THE INFLUENCE OF A METHYLATED-β-CYCLODEXTRIN ON THE SOLUBILITY AND PHOTOSTABILITY OF MIDAZOLAM IN AQUEOUS SOLUTION

A thesis submitted in fulfilment of the
requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

of

RHODES UNIVERSITY

by

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January 2001

ABSTRACT

Midazolam, used clinically as an anticonvulsant, anxiolytic, muscle relaxant and sedative is a photolabile imidazo-benzodiazepine which is marketed under the trade names Dormicum® and Hypnovel® as tablets and injectables. Because of an aqueous solubility of < 0.1 mg/ml above pH 4, the preparation of aqueous dosage formulations near physiological pH, requires a solubilizer. The aim of this study was thus to prepare a 10 mg/ml midazolam aqueous solution for topical application using randomly-methylated-β-cyclodextrin (RAMEB), considered to be a suitable candidate as a solubilizer because of its absorption enhancing properties, and to investigate its effect on both the solubility and the photostability of midazolam. Solubility studies of midazolam (excess of 15 mg/ml) in the presence of 0, 5, 10, 20, 30% m/v of RAMEB at pH 5.0 and pH 5.8 (phosphate buffer) were undertaken and the results analyzed using a UV method validated for linearity, accuracy, precision and specificity. A stability-indicating HPLC method was developed and validated (precision and accuracy, linearity, range, limit of quantitation, specificity, robustness and ruggedness) for application to kinetic photostability studies and the identification of photodegradants by LC-MS. Forced degradation studies were carried out at concentrations of 0.5 mg/ml of midazolam instead of the target concentration of 10 mg/ml because of the acceleratory effect of the decreased concentration on the rate of photodegradation. The solutions of midazolam with and without RAMEB were irradiated at 550 W/m^2 for 12 hrs in order to degrade the drug to ±10% of the original concentration.

The UV method proved to be valid in terms of linearity with a correlation coefficient of 0.9998, precise and accurate, and specific for the determination of midazolam in the presence of RAMEB. The results of the phase solubility studies indicated that desired solubility of 10 mg/ml was achieved with 30% m/v RAMEB at pH 5.0. RAMEB slightly decreased the photostability of midazolam, the rate constants being 0.137 and 0.154 hr^{-1} in the absence and presence of RAMEB, respectively. LC-MS analysis revealed that one of the major photoproducts in the presence and absence of RAMEB was N-desalkylflurazepam, a starting material in the synthesis of midazolam. RAMEB inhibited formation of some photoproducts and introduced two new photoproducts, a dimer and an addition product. The difference in the nature of these photoproducts formed may be attributed to the ability of RAMEB to provide conformational control and to stabilize free radicals.

Although RAMEB improved the solubility of midazolam to the target concentration, photostability is decreased with the presence of different photoproducts. These studies have however provided information on the overall photostability of midazolam, the identity of its photodegradants and the photodegradation pathway in the presence and absence of RAMEB, and may be used for further method development and validation for the analysis of aqueous dosage forms containing RAMEB as a solubilizer.

ACKNOWLEDGEMENTS

It is my pleasure to express sincere gratitude to my supervisor Professor Beverley D. Glass for her guidance, support, motivation and patience, and Professor Michael E. Brown, my co-supervisor, who has been very insightful and dependable.

I wish to give special thanks to:

My mother who has been understanding, tolerant, loving and supportive.

Edith for assisting me with the use of the instruments, especially LC-MS/MS.

Mr. and Mrs. Morley for all the assistance they have granted me, it is well appreciated.

Lesotho Government for financial assistance.

Pharmaceutical Research Laboratories, Port Elizabeth, RSA for photodiode array analysis

All my friends who have always been there for me, encouraging and full of advice: there is no limit to my thankfulness.

For 'Mè Matšeliso,

My mother

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionisation
CBC	2'-Carboxymethoxy-4,4'-bis(3-methyl-2-butenyloxy)chalcone
CDER	Center for Drug Evaluation and Research
DSC	Differentia Scanning Calorimetry
FTIR	Fourier Transform Infrared
GABA	Gamma-amino-butyric-acid
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
ICH	International Committee of Harmonization
LC-MS	Liquid Chromatography-Mass Spectrometry
LOQ	Limit Of Quantitation
MS-MS	Mass Spectrometry-Mass Spectrometry
NMR	Nuclear Magnetic Resonance
RA	Relative Abundance
RAMEB	Randomly methylated β-cyclodextrin
RDS	Relative Standard Deviation
TG	Thermogravimetry
TLC	Thin-Layer Chromatography
USP	United States Pharmacopoeia
UV/VIS	Ultraviolet / Visible

CHAPTER 1: MIDAZOLAM

1.1. Introduction

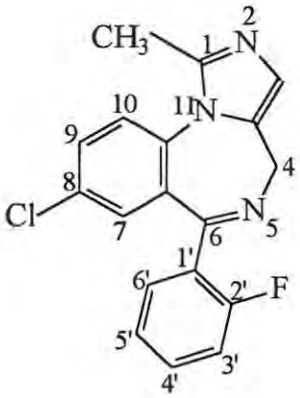
This chapter introduces the benzodiazepine midazolam, its synthesis and explains the effect of the structural differences on the pharmaceutical properties as compared to the classical benzodiazepines. The pharmacology, pharmacokinetics and bioavailability, some physicochemical parameters and dosage forms are reviewed. Included is the project proposal which outlines the aim of the study, the motivation and the proposed methodology. The structure of midazolam is verified and the purity confirmed using spectroscopic, thermo-analytical and chromatographic method, melting and mass spectral determinations.

1.2. Midazolam

1.2.1. Drug Description and Structure-Activity Relationships

Midazolam belongs to a group of benzodiazepines known as the imidazo [1,5a][1,4] benzodiazepines. It was first synthesized in 1976 by Fryer and Walser of the Hoffmann-La Roche company, has the systemic name, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5a][1,4] benzodiazepine (Budavari *et al.*, 1996), and the chemical structure **(1)** as shown in Figure 1.1. Midazolam was found to have sedative, muscle relaxant and anticonvulsant properties similar to that of the conventional benzodiazepines such as diazepam (Pieri *et al.*, 1981) and chlordiazepoxide.

The structural difference between midazolam and the classical benzodiazepines is the imidazole ring that is fused to the 1,2 positions of the diazepine ring in midazolam. This imidazole ring is responsible for the drug's fast action, short elimination half-life, and its basic and soluble nature in aqueous medium.



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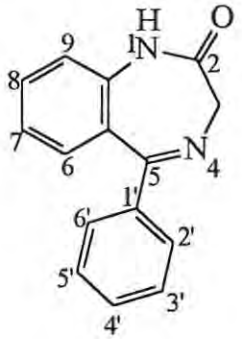


(2)

Figure 1.1. Chemical structure of midazolam(**1**) and the classical benzodiazepine ring system(**2**).

The seven-membered imino-lactam ring is essential for the anxiolytic activity of all the benzodiazepines. Activity is increased by electronegative substituents at positions 7 and 2' of the classical benzodiazepine ring system (**2**). Substitution at position 2' is sterically favourable, while substitution at positions 1 and 3 also enhances the activity (Foye *et al.*, 1995). In midazolam there is a chlorine atom substituted at position 8 (position 7 on the classical benzodiazepine ring system). The substituted chlorine, together with the imidazole ring and the electronegative fluorine at position 2', are responsible for midazolam's high activity. The methyl group on position 1 of the imidazole ring is rapidly oxidised by the hepatic enzymes (Garzone and Kroboth, 1989) and the resulting metabolite eliminated, hence the short duration of action of midazolam.

1.2.2. Synthesis of Midazolam

Preparation of midazolam can be achieved by a series of reactions shown in the reaction scheme in Figure 1.2. The benzodiazepinone (**3**) is treated with methylamine and titanium (IV) chloride, followed by treatment with sodium nitrite to give (**5**). The nitrosamino group of (**5**) is displaced with nitromethane to yield (**6**) which, on reduction and subsequent acylation, results in (**8**). Cyclisation with polyphosphoric acid to the 3,4-dihydro product (**9**), and subsequent dehydrogenation with manganese oxide gives midazolam (**1**) (Fryer and Walser, 1976; Watthey *et al.*, 1984).

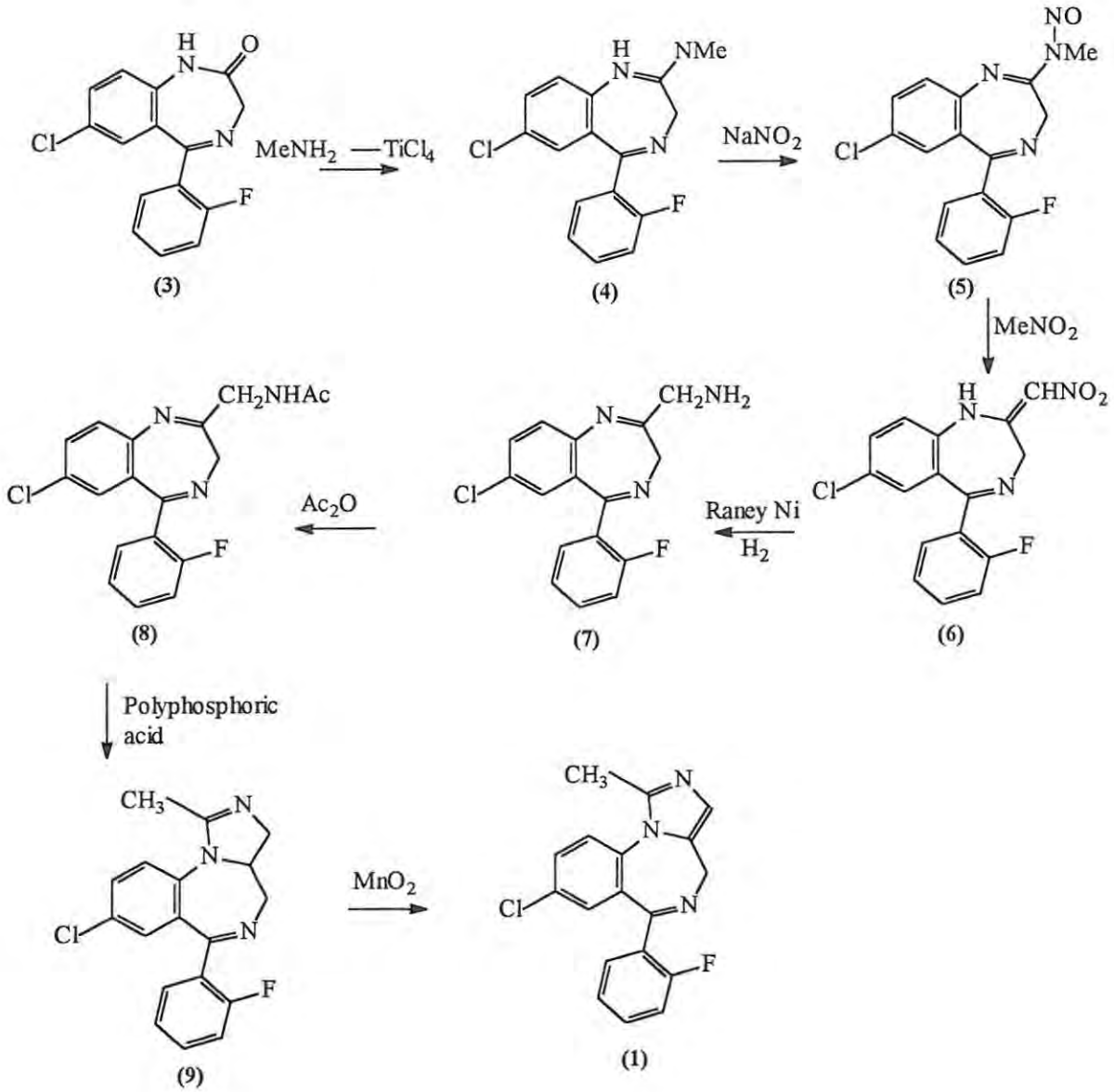


Figure 1.2. Reaction scheme for the synthesis of midazolam.

1.2.3. Mechanism of Action

The area of the brain which regulates the emotional behaviour, the limbic system, is the main site of action of the benzodiazepines, because the benzodiazepine receptors are most highly concentrated there, especially in the amygdala. Benzodiazepines act by potentiating the inhibitory effect of the neurotransmitter gamma-amino-butyric acid (GABA).

Initially it was believed that GABA and the benzodiazepines bind to the same recognition sites, but studies with radioactive labelling have revealed that there are different binding sites. The receptors are situated on the same protein molecule and it is postulated that binding to one receptor changes the shape of the other in a way that affects how the second binds. When GABA binds to GABA-receptor recognition sites on the neurons, it increases the chloride ion conductance of the neuronal cell membrane and therefore slows the rate at which the neurons fire. Binding of benzodiazepines to the benzodiazepine receptors stimulates the binding of GABA to its receptors and therefore enhances the inhibitory effect of GABA (Snyder, 1986; Foye *et al.*, 1995; Ryall, 1989). The increased affinity of GABA for its receptors can aid GABA to compete better and reverse the actions of GABA antagonists (Ryall, 1989).

There are many benzodiazepine receptors in the cerebral cortex and this might be the reason why benzodiazepines have a sedative action (Snyder, 1986).

1.2.4. Clinical Uses

Midazolam has wide clinical application as an anticonvulsant, anxiolytic, muscle relaxant, sedative and sleep-inducing drug. It is used for premedication prior to surgery, but mainly to induce anaesthesia in minor and major surgery (Koch, 1983). Other classical benzodiazepines, diazepam, flunitrazepam and lorazepam, have been used for induction of anaesthesia but they are slow to act and cardiovascular depression may occur (Sanchez-Izquierdo *et al.*, 1998). Midazolam has an advantage over other sedatives such as propofol in that it decreases the occurrence of hypotension, even though wake up time is slightly longer. In induction of anaesthesia, midazolam can be used alone or in combination with other drugs. For example, when midazolam is combined with sufentanil, the haemodynamics and myocardial oxygenation are more acceptable than when sufentanil is combined with enflurane (Murphy, 1998).

The anticonvulsant activity of midazolam makes it suitable for use in status epilepticus (Holmes, 1999). Diazepam has been administered rectally, but this route of administration has proved to be inconvenient for use in emergencies especially in public places as it involves undressing the patient. Use of midazolam by buccal administration is a more acceptable option. Sometimes

patients with generalized convulsive status epilepticus do not respond to conventional agents, but midazolam is always effective and suitable for use in both adults and children (Hanley and Kross, 1998).

