal the use of UV as a method of detection at a wavelength of approximately 220nm. The use of UV detection for the analysis of oxytocin may is a relatively simple procedure without the need for sample derivatization. However, the major limitation of UV detection is its relatively low sensitivity when applied to samples in which particularly low concentrations of oxytocin or other synthetic by-products or degradation products of the molecule are present.

Fluorescence methods with derivatization

The use of post column derivatization methods prior to detection to improve the sensitivity of the analytical methods of oxytocin has been reported [20, 47-49]. It has been shown [48] that spectrofluorometric methods of detection are more sensitive when compared to the use of UV detection at 210 nm with an improvement in sensitivity of between 2- and 5-fold for optimized reaction conditions [47].

The application of post column derivatization with Fluram® has been reported for the HPLC analysis of injection solutions of oxytocin [47, 48]. The method was found to be reproducible with a retention time of less than 10 minutes for oxytocin. The high reproducibility and sensitivity of the reported method permits the accurate characterization and quantitation of oxytocin and related substances in pharmaceutical formulations and is also applicable for the determination and quantitation of by-products that contain free amino functional groups at low concentrations [47].

Derivatization with fluorescamine followed by HPLC has also been applied to the determination of oxytocin in large volume intravenous fluids [20]. Samples were concentrated using an on-line trap C_{18} pre-column concentrator/guard column and switching valve. Separation was achieved using a 5 µm, 4.6 mm i.d. x 125 mm Whatman® C_{18} column and a mobile phase consisting of 21% v/v acetonitrile in 0.1% phosphoric acid. Detection was performed with excitation at 250 nm and measured through a 418 nm cut off filter [20]. The method was used for determining concentrations as low as 40 parts per billion and was validated for selectivity, reproducibility, accuracy and precision.

Fluorometric detection following derivatization was one of the earliest methods applied to the determination of oxytocin (and vasopressin) in biological tissues [50]. Gruber et al., [50] described the extraction of oxytocin from rat pituitary glands and subsequent derivatization with fluorescamine. The peptide reacts with fluorescamine through the free amino functional group to produce a fluorophor. Separation in this case was achieved using a Partisil® ODS reversed-phase bonded column which had been equilibrated with a mobile phase containing 15% v/v acetone, 0.03% ammonium formate and 0.01% thiodiglycol. A 55 minute linear gradient from 15 to 50% v/v acetone with both solutions containing ammonium formate and thiodiglycol at the aforementioned concentration and run at a flow rate of 0.25 ml/minute was used to elute the fluorophors. The retention time for oxytocin under these conditions was approximately 30 minutes and the method was applied to the assay of peptides in tissue samples. The limits of quantitation and detection were in the picomole range with a concentration of 15 pmol of the oxytocin derivative giving a peak with a signal to noise ratio of 15:1.

Photodiode array detection

Rao et al., [51] used a simple isocratic HPLC method with photodiode-array detection (PDA) for the simultaneous detection of oxytocin, lysine vasopressin and arginine vasopressin. Sample concentration was performed using a solid phase extraction technique and analysis was achieved using HPLC following after reconstitution with the mobile phase. A Dynamax® 3009-A C_8 column was used with a mobile phase consisting of 20% v/v acetonitrile with 0.1% trichloroacetic acid, 50 mM heptanesulfonic acid and 30 mM triethylamine in water at pH 2.5. The retention time of oxytocin under these conditions was reported to be 4.6 minutes [51].

Coulometric determination

Although UV detection has been the primary method of choice for the analysis of oxytcon in all sample matrices the use of coulometric detection for the determination of oxytocin in biological samples has also been considered. Samples were prepared using solid phase extraction with an antibody immuno-affinity purification used to extract the oxytocin prior to analysis using HPLC coupled with coulometric detection [52]. The use of dual-electrode coulometric detection permits the oxidation of electro-active amino acids (such as tyrosine and tryptophan) by use of an upstream electrode thereby enhancing the detection capability of the downstream electrode. Consequently the sensitivity of the method is enhanced (up to 5 fold) permitting the analysis of extremely low concentrations of analytes in biological matrices [52]. This method was found to be highly sensitive with the lower limit of detection reported to be 40 pg/ml The retention time for this method was found to be 9.72 minutes.