1.2.5. Adverse Effects

The most common adverse effects are excessive drowsiness, and ataxia (Reynolds, 1993). Laryngospasm may occur although this is not common. This condition can be reversed by flumazenil while sedation is still maintained (Davis *et al.*, 1998). Less common still are cardiorespiratory effects and gastrointestinal depression, but hypovolemic patients can develop hypotension. Use of midazolam results in fewer venous complications compared with other benzodiazepines (Koch, 1983, Bardhan *et al*, 1984) while paradoxical and psychotic reactions are rare. Where long-term infusions are involved, the patient may develop tolerance and tachyphylaxis and if benzodiazepine withdrawal syndrome occurs after three days or more, lorazepam is preferred. Midazolam may also impair coronary vascular autoregulation. (Shafer, 1998). Other side effects include skin reactions, muscle weakening, anterogate amnesia, libido changes and double vision. Midazolam may unmask pre-existing depression (Snyman, 1999), and potentiates effects of central depressant agents (Pieri *et al.*, 1981). Unlike chlordiazepoxide and alprazolam which can cause photosensitivity (Brooks, 1990; Allen,1993), no photosensitivity reactions have been reported with midazolam.

1.2.6. Pharmacokinetics and bioavailability

Midazolam is lipophilic at physiological pH. This property aids rapid absorption of the drug into the systemic circulation following either oral or parenteral administration (Koch, 1983). There is high bioavailability and reliable plasma concentration following oral administration (Schwagmeier *et al.*, 1998). The peak plasma concentration is reached within 20 to 60 minutes of oral administration. The rate and extent of absorption is reduced if the drug is taken one hour after a meal, otherwise, absorption is not affected. An absorption half-life of less than 14 minutes has been reported (Garzone and Kroboth, 1989). Following intramuscular administration, midazolam appears in the systemic circulation within five minutes and reaches peak concentration

in 20 to 30 minutes. In the case of rectal administration, which has been mostly employed in children with status epilepticus, peak plasma concentrations are reached within 20 minutes, 16 minutes on average.

After oral administration the systemic bioavailability is 34 - 68%. The bioavailability is low because of first-pass metabolism (Reynolds, 1993), which is influenced by the age of the patient in men (higher in elderly men than younger men) and is dose dependent. For example, for a dose of 15 mg the bioavailability was found to range between 35 and 65%, while it was 28 - 36% for a 7.5 mg dose. There is complete absorption of midazolam after intramuscular administration, such that bioavailabilities of 40 - 100% have been reported (Garzone and Kroboth, 1989).

The apparent volume of distribution is affected by body weight, pregnancy, gender and renal insufficiency. Some researchers (Garzone and Kroboth, 1989) did not find the volume of distribution to be age-related, while others found a significant increase in subjects over 80 years of age (3.0 L/kg) compared to subjects of 30 years (1.4 L/kg). About 94 -96% of the drug is bound to plasma protein (Reynolds, 1993), and the binding is not affected by smoking, body weight or gender. Midazolam enters red blood cells to a small extent. In the studies that have been carried out (Garzone and Kroboth, 1989; Koch, 1983), midazolam was not found in the cerebrospinal fluid following oral and intramuscular administration, but about 2 - 6 μg/L has been detected in 15% of the patients who had received midazolam intravenously.

Metabolism of midazolam follows hydroxylation to give 1-hydroxymethylmidazolam **(10)**, the major and active metabolite (less active than midazolam), and to a lesser extent 4-hydroxymidazolam **(11)** and 4-hydroxy-1-hydroxymethylmidazolam **(12)** as shown in Figure 1.3. Subsequent glucuronidation gives glucuronide conjugates of these compounds, which are excreted in the urine (Koch, 1983).

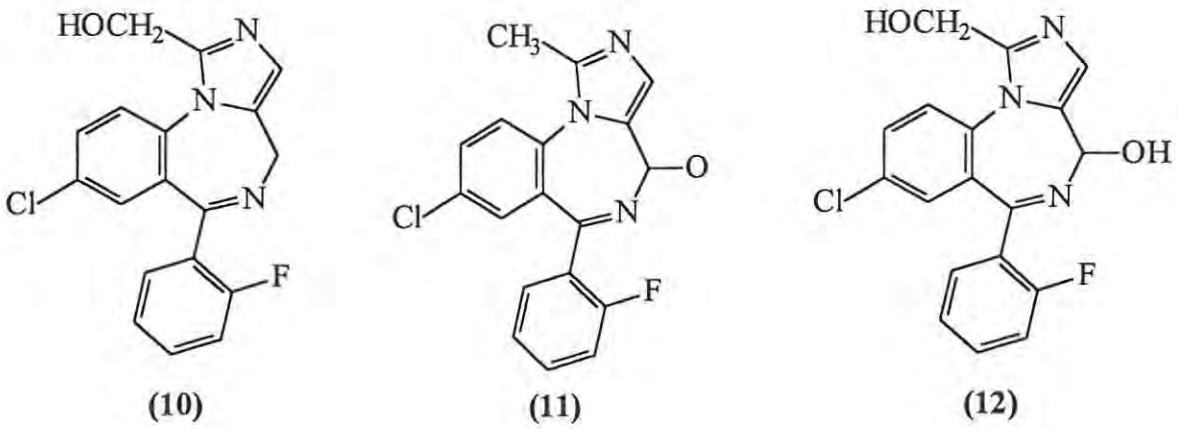


Figure 1.3. Primary metabolites of midazolam.

Midazolam has an elimination half-life of 1 to 2.5 hours in healthy subjects, but the elimination half-life can be quite long (>8 hours) in surgical patients. This is believed to be due to surgical stress or defective hepatic metabolism. The distribution of the drug in the body and its elimination can be greatly affected by physiological changes related to surgery. They include decreased cardiac output, decreased organ perfusion, decreased glomerular filtration, impaired hepatic enzyme activity and haemodilution (Garzone and Kroboth, 1989). The active metabolite has an elimination half-life of about one hour. Elimination is longer in elderly and in obese patients, and in patients with alcoholic liver cirrhosis. Hepatic clearance (first-pass effect) of midazolam is up to 50% of the hepatic blood flow, and plasma clearance is high.

1.3. Dosage Forms

Midazolam marketed in the form of tablets, as the maleate, and injectables as the hydrochloride salt, is distributed under the trade names Dormicum® (tablets or injectable) and Hypnovel® which is an injectable. The tablets are usually prescribed for insomnia at a dose of 7.5 to 15 mg immediately before retiring. For pre-medication, a dose of 15 mg is given 30 to 60 minutes prior to surgery. Injectables are given at a dose of 5 mg intramuscularly, or 10 to 15 mg intravenously, for induction of anaesthesia before operation. Intravenous administration is continued by titration as needed during surgery and usually not more than 5 mg is used (Reynolds, 1993; Snyman, 1999).

1.4. Project Proposal

The solubility of midazolam in aqueous medium makes it suitable for use in preparation of solutions for parenteral administration. However solubility is only achieved at pH ≤ 4 (Koch, 1983), i.e. very acidic solutions due to the reversible conversion to the corresponding open-ring benzophenone (Andersin, 1991). This can be a problem, especially in solutions meant for intramuscular, transdermal or topical application, because irritation may occur. Cyclodextrins have been used to improve the dissolution characteristics of benzodiazepines in solid complexes (Uekama *et al.*, 1983) and in aqueous solution (Andersen and Bundgaard, 1982). The aim of this study was to improve the solubility of midazolam at pH > 4, using a selected cyclodextrin, to enable 10 mg/ml solutions for topical application to be prepared and to investigate the effect of the cyclodextrin on its photostability. The cyclodextrin selected for this study was randomly-methylated-β-cyclodextrin (RAMEB) because its absorption enhancing property makes it suitable for the formulation of solutions intended for topical application.

The drug will be characterized by physical and spectral methods to confirm its identity and purity. Phase solubility studies will be undertaken and the results analysed using a validated UV method in order to determine the amount of RAMEB required to achieve a target concentration of 10 mg/ml of midazolam.

The stability of the drug is to be studied, particularly the effect of RAMEB on the photostability and thermal stability of midazolam. Solutions of the drug, with and without cyclodextrin, once prepared will be irradiated according to the ICH conditions and for sufficient time at 550 W/m^2 in order to degrade the drug to 10% of its original concentration. The kinetics of the photostability of the solutions with and without RAMEB will be studied using a high performance liquid chromatography (HPLC) method of analysis, validated according to the United States Pharmacopoeia (USP 23) standards. If the RAMEB improves the photostability, this can be taken into account in the design of the final package.

Since the HPLC method is suitable for LC-MS application, the photodegradants in the presence and absencece of RAMEB will be identified and a photodegradation pathway proposed. It is of interest to know whether cyclodextrin initiates different degradation pathways that may lead to formation of undesirable products and important that photodegradants only in the presence of RAMEB be identified and this information used in further method development and validation.

1.5. Characterization of Midazolam

Physical properties of a substance offer numerical data useful in distinguishing the compound from other compounds. These include melting point, boiling point, refractive index, intensity of light absorption at different wavelengths, etc. (Hendrickson, 1970). The following experimental procedures were carried out in order to characterize the midazolam.

1.5.1. Thin-Layer Chromatography (TLC)

TLC is a method used to identify compounds (by comparison of R_f value with those of known compounds) or to separate components of a mixture. The R_f values are, however, not always the same (Gunstone, 1970). In this project TLC was used to test the purity of the drug, because it was believed that the method would achieve separation between the drug and any impurities that may be present.

The following method was developed based a method previously used (MacDonald *et al.*, 1972) for the TLC of diazepam, which involved use of chloroform, heptane and ethanol in the ratio 10:10:1 v/v and a Brinkmann F 254 silica gel plate. A solution of miadazolam in acetone was spotted on a Merck Silica gel 60 F_{254} plate which was then placed in a saturated tank containing chloroform, n-heptane and ethanol (ratio 3:3:4, v/v). The solvent front was allowed to ascend for 10 cm. The plate was then removed from the tank and air dried. The plate was visualized under 254nm UV light and the R_f value determined. The R_f value was found to be 0.726. There were no other spots besides the drug spot, which could be an indication that the drug is pure.

1.5.2. Ultraviolet/Visible (UV/VIS) Spectroscopy

Excitation of electrons from the ground state to excited states involves absorption of energy in the ultraviolet and visible regions of the electromagnetic spectrum (Williams, 1966). This energy is characteristic of the chromophore involved. The wavelength at which the substance absorbs can give an indication of the electron system present. Highly conjugated systems need less energy for transition and therefore absorb energy of longer wavelength.

The UV/VIS spectra of a 0.005% m/v solution of midazolam in 0.1M HCl, was determined over the range 200 to 400 nm (USP 23 method for determining UV/VIS spectrum) using a GBC spectrophotometer UV/VIS Model 916 (GBC Scientific Equipment Pty Ltd, Australia). The solution of the drug in 0.1M HCl was found to exhibit a maximum, λ_{max} at 258 nm (Figure 1.4).

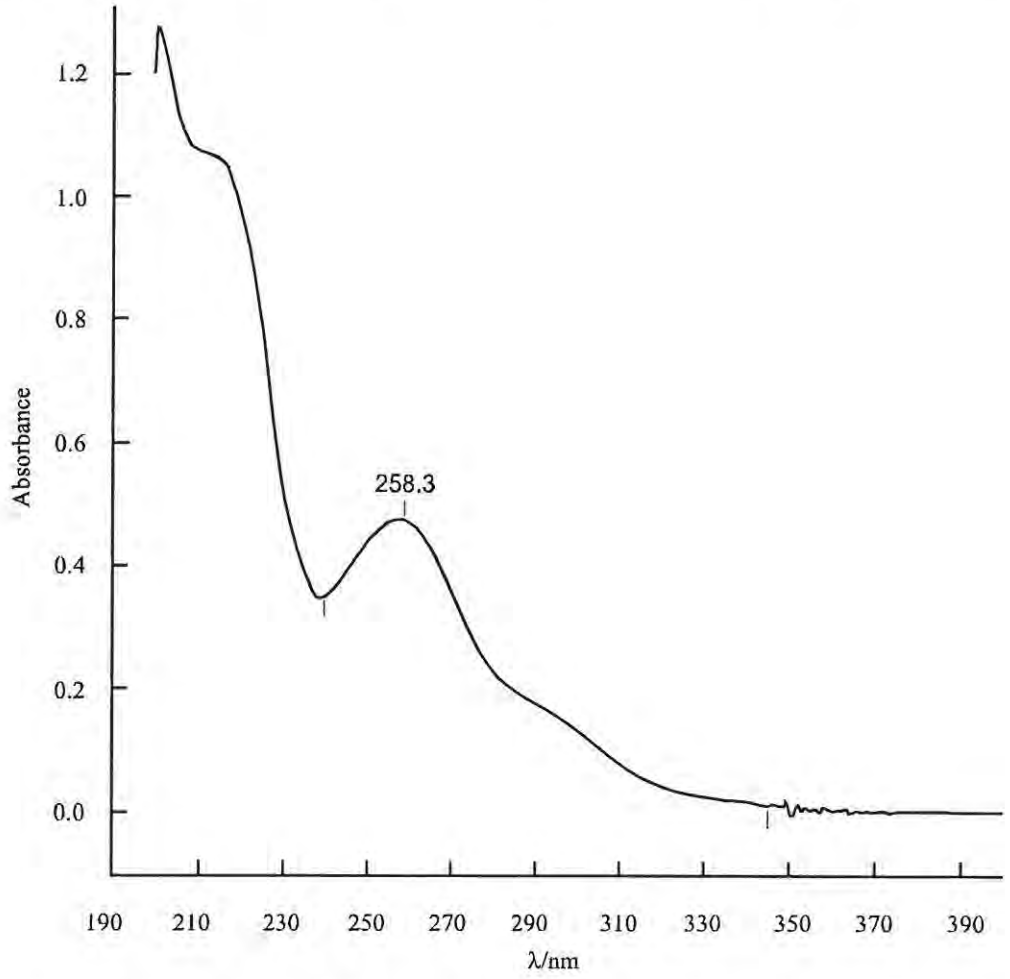


Figure 1.4. The UV/VIS spectrum of midazolam in 0.1M HCl

1.5.3. Fourier Transform Infrared Spectroscopy (FT-IR)

The bending and stretching of bonds in organic compounds occur at specific frequencies that depend on the type of bond involved. An organic group in different compounds will absorb in the same region of the infrared absorption spectrum. If a peak is present in the region characteristic of a certain functional group, it can be assumed that the sample contains that functional group (Gunstone, 1970; MacKenzie, 1971). This method of analysis can therefore be used to identify the functional groups present in a molecule.