LC MS Methods

The use of mass spectrometry has recently been reported for the detection of very low concentrations of oxytocin [42]. The use of liquid chromatography with mass spectrometry provides additional selectivity and sensitivity thereby which eliminating the need for time consuming sample preparation methods that are usually required for concentrating dilute samples. The separation and characterization of oxytocin (and other peptides) using reversed-phase liquid chromatography – mass spectrometry has been performed successfully, indicating the potential usefulness of this technique for the detection of oxytocin [53] Furthermore, the degradation of oxytocin (and other peptides) has also been studied [54] using mass spectrometry and shows the application of this method to monitor oxytocin levels in pharmaceutical dosage forms. The fragmentation patterns of oxytocin were used to identify oxytocin and degradation products [42]. Karbiwnyk et al., [42] used LC-MS to determine the concentration of oxytocin in dilute intravenous solutions. An LC-MS ion trap instrument with an electrospray ionization interface in a positive ion mode was used for the analysis. The isocratic method used an Agilent Zorbax® SB C_{18}, 5µm, 150 mm x 2.1 mm i.d., stationary phase with a mobile phase of 50% acetonitrile (v/v) and water containing 0.05% formic acid at a flow rate of 0.25 ml/minute. Under these conditions the limits of quantitation and detection were 7 and 2ng/ml, respectively.

Cation Exchange Chromatography

Radhakrishnan et al., [49] used cation-exchange chromatography with a Partisil® SCX cation exchange resin, volatile pyridine acetate buffers and an automated fluorescamine column monitoring system to separate oxytocin from related peptides. Separation was achieved using a 50 minute gradient with a mobile phase was varying from 5 x 10^{-3}M pyridine at pH 3.0 to 5 x 10^{-2} M pyridine at pH 4.0 and from which oxytocin was eluted between 40 and 50 minutes.

Ion exchange chromatography has also been reported to be applicable for the analysis of the degradation products from enzymatic degradation of oxytocin [55]. Degradation products from enzymatic degradation were synthesized and were separated on a Partisil® SCX, 10 µm (4.6 mm i.d. x 250 mm) columns. Typical degradation products that were isolated included the peptides Asn and Gln, dipeptides such as Gln-Asn, Leu-Gly, aminated tripeptides including Pro-Leu-Gly-NH2, and other peptides. The mobile phase consisted of 10% v/v methanol in 0.02 M aqueous potassium dihydrogen phosphate of pH 5 and the flow rate was set at 0.5 ml/minute. Samples were monitored with UV detection at 209 nm and the retention time of oxytocin under these conditions was approximately 18 minutes.

Radio-immunoassay

It has been reported that a coupling of ion-pair HPLC and post column detection by RIA may be used for the determination of nonapeptides, including oxytocin in the pineal and pituitary glands [56].

The majority of methods used for the determination of oxytocin levels in pharmacological applications have used radio-imunno assay (RIA) techniques [57-62]. The major advantage of RIA is that very low levels of analyte can be determined and the limits of quantitation and detection are in the sub picogram range [60].

Enzyme-Linked Immuno-Sorbent Assay

A simple Enzyme-Linked ImmunoSorbent Assay (ELISA) method for the determination of oxytocin levels and changes of oxytocin levels during parturition in monkeys was described by Kawasaki et al., [63]. The method was considered to be effective since it had a short run time, permitting multiple analyses to be carried out expediently. Furthermore, the method

was sensitive and it was possible to determine concentrations of oxytocin as low as 8 pg/ml in serum.

Conclusion

Oxytocin is an important neurohypophyseal hormone with a diverse pharmacological profile and important clinical function in preventing and controlling post partum hemorrhage. The analysis of oxytocin has been performed using a diverse range of methods including bioassay methods that measure the drop in the blood pressure of chickens or the effect of oxytocin on uterine contractions. However with advances in analytical technology methods for the assay of oxytocin have become complex and range from classic partition chromatography to non-derivatized HPLC with ultraviolet detection and HPLC with derivatization and fluorescence detection. The need for sensitive methods that are applicable to the detection of very low levels of analyte in dosage forms, solutions for use and biological matrices has resulted in the use of alternate detection methods for analysis. Spectrofluometric and coulometric detectors have been successfully applied to the determination of oxytocin as has the use of HPLC with mass spectrometry to facilitate the detection of extremely low concentrations of oxytocin in samples of interest.

References

1. Pituitary hormones and their hypothalamic releasing factors. In: Hardman JG, Limbird LE, Goodman Gilman A, eds. *Goodman and Gilman's the Pharmacological Basis of Therapeutics*. New York: McGraw Hill Medical Publishing Division, 2001; 1541-62.

2. Hruby VJ, Chow MS, Smith DD. Conformational and structural considerations in oxytocin-receptor binding in biological activity. *Annual Review of Pharmacology and Toxicology* 1990; **30:** 501-34.