The infrared absorption spectrum of midazolam was determined in the following manner: A potassium bromide pellet of the pre-dried dug was prepared as per the USP 23 (1994) and the infrared absorption spectrum determined over the range 400 to 4000 cm^{-1} using a Perkin-Elmer FT-IR spectrometer, SPECTRUM 200 (Perkin-Elmer Limited, England). The absorption bands were assigned to functional groups present in the sample as shown in Table 1.1. The FT-IR results confirm the presence of all the functional groups of midazolam.

Table 1.1. FT-IR Spectral Assignments of Midazolam

Vibrational Mode	Wave number /cm^{-1}	
	Experimental Value	Literature Value
Aromatic C-H stretch	3031	~3030[a,b]
Aliphatic C-H stretch	2924, 2847	3000 - 2850[a]
Imine	1611	1615[a]
Aromatic C=C stretch	1490	1490[a]
C-F stretch	1310, 1215	1400 - 1000[b]
Aromatic out-of-plane bending	823	Below 900[b]
C-Cl stretch	770	800 - 600[b]

[a]Andersin (1991)

[b]Nakanishi (1964)

1.5.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is used to examine the magnetic properties of atoms in a molecule, in order to determine the structures of molecules. ^{1}H NMR, which focuses on the hydrogen nuclei, and ^{13}C NMR are the most commonly used techniques in structural elucidation (Pine *et al.*, 1980). The ^{1}H and ^{13}C NMR spectra of midazolam dissolved in deuteriochloroform ($CDCl_3$) were determined using Bruker Avance 4000 (Bruker Analytische Messtechnik GmbH, Germany) NMR spectrometer. Chemical shift COrrelated SpectroscopY (COSY) and Heteronuclear Multiple Quantum Coherence (HMQC) were also carried out to aid in the assignment of peaks in the ^{1}H NMR spectra. The peaks in the spectrum obtained were assigned as shown in Tables 1.2 and 1.3, with reference to the literature values (Andersin, 1991). The two dimensional NMR techniques were particularly useful in assigning the aromatic and the imidazole ring H's. These results further confirm the presence of a methyl group, a CH_2, one of the protons of which is deshielded (by N), an imidazole ring and aromatic rings.

Table 1.2. ^{1}H NMR Spectral Assignments of Midazolam with Reference to Andersin (1991)

Proton	Chemical Shift (ppm)		Multiplicity
	Experimental Value	Literature Value	
$-CH_3$	2.55	2.56	Singlet
$-CH_2$	4.05, 5.10	4.05, 5.13	Doublet
Imidazole -CH	6.92		Singlet
Aromatic -CH (5')	7.01	7.67 - 6.93	Triplet
" (6')	7.22		Triplet
" (7)	7.25		Singlet
" (10)	7.37		Doublet
" (4')	7.42		Multiplet
" (9)	7.54		Doublet
" (3')	7.61		Triplet

Table 1.3. ^{13}C NMR Spectral Assignments of Midazolam with Reference to Andersin (1991)

Carbon	Chemical Shift (ppm)	
	Experimental Value	Literature Value(Andersin,1991)
C-6	164.3	164.3
C-2'	161.6	160.3
Aromatic and imidazole ring C's	144.1 - 124.0	144.1 - 124.0
C-3'	116.3	116.2
C-4	46.1	46.1
CH_3	15	15

1.5.5. Gas Chromatography- Mass Spectrometry (GC-MS)

Gas chromatography is a method of separation and can thus indicate the presence of other substances beside the drug of interest. Mass spectrometry is used to determine the molecular mass of a substance. Ion bombardment causes the molecule to lose an electron forming a molecular ion. This can then be detected as the highest *m/e* (*m/z*) peak (Pine *et al.*, 1980). The fragmentation pattern can also assist in determining the molecular structure.

The GC-MS system was a Finnigan Mat GCQ (Finnigan Corporation, Austin, USA), fitted with a DB-1 column of dimensions 30 m x 250 μm x 0.25 μm. The injector and the detector were set at 250°C. The system was equilibrated at 200°C for 2 min and then the temperature was increased to 295°C at 30°C / min. The GC chromatogram showed one peak due to midazolam with a retention time of 10.53 min. The mass spectrum obtained (Figure 1.5) shows a molecular ion peak at *m/z* = 325 (confirming the molecular mass of 325), 24.1% relative abundance and a base peak at *m/z* = 310 (RA = 100%, - CH_3). The other mass fragments are the same as appear in the reported mass spectrum (Jones *et al.*, 1989). The most abundant fragment is that due to the loss of $CH_3CH\cdot$ which appears at *m/z* = 297 with relative abundance of 9.4%. Other fragments are *m/z* = 283 (5.3%, - CH_3CHN) and m/z = 249 (3.7%, - $H_2NC_2H_4NH_3\cdot$). Thus midazolam has been successfully identified by comparison of the m/z peak values with the literature values.

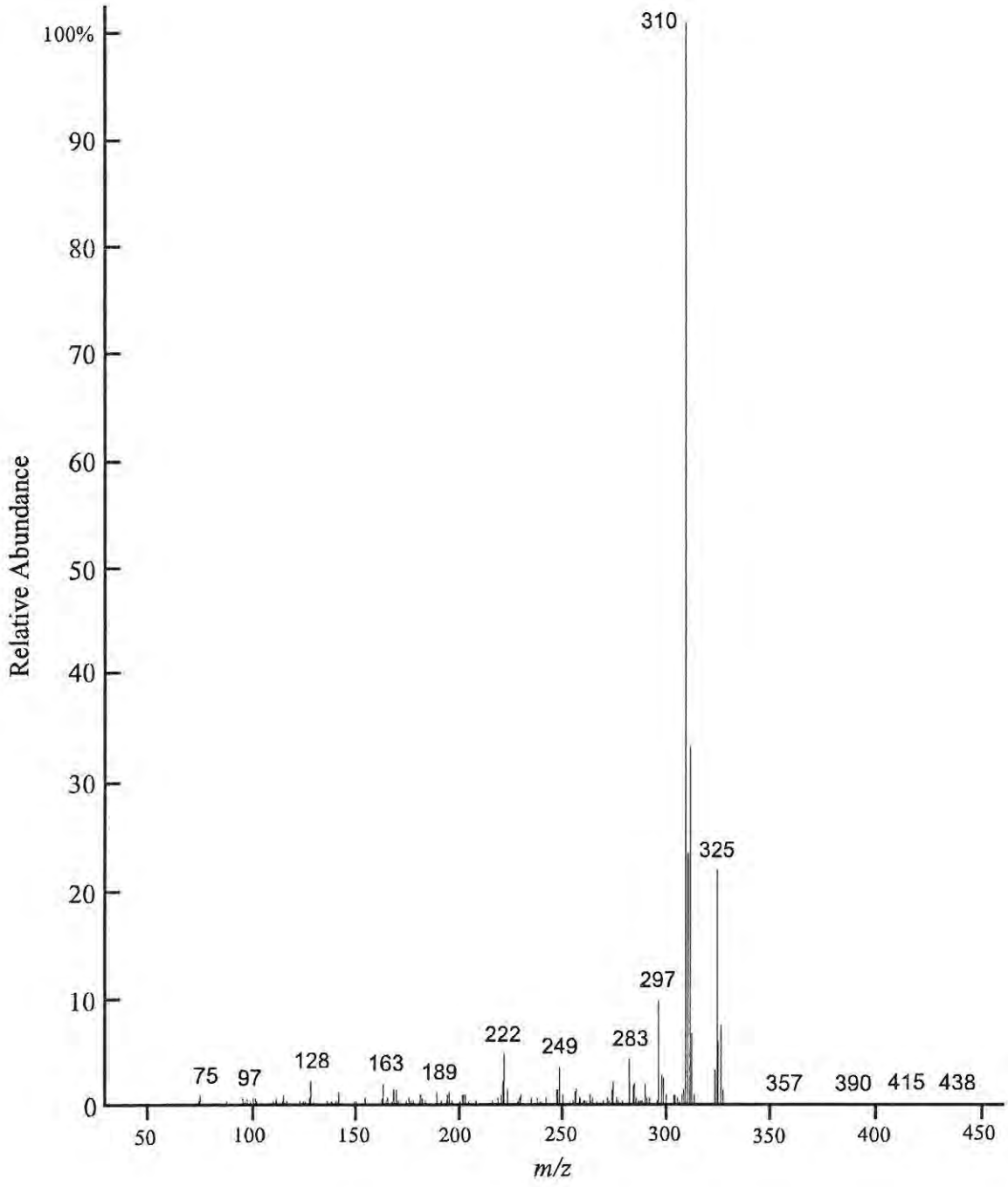


Figure 1.5. Mass spectrum of midazolam

1.5.6. Melting Range

The melting point is a means of identification and a measure of purity of a compound. Pure organic solids have sharp melting points while mixtures of compounds melt over a range of temperatures. The melting range of a compound of ordinary purity is 1-2^{0}C. Presence of an impurity can lower the melting point by as much as 20 to 30^0, so the melting range of can give an indication of purity of the compound (Gunstone, 1970; MacKenzie, 1971). The melting range, determined using a Gallenkamp Melting Point Apparatus (England), was found to be 158-160^{0}C, which agrees with the values reported in the Merck Index 12th ed. The narrow melting range also implies that the compound is pure.

1.5.7. Differential Scanning Calorimetry (DSC)

DSC is used to monitor changes that occur in a sample when heated. As the sample is heated the changes can be detected as deviations from the baseline, indicating the amount of energy supplied to the sample (Brown, 1988) compared to that supplied to an inert reference. Melting of substances is indicated by an endothermic peak. A DSC scan of midazolam was performed from 50^0 to 250^{0}C at 10^{0}C / min in nitrogen, using a Perkin-Elmer DSC 7 Differential Scanning Calorimeter (Perkin-Elmer Limited, England). The scan (Figure 1.6) shows an endothermic peak with onset temperature of 158^{0}C and ΔH 83.7 J/g.

1.5.8. Thermogravimetry (TG)

In thermogravimetry, the events that take place when a sample is heated are monitored as changes of mass. The sample may decompose in a single step or in stages showing several steps of weight loss with time (Brown, 1988). For example, decompositions of hydrated substances usually start with loss of water. Thermogravimetry was performed using a Perkin-Elmer TGA 7 Thermogravimetric Analyzer (Perkin-Elmer Limited, England), over the range 50^0 to 400^{0}C at a heating rate of 10^{0}C / min. The drug started decomposing at 236^{0}C. The decomposition process appeared to take place in one step, although concurrent evaporation of the molten drug cannot be ruled out and the step was complete at 300^{0}C leaving a small amount (4%) of unidentified,

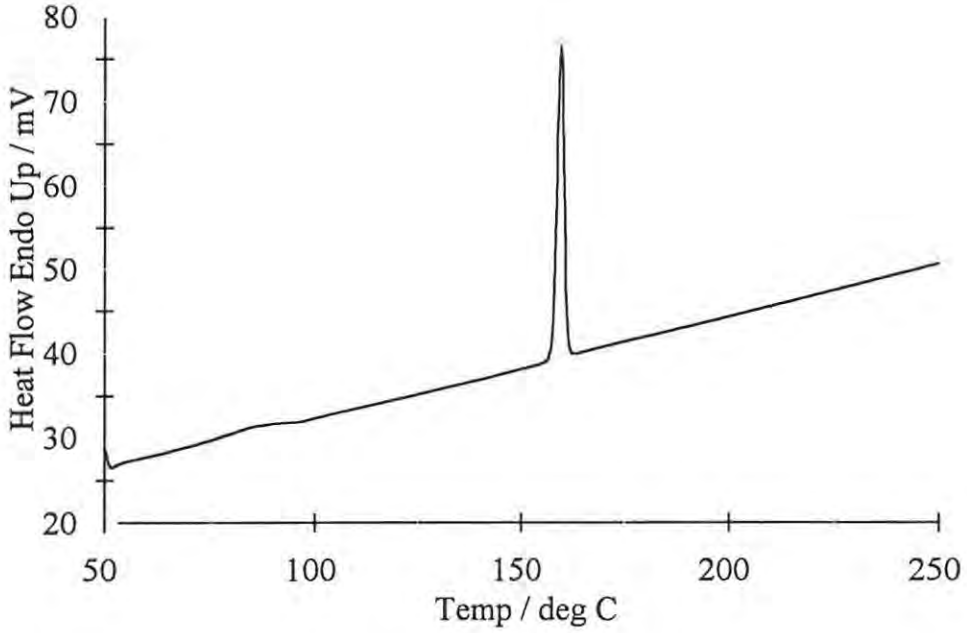


Figure 1.6. DSC curve of midazolam heated at 10°C / min in nitrogen.

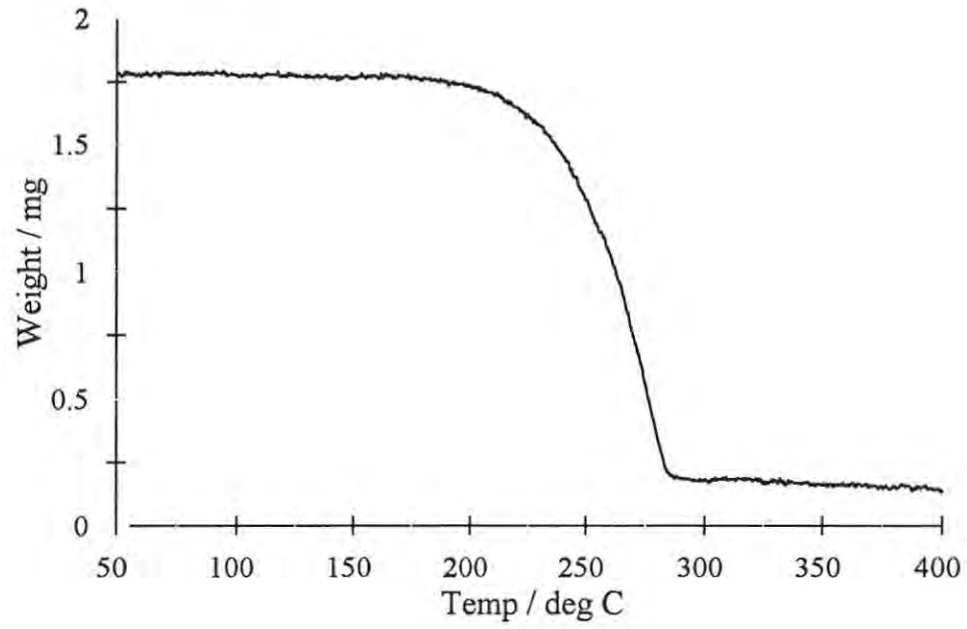


Figure 1.7. TG curve of midazolam heated at 10°C / min in nitrogen.

charred residue. (Figure 1.7).

1.6. Conclusion

Midazolam is effective in all its pharmaceutical applications including use in surgery and in status epilepticus, because of its quick onset and high bioavailability. It has a short elimination half life of 1 to 2.5 hrs. The short duration of action is good for recovery after operation. Midazolam causes less adverse effects than other benzodiazepines, but in long-term infusions lorazepam can be used instead to avoid benzodiazepine withdrawal syndrome. The aqueous-soluble nature of midazolam makes possible the preparation of aqueous solution formulations.

In the characterization of midazolam used for this study, a TLC method was developed which gave an R_f value of 0.726 for midazolam. No other spots appeared on the plate, showing that the drug was pure. UV spectrophotometric studies were used to determine the λ_{max} which was found to be 258 nm and NMR data confirmed the structure of midazolam. GC-MS confirmed the purity and the mass spectrum obtained has the same fragmentation pattern as the one reported in the literature (Jones *et al.*, 1989). The molecular ion peak occurring at m/z 325 confirmed the molecular mass, and the base peak appeared at m/z 310 consistent with the literature findings. The melting range of not more than 2^0C at 158 - 160^0C (Budavari *et al.*, 1996), was confirmed by DSC with onset temperature of 158^0C. All these techniques have confirmed both the identity and the purity of the midazolam to be used in this study.

CHAPTER 2: MIDAZOLAM-CYCLODEXTRIN PHASE SOLUBILITY STUDIES

2.1. Introduction

Cyclodextrins have been used for the improvement of drug solubility (Frank, 1975; Loftsson and Brewster, 1996). Most drugs are hydrophobic in nature and, while this is good for their pharmacological activity, it may be a disadvantage in that it can affect their absorption into the systemic circulation. The improvement of solubility by cyclodextrins can aid in the gastrointestinal absorption and hence improve bioavailability of drugs. It can also make possible preparation of aqueous formulation injectables. For this study there was a requirement to prepare 10 mg/ml aqueous solutions of midazolam for topical application, which is at a concentration is well above the solubility of midazolam of < 0.1 mg/ml at neutral pH (Andersin, 1991), therefore there is a need to use a solubilizer.

Cyclodextrins are considered to be good candidate for the improvement of the aqueous solubility of midazolam close to physiological pH, because previous studies (Andersen and Bundgaard, 1982) have shown that cyclodextrins can improve the aqueous solubility of benzodiazepines. The aqueous solubility of all drugs studied was found to increase linearly with the concentration of the cyclodextrins. The apparent stability constants for the formation of 1:1 complexes ranged from 25 M^{-1} for a diazepam-α-cyclodextrin complex to 1790 M^{-1} for a medazepam-β-cyclodextrin complex. A low stability constant implies poor association of the drug molecule with the cyclodextrin molecule and this may be dependent on the size and the lipohilicity of the drug, the smaller molecules and the more lipophilic molecules forming more stable complexes. Cabral (1994) suggested that the poor association where the stability constant is less than 100 M^{-1} may lead to premature release of the drug, affecting the improvement of physico-chemical properties, although improved physico-chemical properties have been reported for complexes having less than 100 M^{-1} (Veiga and Español, 1995).

Randomly methylated β-cyclodextrin (RAMEB) was chosen for this study because it is an absorption enhancer (Legendre *et al.*, 1995; Marttin *et al.*,1998) and is therefore ideal for formulations intended for topical application. RAMEB has the added advantage of being relatively cheap compared to other methylated cyclodextrins since its preparation is non-specific. A brief overview of cyclodextins including structure, physicochemical properties, toxicity and use for improvement of solubility and stability in the pharmaceutical industry, is included. A UV spectrophotometric method for the analysis of midazolam in the phase solubility studies was developed and validated. Equilibrium solubility studies were carried out to determine the time needed to agitate midazolam to achieve equilibrium solubility. The phase solubility studies that followed determined the amount of RAMEB required to achieve the desired midazolam concentration of 10 mg/ml at pH 5.0 and 5.8.

2.2. Cyclodextrins

2.2.1. Structure and Physicochemical Properties of Cyclodextrins

Cyclodextrins are cyclic oligosaccharide molecules composed of glucopyranose units joined together by α-1,4-linkages. The units are arranged in such a way that the molecule forms a cone shape with the hydroxyl groups on the open ends of the cone, the primary groups on the narrow end and the secondary hydroxyl groups on the wide end, oriented towards the outer surface. This arrangement results in the molecule having a hydrophobic cavity, suitable for inclusion of hydrophobic molecules, and a hydrophilic outer surface which renders cyclodextrins soluble in aqueous medium. The natural cyclodextrins, α-, β-, γ- and δ-cyclodextrin consist of six, seven, eight and nine glucopyranose units, and have the internal diameter of about 5, 6, 8 and 11Å, respectively (Frank, 1975).

β-Cyclodextrin has been by far the most intensively studied and commonly used in pharmaceutical formulations because of its internal diameter which is a suitable size for inclusion of most drug molecules, because it is easily produced (Uekama, 1985) and also has regulatory acceptance. β-Cyclodextrin is less soluble in an aqueous medium than the other natural cyclodextrins and the low solubility is believed to be caused by intra-molecular hydrogen bonding which hinders solvation

by surrounding water molecules. The problem has been alleviated by substitution of the hydroxyl groups to eliminate hydrogen bonding. Alkylation of β-cyclodextrin as well as α- and γ-cyclodextrin has been carried out to produce methyl, hydroxypropyl and hydroxyethyl derivatives (Loftsson and Brewster, 1996). These derivatives have much greater water solubility than the original cyclodextrins, but partial substitution results in more soluble derivatives than complete substitution. For example, the water solubilities of β-cyclodextrin, dimethyl β-cyclodextrin and trimethyl β-cyclodextrin are 1.85, 57 and 31 g/100 ml, respectively (Duchêne and Wouessidjewe, 1990b). Methyl derivatives of β-cyclodextrin are less hygroscopic than β-cyclodextrin. The methyl groups also cause steric hinderance so that the methyl derivatives complex with smaller molecules (Uekama, 1985).

Cyclodextrins form inclusion complexes with many compounds including drug molecules, in which the cyclodextrin is the host and the included molecule is the guest. An inclusion complex may be formed in a 1:1, 2:1 or 1:2 (drug: cyclodextrin) ratio depending on the size of the drug relative to the cyclodextrin. Complex formation may involve total or partial inclusion of the drug molecule. Smaller guest molecules have greater complexing activity while large molecules are only partially complexed if they have a suitable group for inclusion in the cavity (Frank, 1975). Inclusion complexation may result in changes in the physicochemical properties of the guest, e.g. solubility and stability.

2.2.2. Cyclodextrins and Drug Solubilization and Stabilization

Cyclodextrins and derivatives are used in pharmaceutical formulations to enhance the aqueous solubility, stability and bioavailability of drugs. Improvements in the solubility of several drugs such as oxodipine (Veiga and Español, 1995), benzothiazides, barbiturates, steroids, benzodiazepines, naproxen and indomethacin have been demonstrated (Duchêne and Wouessidjewe, 1990a). In addition, cyclodextrins can prevent drug-drug interactions and eliminate unpleasant odour or taste. The solubility of the drug in aqueous medium is improved more for the least soluble drugs. The most soluble natural cyclodextrin is γ-cyclodextrin which happens to be the best solubilizer and dissolution enhancer (Duchêne and Wouessidjewe, 1990a). For substituted cyclodextrins, molar substitution is defined as the average number of substituents

per glucopyranose unit (Loftsson and Brewster, 1996). The derivatives of lower molar substitution enhance the solubility of the guest more than the higher molar substitution derivatives.

Solubility enhancement usually increases when the amount of cyclodextrin is increased. Usually the apparent solubility of the drug increases linearly due to the formation of a soluble inclusion compound (Duchêne, 1987). The apparent stability constant for the formation of drug-cyclodextrin complex, K_c, in solution is given by:

$$K_c = \frac{[D.CD]}{[D][CD]} \tag{2.1}$$

where the [ ] represents the molar concentrations of the drug, D, cyclodextrin, CD and the complex D.CD. A phase solubility diagram is constructed by plotting the total molar concentration of the drug found in solution versus the molar concentration of cyclodextrin. When a 1:1 complex is formed the phase solubility diagram is first order with respect to the cyclodextrin concentration and, the apparent stability constant, $K_{1:1}$ can then be calculated from the plot according to equation 2.2.

$$K_{1:1} = \frac{\text{Slope}}{D_o(1-\text{Slope})} \tag{2.2}$$

where D_o is the equilibrium solubility of the drug in the absence of cyclodextrin. However, phase solubility diagrams of some compounds are not linear, i.e exhibiting an A_L type phase solubility diagram, as described by Duchêne (1987). This is because as the cyclodextrin concentration increases, higher-order inclusion compounds are formed resulting in an upward curvature in the initially linear plot. Thus, the A_p type phase solubility diagram results (Duchêne, 1987). Stability constants can then be calculated by the iteration method as described by Higuchi and Kristiansen (1970). Assuming that only two complexes are formed, namely the 1:1 complex and 1:2 complex, the $K_{1:1}$ stability constant can be expressed as K_c above, and $K_{1:2}$ is given by:

$$K_{1:2} = \frac{[D.CD_2]}{[D.CD][CD]} \tag{2.3}$$

where $D.CD_2$ is the 1:2 complex. The total concentrations of drug, D_t, and cyclodextrin, CD_t in all forms are expressed as:

$$D_t = [D] + [D.CD] + [D.CD_2] \tag{2.4}$$

$$CD_t = [CD] + [D.CD] + 2[D.CD_2] \tag{2.5}$$

Since $[D] = D_o$, combining equations 2.1, 2.3 and 2.4 gives:

$$\frac{D_t - D_o}{[CD]} = K_{1:1}D_o + K_{1:1}K_{1:2}D_o[CD] \tag{2.6}$$

A plot of the left hand side of equation 2.6 versus [CD] should ideally give a straight line, the slope and intercept of which can be used to calculate the values of $K_{1:1}$ and $K_{1:2}$. The free cyclodextrin concentration, [CD], which is not known can be estimated by first assuming that all cyclodextrin complexed is in the D.CD form. Thus, equation 2.5 becomes:

$$[CD] = CD_t - (D_t - D_o) \tag{2.7}$$

and equation 2.6 is transformed into:

$$\frac{D_t - D_o}{CD_t - (D_t - D_o)} = K_{1:1}D_o + K_{1:1}K_{1:2}D_o\,[CD_t - (D_t - D_o)] \tag{2.8}$$

To obtain the first estimates of $K_{1:1}$ and $K_{1:2}$, the left hand term of equation 2.8 is plotted against $CD_t - (D_t - D_o)$. Combining equations 2.1, 2.3 - 2.5 and solving for [CD],

$$[CD] = \frac{-(K_{1:1}D_o + 1) + \sqrt{(K_{1:1}D_o + 1)^2 + 8K_{1:1}K_{1:2}D_oCD_t}}{4K_{1:1}K_{1:2}D_o} \tag{2.9}$$

Knowing the value of [CD] makes possible the use of equation 2.6 to obtain better values of $K_{1:1}$ and $K_{1:2}$. A few iterations, i.e., repeated computations using equations 2.9 and 2.6 successively, result in convergent values for the slope and intercept.

Methyl-β-cyclodextrin may have better solubilizing and stabilizing effect than hydoxypropyl-β-cyclodextrin. For example, the solubility of medroxyprogesterone acetate in aqueous solution is increased more by complexation with methyl-β-cyclodextrin than with hydoxypropyl-β-cyclodextrin (Loftsson *et al.*, 1993).

Unlike with solubility where the use of cyclodextrin has an enhancing effect and very rarely has no effect at all, cyclodextrin can affect the stability of drugs positively or negatively, or have no effect. Carstensen (1995) has indicated that in several instances complexation affects the stability of a drug negatively. Cyclodextrins can protect drugs from oxidation, hydrolysis, isomerization and polymerization. Methyl cyclodextrin slows down ester hydrolysis because the hydroxyl groups of these cyclodextrins are blocked and cannot initiate degradation reactions. For example, methylated cyclodextrins retard the hydrolysis rate of steroidal hormones, ethyl aminobenzoate and indomethacin in aqueous solution. Methyl β-cyclodextrins have been found to increase the stability in some cases where β-cyclodextrin has proved to have destabilizing effect, e.g. degradation of prostagladins where dimethyl β-cyclodextrin has greater stabilizing capacity than trimethyl β-cyclodextrin. β-cyclodextrin being more hygroscopic tends to retain moisture which may initiate hydrolytic degradation (Uekama, 1985).

Cyclodextrins can accelerate some reactions and retard others in the degradation of the same drug. Dimethyl β-cyclodextrin inhibits chlorpromazine photooxidation reactions but accelerates dechlorination to promazine (Uekama, 1985). The reason for this behaviour has not been established. RAMEB is more effective in stabilizing peptide salmon calcitonin against dimerization than other cyclodextrins tested, including 2-hydroxypropyl-β-cyclodextrin and 2-hydroxytrimethylammonio-propyl-β-cyclodextrin, but all the cyclodextrins tested, including RAMEB accelerated enzymatic degradation (Sigurjónsdóttir *et al.*, 1999).

2.2.3. Cyclodextrins and Toxicity

When cyclodextrins are administered orally they are less likely to cause toxicity, since they are rarely absorbed intact and modified cyclodextrins are mostly excreted intact in the faeces (Irie and Uekama, 1997). Parenteral administration results in ulcerations, nephrotoxicity and haemolytic effects. While the methyl derivatives of β-cyclodextrin are more haemolytic than β-cyclodextrin

when administered parenterally, the hydroxypropyl derivatives have less haemolytic effect and do not cause muscle irritation (Duchêne and Wouessidjewe, 1990c). Parenteral administration of hydroxypropyl-β-cyclodextrin results in low toxicity and its effects on the kidneys are reversible (Irie and Uekama, 1997). However, RAMEB and dimethyl-β-cyclodextrin enhance the nasal absorption of drugs without damaging the nasal mucosa. These cyclodextrins interact with the epithelial membranes and increase their permeability to large molecules, such as peptide and protein drugs. Toxicological studies have shown that these cyclodextrins have less local effects than benzalkonium chloride (Marttin *et al.*, 1999; Romeijn *et al*, 1996). In their study, Agu *et al*, (2000) further confirmed that cyclodextrins are safe nasal absorption enhancers.