3. Sokol HW. Evidence for oxytocin synthesis after electrolytic destruction of the paravenricular nucleus in rats with hereditary hypothalamic diabetes insipidus. *Neuroendocrinology* 1970; **6:** 90-7.

4. Nachtmann F, Krummen K, Maxl F, Riemer E. Oxytocin. *Analytical Profiles of Drug Substances* 1981; **10:** 563-600.

5. United States Pharmacopeia/National Formulary. 31, 2897-2898. 2008. Rockville, MD, United Stated Pharmacopeial Conventionn Inc. Twinbrook Parkway.

6. British Pharmacopoeia. Volume 1, 1635-1636. 2009. London, The Stationary Office.

7. Oxytocin. Sweetman, S. Electronic Version. 2009. London, Pharmaceutical Press. Martindale: The Complete Drug Reference. 1-27-2009.

8. Gimpl G, Fahrenholz F. The oxytocin receptor system: Structure, function, and regulations. *Physiological Reviews* 2001; **81:** 629-83.

9. Zingg HH, Laporte SA. The oxytocin receptor. *Trends in Endocrinology and Metabolism* 2003; **14:** 222-7.

10. Syntocinon. *Monthly Index of Medical Specialities (MIMS)* 2008; **48:** 239.

11. WHO International Biological Reference Preparations. http://www.who.int/biologicals/reference_preparations/catalogue_no/en/index.html . 2009. Access date: 13 May 2009.

12. Fjellestad-Paulsen A, Lundin S. Metabolism of vasopressin, oxytocin and their analogues [Mpa1, -Arg8]-vasopressin (dDAVP) and [Mpa1, -Tyr(Et)2, Thr4, Orn8]-oxytocin (antocin) in human kidney and liver homogenates. *Regulatory Peptides* 1996; **67:** 27-32.

13. Deptula S. Evaluation of oxytocin determination methods. *Acta Poloniae Pharmaceutica (Abstract on Science Direct®)* 1966; **23:** 51-4.

14. Houvenaghel A. Biological determination of plasma oxytocin. *Memoires de l'Academie Royale de Medecine de Belgique (Abstract on Science Direct®)* 1979; **7:** 78.

15. Van Dongen CG, Hays RL. A sensitive in vitro assay for oxytocin. *Endocrinology* 1966; **78:** 1-5.

16. Krummen K, Frei RW. The separation of nonapeptides by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* 1977; **132:** 27-36.

17. United States Pharmacopeia. 22, 1005-1006. 1990. Twinbrook Parkway, Rockville MD, United States Pharmacopeial Convention, Inc.

18. British Pharmacopoeia. Volume 1, 475-478. 1993. London, The Pharmaceutical Press.

19. Andre M. Effects of mobile phase and stationary phase on the quantitative determination of oxytocin. *Journal of Chromatography A* 1986; **351:** 341-5.

20. Brown DS, Jenke DR. Determination of trace levels of oxytocin in pharmaceutical solutions by high-performance liquid chromatography. *Journal of Chromatography A* 1987; **410:** 157-68.

21. Larsen B, Fox BL, Burke MF, Hruby VJ. The separation of peptide hormone diasteroisomers by reverse phase high pressure liquid chromatography. Factors affecting separation of oxytocin and its diastereoisomers - structural implications. *International Journal of Peptide and Protein Research* 1979; **13:** 12-21.

22. Hase S, Walter R. Symmetrical disulphide bonds as sulphur-protecting groups and their cleavage by dithiothreitol. Synthesis of oxytocin with high biological activity. *International Journal of Peptide and Protein Research* 1973; **5:** 283-8.

23. Mühlemann M, Titov MI, Schwyzer R, Rudinger J. The use of intermediates with preformed disulphide bridge for the synthesis of oxytocin and deamino-oxytocin. *Helvetica Chimica Alta* 1972; **55:** 2854-60.

24. Khan SA, Sivanandaiah KM. Solid-phase synthesis of oxytocin, desaminooxytocin and 4-Thr-oxytocin using active esters in the presence of 1-hydroxybenzotriazole. *International Journal of Peptide and Protein Research* 1978; **12:** 164-9.

25. Live DH, Agosta WC, Cowburn D. A rapid, efficient synthesis of oxytocin and [8-arginine]-vasopressin. Comparison of benzyl, p-methoxybenzyl, and p-methylbenzyl as protecting groups for cysteine. *Journal of Organic Chemistry* 1977; **42:** 3556-61.