Although cyclodextrins have toxic effects when administered parenterally, studies carried out with rabbits have shown that cyclodextrins can in fact reduce toxic effects of certain drugs. For example, β-cyclodextrin and γ-cyclodextrin alleviate skin irritation caused by prochlorperazine, and β-cyclodextrin lessens red blood cell haemolysis caused by the chlorpromazine hydrochloride intramuscular injection. Gastric injury caused by drugs following oral administration can be alleviated by complexation of the drug with cyclodextrin. Oral administration of β-cyclodextrin complexes of both indomethacin and piroxicam has been demonstrated to result in less gastric lesions than administration of the drugs alone (Rajewski and Stella, 1996).

The haemolytic effect of cyclodextrin is attributed to their ability to include membrane lipids such as cholesterol in their cavities. γ-Cyclodextrin is, however, more susceptible to enzymatic degradation and interacts to a lesser extent with the membrane lipids and it is therefore less toxic. When the cyclodextrin cavities are occupied by molecules there is no interaction between cyclodextrins and the membranes (Irie and Uekama, 1997).

2.3. UV Method Development and Validation

The solvent used is 0.1 M HCl and is considered suitable because of previous studies (section 1.5.2). A 100 mg quantity of midazolam was dissolved and diluted to 100ml with 0.1M HCl (solvent). 0.3 ml of the solution was diluted to 20 ml with the solvent and the resultant solution

scanned over the range 200 to 400 nm using a GBC spectrophotometer UV/VIS 916 (GBC Scientific Equipment Pty Ltd., Australia) exhibited a maximum at 258 nm. This is the wavelength at which the absorbances were measured for analysis of the samples in the phase solubility studies.

The UV method was validated in terms of linearity, precision and accuracy to test the system response, and to ensure that the method is suitable and reliable for use during the phase solubility studies. Specificity tests were carried out to ensure that RAMEB did not interfere with the UV absorption of the drug at the analytical wavelength.

2.3.1. Linearity

The method linearity deduces the relationship between instrumental response and the known concentration of sample over a given range. It is determined using linear regression analysis and the correlation coefficient of a linear method should be between 0.99 and 1.01 for it to be valid (Brittain, 1998).

About 50 mg of midazolam was weighed, dissolved in 0.1M HCl and diluted to 100 ml with the same solvent. The resultant solution was diluted to achieve concentrations of 0.005, 0.01, 0.015, 0.02 and 0.025 mg/ml. The absorbances of these solutions were measured at 258 nm and a standard calibration curve (Figure 2.1) was constructed by plotting absorbance versus concentration. The response was linear over the range tested and the correlation coefficient was 0.9998. The regression equation obtained from the calibration curve,

$$\text{conc} = -5.105 \times 10^{-5} + 2.746 \times 10^{-2}\ \text{Abs} \qquad (2.11)$$

indicates that the slope is 0.02746 and the y intercept is -0.00005. The linearity of this method indicates that Beer's Law is obeyed over the working range.

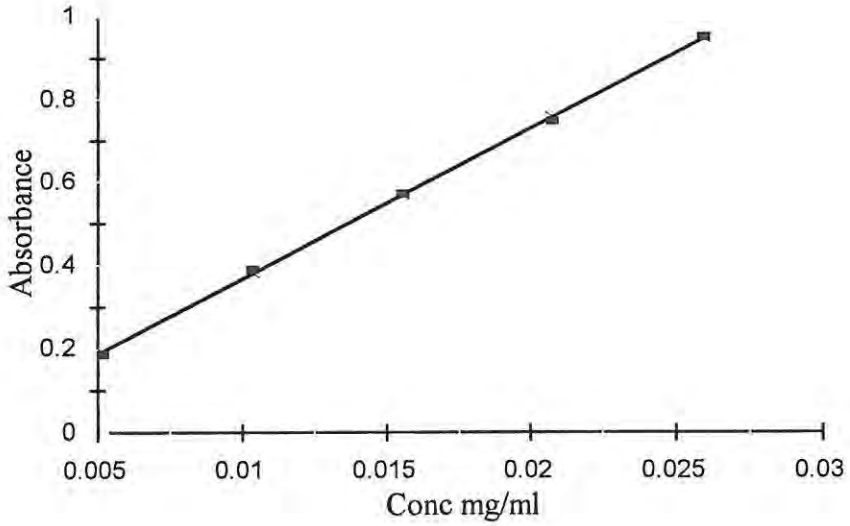


Figure 2.1. Midazolam calibration curve.

2.3.2. Accuracy

Accuracy of a method is a measure how of close the result obtained using the method can be to the theoretical value. It is determined by spiking a known quantity of an analyte into a placebo medium and it is expressed as percent recovery.

Six RAMEB solutions were spiked with midazolam 35.2 mg, 40.3 mg, 44.3 mg, 55.1 mg, 61.5 mg and 65.1 mg and diluted to 100 ml with 0.1 M HCl. The resultant solutions were diluted 3ml to 100 ml with 0.1M HCl and the absorbances were measured at 258 nm. The standard solution was prepared by dissolving 50.7 mg midazolam in 100 ml 0.1 M HCl and diluting a 3 ml to 100 ml with 0.1M HCl. The absorbance of the resultant solution was found to be 0.539. The percent recoveries (Table 2.1) were within 98% to 102% which shows that the method is accurate over the range tested (Jenke, 1996). The calculations were performed using the equations:

$$\text{Amount added} = \frac{\text{spl. wt. (mg)} \times 3}{100 \times 100} \qquad (2.12)$$

$$\text{Amount found} = \frac{\text{abs. spl. x std. wt.(mg) x 3}}{\text{abs. std. x 100 x 100}} \quad (2.13)$$

$$\% \text{ Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \text{ x } 100 \quad (2.14)$$

where spl. = sample, wt. = weight, abs. = absorbance, std. = standard.

Table 2.1. Accuracy of the UV Method For Analysis of Midazolam

Mass (mg)	Absorbance (Abs units)	Amount added (mg/ml)	Amount found (mg/ml)	% Recovery
35.2	0.3771	0.01056	0.01064	100.8
40.3	0.4351	0.01209	0.01228	101.6
44.3	0.4685	0.01329	0.01322	99.5
55.1	0.5808	0.01653	0.01639	99.2
61.5	0.658	0.01845	0.01857	100.7
65.1	0.6846	0.01953	0.01932	98.9
Average recovery = 100.1%; standard deviation = 1.069; %RSD = 1.068				

2.3.3. Precision

Precision is the degree of agreement between a series of measurements of the same concentration of analyte. It is determined by calculating the relative standard deviation (RSD), which must be < 1% for adequate precision (Brittain, 1998). A standard solution was prepared in the same way as in the accuracy test using 50.2 mg of midazolam. The first set of results was obtained by measuring the absorbance of one solution six times. For the second set of results, six replicate dilutions of the stock solution were performed and the absorbance of each solution measured. The results (Table 2.2) show adequate precision with an %RSD less than 1%.

Table 2.2. UV Method Precision

Mass (mg)	Absorbance	
	Replicate Measurement	Replicate Dilution
50.2	0.5391	0.5369
50.2	0.5393	0.5408
50.2	0.5393	0.5408
50.2	0.5394	0.5409
50.2	0.5394	0.5409
50.2	0.539	0.5409
Average	0.5393	0.5402
Standard deviation	0.0002	0.002
%RSD	0.030	0.299

2.3.4. Specificity

It is very important to test the specificity of a method when measuring the concentration of the drug in the presence of its excipients. Specificity of a method is its ability to yield reliable results even in the presence of potentially interfering substances (ICH, 1994a).

In this study the interference of RAMEB as an excipient was investigated. About 100 mg RAMEB was dissolved in enough 0.1M HCl to produce 100 ml. This solution was diluted 0.45 ml to 10 ml with 0.1M HCl to produce 0.045% m/v RAMEB (equivalent to the amount, after dilution, in phase solubility samples containing 30% m/v RAMEB). The final solution was scanned over the range 200 to 400 nm with no absorption observed. The presence of photodegradation products was ruled out by covering the flasks with foil during the phase solubility studies. Thermal stability of midazolam at 25^{0}C was confirmed by HPLC method (Table 4.2)

The effect of RAMEB on the absorption of midazolam was evaluated by preparing solutions of midazolam, three of about 0.005 mg/ml, and three of the concentration 0.025 mg/ml (at the lower

end and the upper end of the working range) and measuring their absorbances at 258 nm. The amount of RAMEB enough to achieve the concentration of 30% m/v was added to the solutions and their absorbances were measured again. The results expressed as %deviation from the absobance of midazolam without RAMEB (Table 2.3) show that RAMEB does not have significant effect on the absorbance of midazolam at 258 nm.

Table 2.3. Absorbances of Solutions of Midazolam With and Without RAMEB

Concentration (mg/ml)	Abs without RAMEB	Abs with RAMEB	% Deviation
0.005	0.891	0.8948	0.43
0.005	0.8558	0.8531	0.31
0.005	0.8818	0.8799	0.33
0.025	0.2151	0.2167	0.74
0.025	0.221	0.219	0.9
0.025	0.2198	0.2205	0.32

2.4. Phase Solubility Analysis

RAMEB was supplied by Wacker-CHEMIE GMBH, Germany and has a degree of substitition of 1.8.

2.4.1. Water Content of RAMEB

The water content of RAMEB under investigation was determined using a Mettler DL18 Karl Fischer titrator (Mettler-Toledo, Switzerland). About 100 mg RAMEB was dissolved in methanol that had been pre-titrated with hydranal composit 5. The resultant solution was titrated with the same reagent. Three different readings were taken and the average is reported. The RAMEB was found to contain 9.2 % moisture (Table 2.4). This value was used to make the necessary adjustments for the amount of RAMEB used in the phase solubility studies.

Table 2.4. Water Content of RAMEB

% Moisture	Mean Deviation
9.455	0.271
8.919	0.265
9.178	0.006
Average = 9.184, Standard deviation (n=3) = 0.268, %RSD = 2.9 %	

2.4.2. Preparation of Solubility Medium

The phase solubility studies were carried out at three different pH values, namely pH 4, and 5.8. The pH 4 medium was achieved by adjustment of pH with dilute hydrochloric acid. The other two media were achieved by phosphate buffers which were prepared in the following manner. A 0.2M sodium hydroxide solution was prepared by dissolving 8 g of sodium hydroxide in enough water to produce 1000 ml (USP, 1994). Potassium phosphate solution (0.2M) was prepared by dissolving 27.22 g potassium dihydrogen orthophosphate in enough water to produce 1000 ml. To prepare each buffer, 250 ml of the potassium phosphate solution was added to about 250 ml water, adjusted to the desired pH, either 5.0 or 5.8, with sodium hydroxide solution and diluted to 1000 ml with water.

2.4.3. Determination of Equilibrium Solubility Time

Equilibrium solubility time determinations were performed with the highest concentration of RAMEB to be studied. Each sample was prepared by suspending about 75 mg midazolam (in excess of 15 mg/ml) in 5 ml of 30% m/v RAMEB solution that had been adjusted to pH 4.16 with dilute HCl. The suspensions in stoppered quickfit flasks, that had been covered in foil in order to prevent degradation due to light, were shaken in a water bath at 25±1^{0}C and the samples were analysed at 6 hrs, 18 hrs, 30 hrs and 48hrs. The procedure was repeated with 30% m/v RAMEB solutions that had been adjusted to pH 5.03 and pH 5.79 using phosphate buffers at pH 5.0 and pH 5.8.

2.4.4. Sample Analysis

The equilibrated solutions were centrifuged for at least 30 min. The supernatant solutions were diluted 1 ml to 100 ml, then 1.5 ml to 10 ml using the respective buffer solutions and dilute hydrochloric acid for the pH 4 solutions. The absorbances of the final solutions were measured at 258 nm and the respective concentrations were calculated using equation (2.11).

The results are shown in Table 2.5 and the equilibrium solubility curves (Figure 2.2.), constructed by plotting time in hours versus concentration in milligrams, show that equilibrium solubility was already reached at 6 hrs (9.9 mg/ml) at pH 4.2, 18 hrs (10 mg/ml) at pH 5.0 and 6 hrs (10.8 mg/ml) at pH 5.8. Since the equilibrium solubility was reached for all media by 18 hrs, 24 hrs was chosen as the convenient time for analysis of the phase solubility studies samples. The studies were carried out with two pH media, i.e., pH 5.0 and pH 5.8.

Table 2.5. Equilibrium Solubility of Midazolam in 30% m/v RAMEB at pH 4, 5 and 5.8

	Midazolam Concentration (mg/ml)				
Time (hrs)	0	6	18	30	48
pH 4.2	0.000	10.545	9.581	10.080	9.379
pH 5.0	0.000	9.615	10.033	10.573	9.883
pH 5.8	0.000	10.767	10.776	11.109	10.805

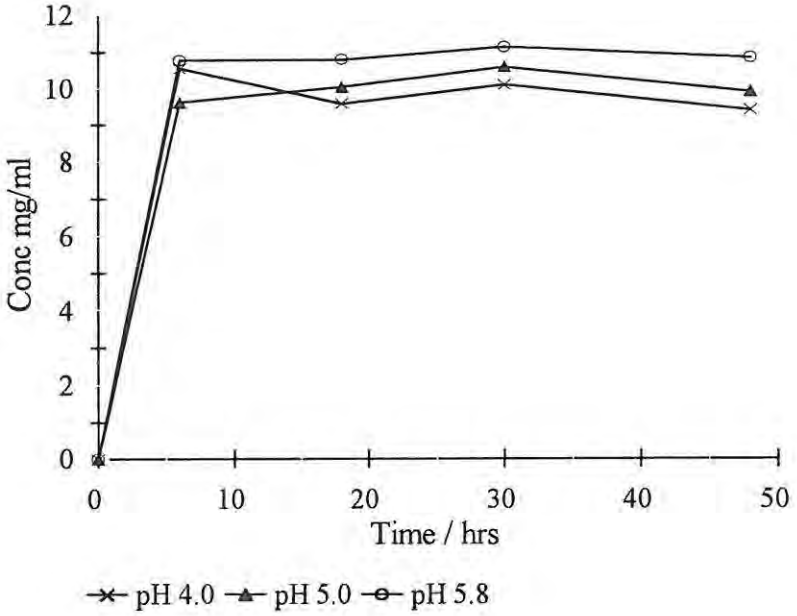


Figure 2.2. Equilibrium solubility curves of midazolam in 30% m/v RAMEB solution.

2.4.5. Phase Solubility Studies

The phase solubility studies were performed in triplicate. A 75 mg quantity of midazolam was weighed into a conical flask and suspended in 5 ml of phosphate buffer solution pH 5.8 containing 0, 5, 10, 20, 30% m/v RAMEB (0.042, 0.084, 0.168, 0.252 mol/L). The suspensions in stoppered conical flasks, covered in foil, were shaken in a water bath at 25 ± 1^{0}C and the samples were analysed after 24 hrs, using the UV method which has been developed and validated (section 2.4.3). The procedure was repeated with phosphate buffer at pH 5.0 and the results are shown in (Table 2.6).

The highest concentration reached with phosphate buffer pH 5.8 was 8.4 mg/ml (below the target concentration), while at pH 5.0 the concentration of 10.6 mg/ ml was achieved, with 30% m/v RAMEB. A plot of the total molar concentration of midazolam found in solution, D_t, versus the total concentration of RAMEB, CD_t gave the phase solubility diagrams (Figure 2.3). In both cases the phase solubility curves obtained were of the A_p type, showing that if RAMEB enhanced the solubility by complexation, not only 1:1 complexes but higher-order complexes were also

formed. An assumption was made that two complexes were formed, a 1:1 and 1:2 complex. The apparent stability constants for the formation of drug-cyclodextrin complex were calculated as described in section 2.2.2.

Table 2.6. Solubility of Midazolam in Phosphate Buffers with Different RAMEB Concentrations

RAMEB Concentration (mol/L)	Midazolam Concentration (mg/ml) ± RSD (n = 3)		Midazolam Concentration mol/L	
	pH 5.0	pH 5.8	pH 5.0	pH 5.8
0	0.275 ± 6.9	0.139 ± 18.1	0.00085	0.00043
0.042	1.025 ± 8.2	0.813 ± 5.4	0.00315	0.0025
0.084	2.440 ± 9.9	1.972 ± 7.8	0.0075	0.0061
0.168	7.431 ± 6.5	5.295 ± 17.2	0.0229	0.0163
0.252	10.626 ± 5.5	8.351 ± 7.9	0.0327	0.0257

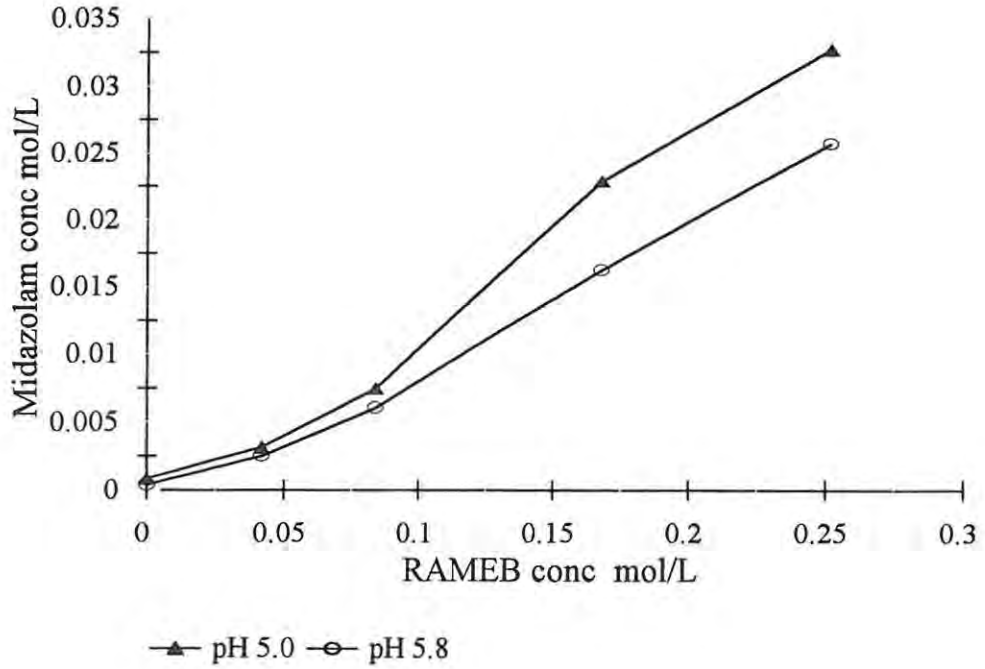


Figure 2.3. Phase solubility diagrams of midazolam at pH 5.0 and 5.8.

The high cost of midazolam made it difficult to carry out the experiment with as many RAMEB concentrations, in between the lowest and the highest, as would have been desired in order to have well representative points. Therefore calculation of apparent stability constants was accomplished with some difficulty. Three iterations were performed to obtain the stability constants for complex formation at both pH values . The first estimates of $K_{1:1}$ and $K_{1:2}$ values at pH 5.0 were obtained by calculations with equation 2.8, which gave the co-ordinates shown in Table 2.7. $K_{1:1}$ and $K_{1:2}$ values obtained from a plot of the co-ordinates were 56.7 and 10.7, respectively. The three iteration with equations 2.9 and 2.6 successively, gave the co-ordinates shown in Tables 2.8, 2.9 and 2.11. Table 2.12 shows the co-ordinates obtained by use of equation 2.8 for the first estimates of $K_{1:1}$ and $K_{1:2}$ values at pH 5.8. The $K_{1:1}$ and $K_{1:2}$ values found were 104.4 and 7.3, respectively. Iterations at pH 5.8 resulted in the co-ordinates shown in Tables 2.13, 2.14 and 2.15. The final $K_{1:1}$ and $K_{1:2}$ values obtained at pH 5.0 were 46.05 and 18.78, respectively; and 96.16 and 10.03, respectively, at pH 5.8.

Table 2.7. Co-ordinates for the First Estimates of $K_{1:1}$ and $K_{1:2}$ Values At pH 5.0

$CD_t - (D_t - D_o)$	$\frac{D_t - D_0}{CD_t - (D_t - D_0)}$
0.0397	0.058
0.0773	0.086
0.1459	0.1511
0.2201	0.1447

Table 2.8. Co-ordinates for the First Iteration with Equations 2.9 and 2.6 At pH 5.0

[CD]	$\frac{D_t - D_0}{[CD]}$
0.0386	0.0597
0.0747	0.0891
0.1409	0.1566
0.2006	0.1588

Table 2.9. Co-ordinates for the Second Iteration At pH 5.0

[CD]	$\frac{D_t - D_0}{[CD]}$
0.0387	0.0595
0.0738	0.099
0.1378	0.16
0.1945	0.1638

Table 2.10. Final Iteration Co-ordinates At pH 5.0

[CD]	$\frac{D_t - D_0}{[CD]}$
0.0383	0.0602
0.0734	0.0907
0.1363	0.1618
0.1924	0.1656

Table 2.11. Co-ordinates for the First Estimates of $K_{1:1}$ and $K_{1:2}$ Values At pH 5.8

$CD_t - (D_t - D_o)$	$\frac{D_t - D_0}{CD_t - (D_t - D_0)}$
0.0399	0.0519
0.0784	0.072
0.1521	0.1043
0.2267	0.1115

Table 2.12. Co-ordinates for the First Iteration with Equations 2.9 and 2.6 At pH 5.8

[CD]	$\frac{D_t - D_0}{[CD]}$
0.0392	0.0528
0.0768	0.0735
0.1474	0.1077
0.2131	0.1186

Table 2.13. Co-ordinates for the Second Iteration At pH 5.8

[CD]	$\frac{D_t - D_0}{[CD]}$
0.0392	0.0529
0.0763	0.0739
0.1455	0.109
0.2094	0.1207

Table 2.14. Final Iteration Co-ordinates At pH 5.8

[CD]	$\frac{D_t - D_0}{[CD]}$
0.0391	0.0529
0.0761	0.0741
0.145	0.1094
0.2082	0.1214

2.5. Conclusion

The desired concentration of 10 mg/ml is not easy to achieve with midazolam base. RAMEB was a good candidate for this study because it is easily produced, since its preparation involves non-specific substitution. Potential use of RAMEB was investigated with possible toxicity in mind and therefore the highest concentration used was 30% m/v. Toxicity studies that have been carried out report least toxicity in comparison to sodium glycocholate, sodium taurodihydrofusidate and L-α-phosphatidylcholine, but these were in low concentrations of RAMEB at 2% to 5% (Marttin *et al*, 1998). High concentrations should, therefore be used with caution.

A simple UV method was successfully developed and validated for use in the phase solubility studies. The UV method used proved to be suitable for this kind of study as validated. It was linear over the working range, with a correlation coefficient of 0.9998. The accuracy of the method was demonstrated by the recovery values ranging between 98.9% and 101.6%. The effect of cyclodextrin on the absorbance readings of the drug was not significant, showing that the concentration of midazolam was selectively measured in the presence of RAMEB.

Equilibrium solubility studies showed that at 18 hrs, equilibrium solubility was reached at all pH values investigated. Twenty-four hours was, therefore, considered to be sufficient time for achieving equilibrium solubility. Phase solubility studies revealed that even with 30% m/v

RAMEB the target concentration of 10 mg/ml midazolam in aqueous solution was not reached at pH 5.8, but occured at pH 5.0 only with 30% m/v RAMEB. Midazolam solubility increased with increasing RAMEB concentration. The relationship was not completely linear, but an A_p type phase solubility diagram was obtained, indicating complex association of midazolam molecules with RAMEB molecules, and formation of higher-order complexes than 1:1 complex.

The values of the apparent stability constants for the formation of 1:2 complexes, $K_{1:2}$, were very low compared to $K_{1:1}$ constants, showing that the possibility of such association is very low. The $K_{1:1}$ constants, however, are on the lower end of the range reported by Andersin and Bundgaard (1982) for other benzodiazepines, suggesting poor association of midazolam molecules within the RAMEB cavities. This was to be expected because midazolam molecule may be too large to fit in the RAMEB cavity. However, the low $K_{1:1}$ values does not necessarily mean low solubility, for example, the apparent stability constant for medazepam-β-cyclodextrin complex was about three times as much as that for chlordiazepoxide-β-cyclodextrin, but the chlordiazepoxide complex had the highest solubility. The same behaviour was observed with midazolam, where $K_{1:1}$ at pH 5.8 is higher than that obtained at pH 5.0, while the solubility is higher at pH 5.0. The improved solubility can be attributed to non-specific interactions of the drug molecules with RAMEB. As already mentioned (section 2.1) the low stability constants do not necessarily mean that the stability of midazolam in these solutions will not be increased.

CHAPTER 3: HPLC METHOD DEVELOPMENT AND VALIDATION

3.1. Introduction

High Performance Liquid Chromatography (HPLC) is a very powerful analytical technique because of its unique capability to separate complex mixtures. The most important development in the field of analytical chemistry has been the coupling of reverse phase HPLC with atmospheric pressure chemical ionization (APCI) mass spectrometry (LC - MS). The convenience of this arrangement is that it allows identification by molecular mass of the components of the mixture as they elute from the chromatographic column. The HPLC technique is also very useful in quantitative analysis because the detector response is proportional to the concentration of the respective component, is simple to use, precise and accurate (Katz, 1978). The advantage of using the technique in the photostability studies is that it provides information about the number of photoproducts formed and whether some of the photoproducts subsequently undergo degradation (Moore, 1987).

Quantitative HPLC methods have been used for studies on the decomposition of midazolam due to the presence of various excipients in the formulations (Steedman *et al.*, 1992; Bhatt-Mehta *et al.*, 1993). Andersin and Tammilehto (1995) used an HPLC method to separate midazolam from its photodegradation products in aqueous solution, and Carrillo *et al.* (1998) have used another HPLC method to analyse midazolam and its metabolites in blood plasma. However these methods do not make allowance for subsequent identification of the other compounds present with midazolam. The aim of the study at this point was to develop and validate a stability-indicating HPLC method that would be used quantitatively for kinetic studies of the photodegradation of midazolam, and qualitatively for identification of the degradation products by LC-MS.

A stability-indicating assay is established once the parent drug can be separated from all potential degradation products and excipients (Dong *et al.*, 1990). It must be able to selectively determine small changes in the drug concentration throughout the duration of the investigation and it must have good intermediate precision ($\leq 1\%$ RSD). Resolution should be high in order to identify and quantitate degradation products, both primary and secondary (Jenke, 1996b).

3.2. Method Development

Methanol was used as the organic mobile phase component and the compositions investigated were 65:35 of methanol/water(Andersin and Tammilehto, 1995), 70:30 methanol/water (Vasiliades and Sahawneh, 1982) and 70:30 methanol/water for comparison. For selection of a suitable wavelength, the procedure in section 1.5.2 was used to determine the UV spectrum of midazolam in methanol/water and λ_{max} was found to be 219 nm. In order to avoid methanol interference at 219 nm, the use of a longer wavelength at which the absorbance was above 50% of that at λ_{max} (240 nm) was investigated The optimum organic/aqueous phase ratio was investigated and the mobile phase was modified by addition of ammonium acetate as required for LC-MS studies. Column choice was based on performance between μBondapak C_{18} 10μm and Spherisorb ODS2 5μm (250 mm x 4.6 mm, i.d.) columns to compare and chose based on the best peak resolution.

3.2.1. Equipment

The HPLC system used constituted a Thermo Separation Products isocratic pump, Spectra series P100; UV detector UV100; Waters μBondapak C_{18} 10μm column (250 mm x 4.6 mm, i.d.) or Waters Spherisorb ODS2 column 5μm (250 mm x 4.6 mm, i.d.), Waters Technologies Ireland Limited, Ireland; a Rheodyne injector Model 7125 (Cotati, California) equipped with a 20 μL loop and Rikadenki electronic recorder, Rikadenki Kogyo Co. (Tokyo, Japan).