26. Spatola AF, Cornelius DA, Hruby VJ, Blomquist AT. Synthesis of oxytocin and related diastereomers deuterated in the half-cystine positions. Comparison of solid phase and solution methods. *Journal of Organic Chemistry* 1974; **39:** 2207-12.

27. Flouret G, Terada S, Kato T, Gualtieri R, Lipkowski A. Synthesis of oxytocin using iodine for oxidative cyclization and silica gel adsorption chromatography for purification. *International Journal of Peptide and Protein Research* 1979; **13:** 137-41.

28. SANDOZ AG, Basel Switzerland. Quality control. 1981. In, Nachtmann F, Krummen K, Maxl F, Riemer E. Oxytocin. *Analytical Profiles of Drug Substances*

1981; **10:** 563-600.

29. Nakamura H, Pisano JJ. Derivatization of compounds at the origin of thin-layer plates with fluorescamine. *Journal of Chromatography* 1976; **121:** 33-40.

30. Nachtmann F. High-performance liquid chromatography of intermediates in the oxytocin synthesis. *Journal of Chromatography A* 1979; **176:** 391-7.

31. Sutcliffe N, Corran PH. Comparison of selectivities of reversed-phase high-performance liquid chromatography, capillary zone electrophoresis and micellar capillary electrophoresis in the separation of neurohypophyseal peptides and analogues. *Journal of Chromatography A* 1993; **636:** 95-103.

32. Dudkiewicz W, Snycerski A, Tautt J. HPLC method for the determination of oxytocin in pharmaceutical dosage form and comparison with biological method. *Acta Poloniae Pharmaceutica - Drug Research* 2000; **57:** 403-6.

33. Krummen K, Maxl F, Nachtmann F. The use of HPLC in the quality control of oxytocin. *Pharmaceutical Technology International* 1979; **2:** 37-43.

34. Krummen K, Maxl F, Nachtmann F. The use of high-performance liquid chromatography (HPLC) in the quality control of oxytocin. *Pharmaceutical Technology* 1979; **3:** 77-83.

35. Maxl F, Siehr W. The use of high-performance liquid chromatography in the quality control of oxytocin, vasopressin and synthetic analogues. *Journal of Pharmaceutical and Biomedical Analysis* 1989; **7:** 211-6.

36. Ohta M, Fukuda H, Kimura T, Tanaka A. Quantitative analysis of oxytocin in pharmaceutical preparations by high-performance liquid chromatography. *Journal of Chromatography A* 1987; **402:** 392-5.

37. Pask-Hughes RA, Hartley RE, Gaines Das RE. A comparison of high performance liquid chromatographic assays with the current pharmacopoeial assays for the combined formulation of ergometrine and oxytocin. *Journal of Biological Standardization* 1983; **11:** 13-7.

38. Krummen K, Frei RW. Quantitative analysis of nonapeptides in pharmaceutical dosage forms by high-performance liquid chromatography. *Journal of Chromatography A* 1977; **132:** 429-36.

39. Wang G, Miller RB, Melendez L, Jacobus R. A stability-indicating HPLC method for the determination of oxytocin acetate in oxytocin injection, USP, synthetic. *Journal of Liquid Chromatography and Related Technologies* 1997; **20:** 567-81.

40. Gard JW, Alexander JM, Bawdon RE, Albrecht JT. Oxytocin preparation stability in several common obstetric intravenous solutions. *American Journal of Obstetrics and Gynecology* 2002; **186:** 496-8.

41. Chaibva FA, Walker RB. Development and validation of a stability-indicating analytical method for the quantitation of oxytocin in pharmaceutical dosage forms. *Journal of Pharmaceutical and Biomedical Analysis* 2007; **43:** 179-85.

42. Karbiwnyk CM, Faul KC, Turnipseed SB, Andersen WC, Miller KE. Determination of oxytocin in a dilute IV solution by LC-MS. *Journal of Pharmaceutical and Biomedical Analysis* 2008; **48:** 672-7.

43. O'Hare MJ, Nice EC. Hydrophobic high-performance liquid chromatography of hormonal polypeptides and proteins on alkylsilane bonded silica. *Journal of Chromatography* 1979; **171:** 209-26.

44. Pask-Hughes RA, Corran PH, Calam DH. Assay of the combined formulation of ergometrine and oxytocin by high-performance liquid chromatography. *Journal of Chromatography A* 1981; **214:** 307-15.