3.2.2. Materials

RAMEB (Wacker-CHEMIE GMBH, Germany), of the molar substitution of 1.8, and midazolam were donated by Aspen-Pharmacare (Port Elizabeth, RSA). The water was purified by passing through Milli-Q RG (Millipore S.A., France), fitted with a Super C® carbon cartridge, two Ion-X® exchange cartriges and an Organex-Q® cartridge. All other materials were of analytical grade. Sodium hydroxyde and ammonium acetate were obtained from Saarchem-Holpro (Krugersdorp, RSA), Hydrochloric acid and HPLC methanol from BDH Laboratory Supplies (England) and monobasic potassium hydrogen phosphate was form Merck, E Merck (Germany).

3.2.3. Preparation of Solutions

Standard solution: 100mg of midazolam was dissolved in 50 ml methanol and diluted to 100ml with water. The resultant solution was diluted 1 ml to 10 ml, then 1 ml to 5 ml with a 50:50 mixture of methanol and water to a final concentration of 0.02 mg/ml.

Sample solution (0.5 mg/ml midazolam): Water (60 ml) was acidified to pH 3.0 with a dilute HCl solution. About 50 mg of midazolam was added slowly with stirring and the HCl solution was added dropwise to aid solubility while the pH was not allowed to drop below pH 2.90. The clear solution was stirred for 30 minutes protected from light, pH adjusted to 5.0 with a 0.2 M NaOH solution and the solution was diluted to 100 ml with phosphate buffer pH 5.0 (prepared as in section 2.4.2). For solutions containing RAMEB, respective amounts of RAMEB were dissolved in water, which was then acidified as above and the procedure continued from the addition of midazolam.

Mobile phases: For 65:35, 70:30 and 75:25 methanol/water mobile phases containing 30 mM ammonium acetate, 1.156g of ammonium acetate was dissolved in 150 ml water and 350 ml methanol was added. For 20 mM and 40 mM ammonium acetate mobile phases, 0.6168 g and 1.2336 g, respectively, were used instead. The pH's were adjusted by dropwise addition of either orthophosphoric acid or 0.2 M NaOH solution as desired to achieve pH 6, 7 or 8. The mobile phases were stirred and degassed by filtration through Millipore 0.45 μ filter.

3.2.4. Chromatographic Procedure

The detector wavelength was set at 240 nm, flow rate at 1.0 ml $min,^{-1}$ range at 0.5 AUFS, and chart speed at 30 cm $hr.^{-1}$ The method was developed by injecting 20μL of standard solution of midazolam into the system with different component mobile phases. The effect of the ammonium acetate concentration, pH of mobile phase and organic/aqueous phase ratio was investigated by comparing the respective retention times. The retention times obtained with μBondapak C_{18} column were also compared with those of Spherisorb ODS2 column. A low concentration of midazolam solution (0.5 mg/ml) was irradiated (section 4.4.1), for 8 hrs to generate degradation products and the degraded solution was injected to test for good resolution of the drug peak and

the degradation products. The final mobile phase composition and chromatographic parameters were based on the results obtained.

3.2.5. Results and Discussion

The results (Table 3.1) show a great difference for the retention time of midazolam (7.8 min) between μBondapak C_{18} and Spherisorb ODS2 columns. When μBondapak C_{18} column was used the retention time of midazolam was too short, to allow for the degradation products. This is because the μBondapak C_{18} packing material is of larger particle size, 10 μ as opposed to 5 μ of the Spherisorb ODS2 column and the Spherisorb ODS2 has spherical particles while μBondapak C_{18} has irregular shaped particles. The small particle size and shape of Spherisorb ODS2 results in more regular packing and increased plate count, hence the separation efficiency. Change in the concentration of ammonium acetate and the pH of the mobile phase resulted in negligible variations in the retention time. Therefore, the concentration of ammonium acetate can vary between 20mM and 40mM, and the pH of the mobile phase can be maintained at pH 7 ± 1.

Table 3.1. Retention Times of Midazolam for Different Mobile Phases

Variable		Retention Time, R_t (min)	Mean R_t (mim)
Column	μBondapak C_{18}	6.8, 6.0	6.8
(65:35 solvent ratio)	Spherisorb ODS2	14.6, 14.6	14.6
Ammonium acetate conc.	20 mM	10.0, 10.2	10.1
	30mM	10.0, 10.0	10.0
	40mM	9.9, 9.8	9.85
Solvent ratio	65:35	14.6, 14.6	14.6
	70:30	10.0, 10.0	10.0
	75:25	7.0, 7.0	7.0
Mobile phase	pH 5.8	10.4, 10.4	10.4
	pH 6.8	10.0, 10.0	10.0
	pH 7.8	10.2, 10.2	10.2

The effect of the solvent ratio was observed as a difference of 3 min between 75:25 and 70:30 ratios, and 4.6 min between 70:30 and 65:35 ratios. Organic/aqueous phase composition of 75:25 resulted in unresolved peaks, while peaks were too far apart and broad when a 65:35 composition was used. Solvent ratio of 70:30 resulted in the best resolution between midazolam and its degradation products as shown in Figure 3.1.

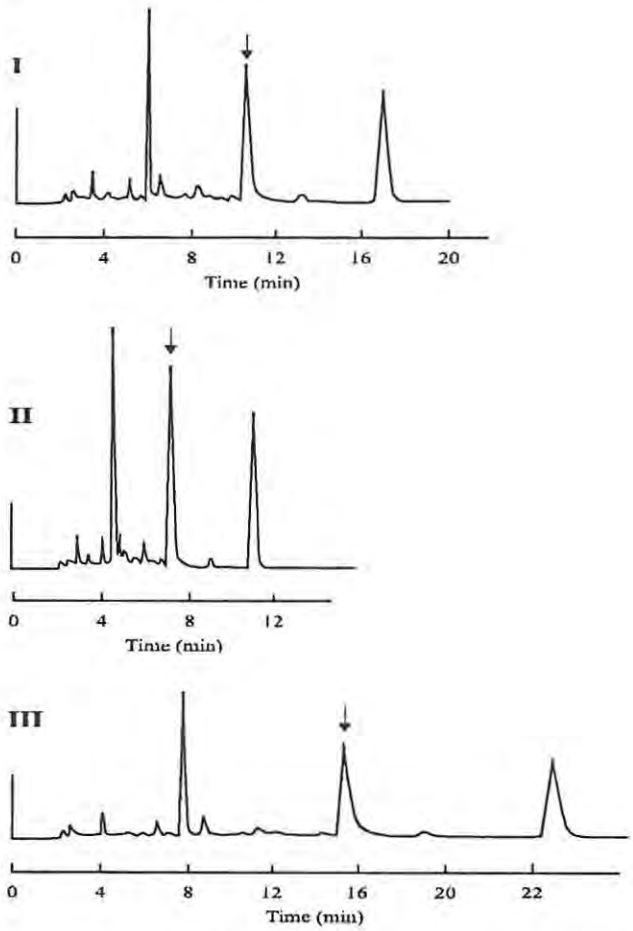


Figure 3.1. HPLC Chromatograms of degraded midazolam solution with mobile phase solvent ratio of (I) 65:35, (II) 70:30, and (III) 75:35. Midazolam peak is indicated by the arrow.

3.2.6. Final Chromatographic Parameters

Mobile phase was made up of 70:30 methanol/water with 30mM ammonium acetate and the resultant pressure was 1990 to 2200 PSI. The wavelength was set at 240 nm, flow rate at 1.0 ml $min,^{-1}$ range at 0.1 AUFS, rise time at 1.0 secs and chart speed at 30 cm $h.^{-1}$ The injection volume was 20μL.

3.3. Method Validation

Method validation is the process of confirming that an analytical method is suitable for its intended use. The analytical performance parameters reported in the literature for the method validation in pharmaceutical analysis include linearity, precision, accuracy, range, specificity, limit of detection and quantitation, stability of analytical solutions, robustness and ruggedness (Carr and Wahlich, 1990; Edwardson *et al.*, 1990; Hokanson; 1994; ICH, 1994/6). A stability-indicating method, which should be able to quantitate the drug substance and degradation compounds, falls into category II as specified by USP 23 (1994), under *Validation of Compendial Methods*. In this case the limit of detection is not included. Method validation in this study was carried out by following the validation parameters for a stability-indicating HPLC method which include all the above parameters except the limit of detection (Russ *et al.*, 1998; Wang, 1997; Zhang *et al.*, 1997).

3.3.1. Accuracy

The accuracy of an analytical method is a measure of the closeness of agreement between the test results found by that method and the value which is accepted as the true value (ICH,1994; USP 23) and is performed across the specified linear range of the analytical procedure. The recommended range for assay of active in a drug substance or drug product is 80 to 120% of the label claim. Accuracy should be assessed in triplicate over a minimum of 3 concentration levels. The whole analytical procedure should be repeated for each determination. Accuracy for the drug product is performed by addition of known amounts of drug to mixtures of excipients, followed by analysis using the method to be validated. The accuracy is then reported as percent recovery by the assay (ICH, 1996; CDER,1994), which should be 98% to 102% of the theoretical value. The plot of recovered versus known concentration should have a correlation coefficient of 1.00, a slope of 1.00 and an intercept of 0.00 (Jenke, 1996a).

The accuracy test was carried out by spiking 30% m/v RAMEB solutions with known concentrations of midazolam and determining recovery over the range 80% to 120% of the test concentration (0.5 mg/ml). Three solutions of concentration 0.4 mg/ml, three of 0.5 mg/ml and

three of 0.6 mg/ml midazolam were prepared, diluted to the final concentrations of about 0.008, 0.01 and 0.012 mg/ml, respectively, with 50:50 methanol/water and analysed for recovery. The standard solution was prepared by dissolving 50.0 mg midazolam in 100 ml and diluting 1 ml to 10 ml, then 1 ml to 5ml with 50:50 methanol/water (0.010 mg/ml). The concentration in the samples injected was calculated using the equation (Katz, 1978):

$$c_{spl} = \frac{c_{std} \times h_{spl}}{h_{std}} \qquad (3.1)$$

where c denotes concentration; spl, sample; std, standard; and h, the peak height. The dilution factor of 50 was used to calculate the original sample concentration. The results (Table 3.2) show good recovery by the method. The results fall within the specifications 100 ± 2%. The average recoveries at 80%, 100% and 120% were 100.1, 100.2 and 100.6%, respectively. A plot of the means of drug recovered vs known concentrations (Figure 3.2) resulted in a correlation coefficient of 1.00, the slope 1.00 and intercept of 0.004≃0.00.

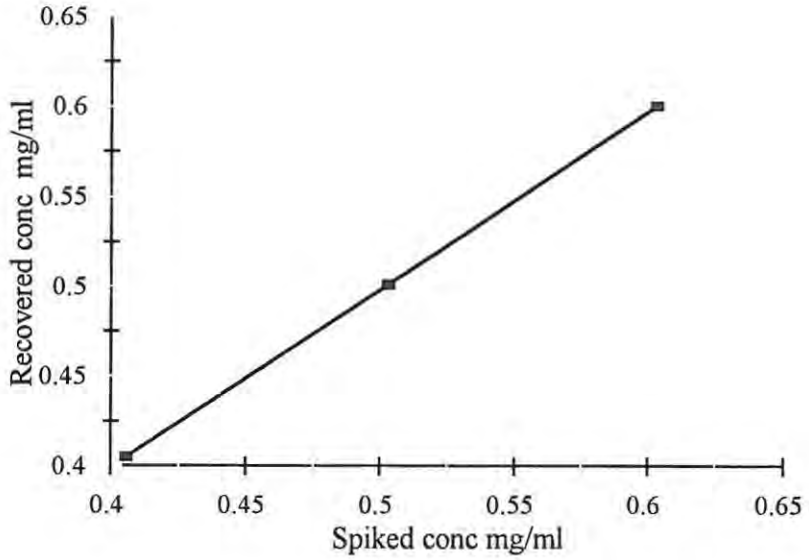


Figure 3.2 A curve of recovered vs spiked concentrations.

Table 3.2 HPLC Method Accuracy for the Analysis of Midazolam

Recovery Level	Spiked Concentration (mg/ml)	Recovered Concentration (mg/ml)	% Recovery
80%	0.402	0.3999	99.5
	0.409	0.4098	100.2
	0.405	0.4078	100.7
100%	0.497	0.4985	100.3
	0.502	0.5070	101.0
	0.508	0.5039	99.2
120%	0.600	0.6066	101.1
	0.598	0.6027	100.8
	0.601	0.6004	99.9

3.3.2. Linearity

The linearity of an analytical procedure is the ability to obtain test results which are directly proportional to the concentration of analyte in the sample in a specified range. Concentrations at which the studies are carried out and the concentrations of the samples tested for accuracy should be in the linear range, i.e., should obey Beer's Law (ICH, 1994).

Linearity may be demonstrated by preparing standard solutions at five concentration levels from 50 to 150% of the target analyte concentration and analysing them at least three times (Green, 1996), or dilution of stock solution five times and evaluation using the method that is being validated. A linear relationship is evaluated by calculation of a regression line by method of least squares. The regression coefficient, r, should be ≥ 0.999 (CDER, 1994). The intercept should be about 2% of target concentration, or correlation coefficient between 0.98 and 1.00 and the intercept not significantly different from zero (Jenke, 1996a).

100 mg midazolam was diluted to 100 ml with 50:50 methanol water and further diluted six times to give concentration 0.004 mg/ml to 0.02 mg/ml with the same solvent. The resultant solutions were analysed under the chromatographic conditions mentioned in section 3.2.3. Each solution was injected twice and a calibration curve (Figure 3.3) constructed by plotting mean peak height in mm versus concentration in mg/ml. The coefficient of correlation was found to be 1.00 showing that the method is linear and that Beer's Law is obeyed.

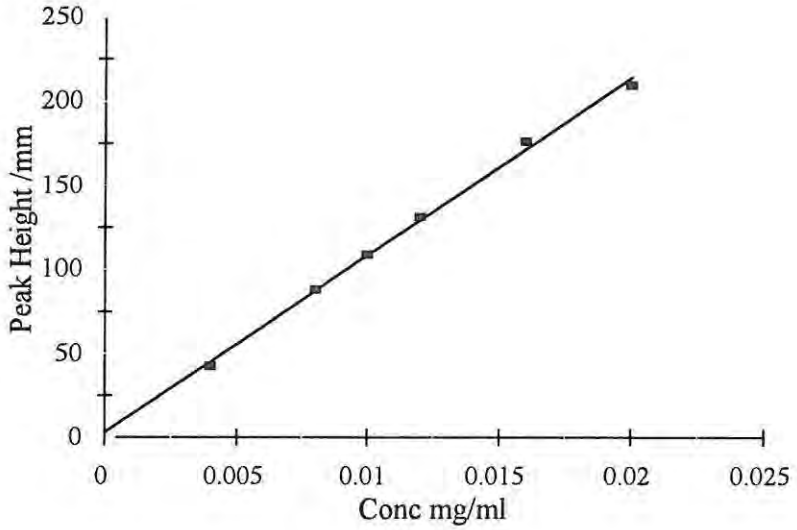


Figure 3.3. Method linearity calibration curve.