45. Kumar V, Madabushi R, Derendorf H *et al.* Development and validation of an HPLC method for oxytocin in Ringer's Lactate and its application in stability analysis. *Journal of Liquid Chromatography and Related Technologies* 2006; **29:** 2353-65.

46. Bridges TE, Marino V. A rapid and sensitive method for the identification and quantitation of sub-picomolar amounts of neurohypophysial peptides. *Life Sciences* 1987; **41:** 2815-22.

47. Frei RW, Michel L, Santi W. Post-column fluorescence derivatization of peptides : Problems and potential in high-performance liquid chromatography. *Journal of Chromatography* 1976; **126:** 665-77.

48. Frei RW, Michel L, Santi W. New aspects of post-column derivatization in high-performance liquid chromatography. *Journal of Chromatography* 1977; **142:** 261-70.

49. Radhakrishnan AN, Stein S, Licht A, Gruber KA, Udenfriend S. High-efficiency cation-exchange chromatography of polypeptides and polyamines in the nanomale range. *Journal of Chromatography A* 1977; **132:** 552-5.

50. Gruber KA, Stein S, Brink L, Radhakrishnan A, Udenfriend S. Flourometric assay of vasopressin and oxytocin: A general approach to the assay of peptides in tissues. *Proceedings of the National Academy of Sciences of the United States of America* 1976; **73:** 1314-8.

51. Rao PS, Weinstein GS, Wilson DW, Rujikarn N, Tyras DH. Isocratic high-performance liquid chromatography--photodiode-array detection method for determination of lysine- and arginine-vasopressins and oxytocin in biological samples. *Journal of Chromatography A* 1991; **536:** 137-42.

52. Kukucka MA, Misra HP. Determination of oxytocin in biological samples by isocratic high-performance liquid chromatography with coulometric detection using C18 solid-phase extraction and polyclonal antibody-based immunoaffinity column purification. *Journal of Chromatography B: Biomedical Sciences and Applications* 1994; **653:** 139-45.

53. Toll Hr, Oberacher H, Swart R, Huber CG. Separation, detection, and identification of peptides by ion-pair reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry at high and low pH. *Journal of Chromatography A* 2005; **1079:** 274-86.

54. Huck CW, Pezzei V, Schmitz T, Bonn GK, Bernkop-Schnürch A. Oral peptide delivery: Are there remarkable effects on drugs through sulfhydryl conjugation? *Journal of Drug Targeting* 2006; **14:** 117-25.

55. Heping W, Pacáková, Stulík K, Barth T. Ion-exchange high-performance liquid chromatographic analysis of the products of the enzymatic degradation of oxytocin. *Journal of Chromatography A* 1990; **519:** 244-9.

56. Fisher LA, Fernstrom JD. Measurement of nonapeptides in pineal and pituitary using reversed-phase, ion-pair liquid chromatography with post-column detection by radioimmunoassay. *Life Sciences* 1981; **28:** 1471-81.

57. Janaky T, Szabo P, Kele Z *et al.* Identification of oxytocin and vasopressin from neurohypophyseal cell culture. *Rapid Communications in Mass Spectrometry* 1999; **12:** 1765-8.

58. Mori M, Vigh S, Miyata A, Yoshihara T, Oka S, Arimura A. Oxytocin is the major prolactin releasing factor in the posterior pituitary. *Endocrinology.* 1990; **126:** 1009-13.

59. Rosenblum LA, Smith ELP, Altemus M *et al.* Differing concentrations of corticotropin-releasing factor and oxytocin in the cerebrospinal fluid of bonnet and pigtail macaques. *Psychoneuroendocrinology* 2002; **27:** 651-60.

60. Schams D. Oxytocin determination by radioimmunoassay. III. Improvement to subpicogram sensitivity and application to blood levels in cyclic cattle. *Acta Endocrinology(Copenh).* 1983; **103:** 180-3.

61. Vecsernyes M, Torok A, Jojart I, Laczi F, Penke B, Julesz J. Specific radioimmunoassay of oxytocin in rat plasma. *Endocrinology Regulation.* 1994; **28:** 145-50.

62. Bosch OJ, Kromer SA, Brunton PJ, Neumann ID. Release of oxytocin in the hypothalamic paraventricular nucleus, but not central amygdala or lateral septum in lactating residents and virgin intruders during maternal defence. *Neuroscience* 2004; **124:** 439-48.

63. Kawasaki K, Mitsui Y, Ono T *et al.* Simple method for assaying serum oxytocin and changes in serum oxytocin level during parturition in cynomolgus monkeys. *Experimental Animals* 2002; **51:** 181-5.