3.3.3. Precision

Precision of an analytical procedure is the measure of how close the results, obtained from multiple sampling of a homogenious sample, are to each other after a series of measurements under the same analytical conditions (ICH, 1994). It is usually expressed as the variance, standard deviation or coefficient of variation. Repeatability is precision determined under the same operating conditions over a short interval of time. Methods for stability studies should have an RSD of ≤ 1 % for at least five injections ($n \geq 5$) for the active drug in a drug substance or drug product (CDER, 1994). Intermediate precision is precision within laboratory variations such as different days, different analysts, different equipment, etc.

The precision tests carried out include repeatability and intermediate precision between days. For repeatability the second highest concentration (0.016 mg/ml) and the second lowest concentration (0.008 mg/ml) solutions in the linearity test were injected six times each and the standard deviations calculated (Table 3.3). The repeatability test was carried out on five different days and the inter-day standard deviations were determined and recorded as intermediate precision (Table 3.4).

Table 3.3. HPLC Method Repeatability

Injection	Peak Height (mm)	
	At 0.016 mg/ml	At 0.008 mg/ml
1	177	83.0
2	179	83.0
3	181	83.5
4	179	83.5
5	179	83.5
6	179	84.0
Mean (mm)	178.9	83.4
Standard Deviation	1.11	0.3
% RSD	0.62	0.4

3.3.4. Range

The range is the interval between the upper and lower concentrations of analyte in the sample for which it has been established that the analytical procedure has a suitable level of precision, linearity and accuracy (CDER, 1994; ICH, 1994). For the analysis of a drug substance or drug product, minimum range should be 80 to 120 % of the test concentration (ICH, 1996). The HPLC method under investigation, as demonstrated so far, has good precision and accuracy, and is linear in the range tested, i.e., 80 to 120% of a 0.05 mg/ml midazolam solution.

Table 3.4. Intermediate Precision of the HPLC Method

Day	0.016 mg/ml concentration		0.008 mg/ml concentration	
	Peak Height (mm)	Mean Deviation	Peak Height (mm)	Mean Deviation
1	178.9	0.28	83.4	0.54
2	180.9	1.72	83.8	0.14
3	180.7	1.52	85.4	1.46
4	178.6	0.58	83.3	0.64
5	179.8	2.38	84	0.06
Mean (mm)	176.8		83.94	
Standard Deviation	1.68		0.43	
% RSD	0.95		0.51	

3.4.5. Specificity / Selectivity

Specificity is the ability to assess unequivocally the analyte in the presence of extraneous components, such as degradants, excipients, impurities, etc., which may be expected to interfere with the analysis (ICH, 1994; CDER, 1994). For chromatographic procedures representative chromatograms should show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte. Peak purity tests must be carried out to show that the analyte chromatographic peak is not attributed to more than one component. This can be done by use of diode array and mass spectrometry. (ICH, 1996). Resolution between midazolam and its photodegradants was demonstrated in the method development section (section 3.2.4). In order to test for peak purity, a 0.5 mg/ml midazolam solution which had been irradiated for photodegradation (section 3.2.3) was analysed by LC-MS (section 4.4.4). The M + 1 peak as determined at different times within the drug peak was *m/z* 326 and no other competitive peaks were observed. Thus, there were no other components in the drug peak, therefore the method is specific for the analysis of midazolam in the presence of

its degradation products. In the presence of RAMEB, however, some ionization of RAMEB occurred on the LC-MS, therefore photodiode array analysis (Performed by Pharmaceutical Research Laboratories, Port Elizabeth, RSA) was used to determine the peak purity and the peak was found to be due to midazolam alone.

3.3.6. Quantitation Limit

The limit of quantitation (LOQ) of an analytical procedure is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy (CDER, 1994; ICH, 1996). The approach used in this study for the determination of quantitation limit is that based on the standard deviation of the response and the slope. The quantitation limit is expressed as

$$\mathrm{LOQ} = \frac{10\sigma}{S} \qquad (3.1)$$

where σ is the standard deviation of the response after six injections at the lowest target concentration, and S is the slope which was estimated from the calibration curve obtained in the test for linearity (section 3.3.2). The LOQ was found to be 0.002 mg/ml (Table 3.5).

It is important to perform experiments near the limit of detection in order to determine whether the results obtained at this level are reliable. To validate the limit of quantitation, three solutions prepared near the quantitation limit were analysed. About 20 mg was weighed and diluted to 100 ml with a 50:50 mixture of methanol and water and then 0.2 ml diluted to 10 ml with the solvent. The standard solution was prepared by weighing 50.5 mg and treating it in the same way as the samples. The results (Table 3.6) show good recovery at the concentrations tested.

Table 3.5 Limit of Quantitation Data for HPLC Analysis

Injection	Peak Height (mm)
1	18
2	18.5
3	19
4	18.5
5	18.5
6	18
Mean	18.4
σ	0.377
LOQ (mg/ml)	0.002

Table 3.6 Validation of the Limit of Quantitation

Known Concentration (mg/ml)	Concentration Found (mg/ml)	% Recovery
0.00416	0.00422	101.4
0.00404	0.00396	98.3
0.00396	0.00401	101.3

3.3.7. Robustness

The robustness of an analytical procedure is its capability to remain unaffected by small but deliberate variations in method parameters, and shows how reliable the method can be during normal use (ICH, 1994). Robustness was evaluated during the development phase, in the test for influence of pH of mobile phase, use of different columns, and variation in the mobile phase composition. As already mentioned, the best column for the method is the Spherisorb ODS2 5μm column, the concentration of ammonium acetate can vary between 20mM and 40mM, and the pH

of the mobile phase has to be maintained at pH 7 ± 1 pH unit, where the resultant retention time is 10 ± 0.2 min.

3.3.8. Sample Solution Stability

Solution stability of the drug substance or product after preparation according to the test method should be evaluated according to the method, especially where overnight runs are employed (CDER, 1994). About 50 mg midazolam was dissolved in enough 50:50 methanol/water to produce 100 ml. 1 ml of the solution was diluted to 10 ml, stored at room temperature, in the dark and injected at times 0, 3, 6, 9, 12 and 24 hrs. According to the results (Table 3.7), the diluted final solution is relatively stable within the day. A maximum deviation of 2% from the original concentration was observed.

Table 3.7 Stability of Sample Solution

Time (hrs)	Mean Peak Height (n = 3)	% Deviation From t_0
0 (t_0)	115.0	0
3	116.0	0.87
6	117.3	2.00
9	114.3	0.60
12	112.9	1.83
24	116.7	1.50

3.3.9 Ruggedness

Ruggedness of an analytical procedure is the measure of reproducibility of the results obtained using the procedure under various normal conditions, e.g., different laboratories, different analysts, different instruments, different days, etc (USP, 1994). Ruggedness is covered once intermediate precision, reproducibility, robustness and sample stability have been established

(CDER, 1994; Hokanson, 1994).

3.4. Conclusion

An HPLC method has been successfully developed for the analysis of midazolam in the presence of its degradation products and RAMEB. The method is suitable for use on LC-MS, without further modification because the mobile phase contains ammonium acetate which aids in MS ionization. It was successfully validated according to international guidelines and was found to have suitable levels of accuracy (average recoveries of 100.1, 100.2 and 100.6% at 80, 100 and 120%, respectively), linearity (correlation coefficient of 1.00) and precision (%RSD of 0.62 and 0.4%) within the range which was 80% to 120% of the concentration of the solution used in the stability studies. The LC-MS proved to be reliable in evaluating the specificity of the assay in the absence of RAMEB, but for the solutions containing RAMEB photodiode array was used and the drug peak was found to be pure.

The limit of quantitation at 0.002 mg/ml was below the working range because photodegradation was followed until about 10% of the original concentration. Thus the lowest measured concentration of midazolam is ±0.05 mg/ml. Robustness of the method was demonstrated during the method development, where it was shown that the pH of mobile phase can be varied between pH 6 and pH 8, and the concentration of ammonium acetate in the mobile phase can be varied between 20 mM and 40 mM, while the organic/aqueous phase ratio has to be maintained at 70:30 methanol/water because these are the conditions at which the best peak resolution was reached. Ruggedness was confirmed by the robustness results and by the results of intermediate precision which had the % RSD of 0.9 and 0.5% which means that the method is precise and suitable for use for the analysis of midazolam.

CHAPTER 4: PHOTODEGRADATION KINETICS AND IDENTIFICATION OF PHOTODEGRADANTS BY LC-MS

4.1. Introduction

Degradation of drugs due to exposure to light can result in loss of pharmacological activity. An example is the loss of antibacterial activity of ciprofloxacin after exposure to UV radiation (Tiefenbacher, *et al.*, 1994). Undesirable side-effects, such as phototoxicity, can occur as a result of photodegradation. One of the photoproducts of benzydamine, a phototoxic anti-inflammatory and analgesic agent, is 2-β-dimethylaminopropyl-1-benzylindalolin-3-one, reported to be an effective photohaemolytic agent and it is presumed that the phototoxicity of the drug is due to the intermediates and photoproducts (Vargas *et al.*, 1993a). Phototoxicity of fenofibrate is attributed to the formation of a peroxyacid as a result of photolysis (Vargas *et al.*, 1993b). It is, therefore, necessary to study the photodegradation of to guard against loss of activity and, if the photoproducts are toxic, appropriate measures can be taken to prevent their appearance in the formulation even after storage.

Selkämaa and Tammilehto (1989) reported that the photochemical decomposition of midazolam in ethanolic solution resembled that of diazepam, while Andersin and Tammilehto (1989) reported apparent first-order kinetics for the photodegradation of midazolam, with the apparent rate constant directly proportional to the initial concentration.

Andersin *et al.* (1994), investigated the influence of pH and the irradiation source on the photochemical decomposition of midazolam in aqueous solution. At very low pH, only one product, 6-(8-chloro-1-methyl-4,5-dihydro-2,5,10*b*-triaza-benzo[*e*]azulen-6-ylidene)-cyclohexa-2,4-dienone was obtained. More products were formed as the pH was increased. These include 6-chloro-2-methyl-4-(2-fluorophenyl)-quinazoline, 6-chloro-2-methyl-4-(1H)-quinazolinone, N-desalkylflurazepam, 2-amino-5-chloro-2'-fluorobenzophenone and 6-(6-chloro-2-methyl-3H-quinazolin-4-ylidene)-cyclohexa-2,4-dienone. Decomposition products caused by irradiation by a high-pressure mercury lamp were different from those formed on exposure to normal daylight,

although there were some trace products which were the same under both types of irradiation. Later Andersin and Tammilehto (1995) studied the effect of pH on the photochemical stability of midazolam solutions and reported that the rate of degradation increased with increasing pH.

The present study is aimed at investigating the effect of RAMEB on the degradation kinetics of midazolam in aqueous solution (at pH 5.0 as established by the phase solubility studies), and to investigate whether RAMEB causes formation of different photodegradants from those formed in the absence of RAMEB.

4.2. Photostability of Drugs in Solution

Photodecompositions of drugs in solution, where there is a large excess of water molecules, are usually pseudo first-order. In concentrated solutions the reaction is apparently zero-order with respect to the drug (Connors, 1979), but there are some complex reactions which do not have a definite order (Laidler, 1965). Photodecomposition of metronidazole studied under different conditions (A/Karim *et al.*, 1991) was found to follow pseudo first-order kinetics, while zero-order kinetics for 0.5% aqueous solution were reported (Barnes and Sugden, 1985). For reliable chemical kinetic data in stability tests, degradation needs to be followed through several half-lives (Carstensen, 1995).

Photodegradation of drugs depends on the intensity of radiation (A/Karim *et al.*, 1996) and the type of irradiation source (Andersin *et al.*,1994). The rate of degradation may be pH dependent, either increasing with pH (Andersin and Tammilehto, 1995), or decreasing with increasing pH (Akhtar *et al.*, 1999). The solvent used for the degradation studies determines the extent of degradation and the type of photoproducts. For example, photoproducts were detected in aqueous solutions of chloramphenicol, but not in solutions of the drug in ethanol or benzene (Shih, 1971). A solvent addition product was detected among the degradation products of midazolam in ethanolic solution (Selkämaa and Tammilehto,1989). Additives can affect the photostability positively or negatively. The photostability of colchicine was enhanced by uric acid and decreased by glycerin (Habib and Asker, 1989).

Photodegradation of some drugs involves formation of a superoxide anion (Mckormick and Thomason, 1978), while others degrade through a triplet-state energy transfer to form singlet oxygen (Tønnesen and Moore, 1991; Bosca *et al.*, 1992). Photodegradation of amiloride occurs predominantly via singlet oxygen, although a free radical mechanism was postulated (Li *et al.*, 1999). Free radical mechanisms were also proposed for the photodegradation of fenofibrate (Vargas *et al.*, 1993b), and benzydamine (Vargas *et al.*, 1993a).

Structurally related compounds often show some similarities in the nature of photoproducts, but photodegradation of benzodiazepine group compounds follows a variety of chemical reactivity which depends on the type of substituents. Nitro-derivatives produce amino reduction compounds, chlordiazepoxide an oxaziridine, and diazepam decomposes into a mixture of benzophenones, 4-phenylquinazolinones and 4-phenylquinazolines (Moore, 1987).

4.3. Cyclodextrins and Drug Photostability

Cyclodextrins can affect the photobehaviour of drug molecules, and have therefore been widely investigated for use in modifying certain photochemical reactions so as to achieve selectivity. Cyclodextrin can alter the photobehaviour of a molecule by changing the ground state distribution of reactive and non-reactive conformers ("conformational control"), whereby translational and rotational motions are restricted. This results is selectivity (Rao, *et al.*, 1986), and thus the reaction can be caused to proceed along one of the competing pathways (Reddy and Ramamurthy, 1987). The "cage effect" of inclusion of molecules in the cyclodextrin cavity may cause radical pairs to undergo reaction possibly even generating the parent drug (Rao *et al.*, 1987) before being able to diffuse into the surrounding medium. This occurs to a lesser extent in the solution outside of the cyclodextrin cavity because of more rapid diffusion (Reddy et al, 1986).

In the photodegradation of chlorpromazine, dimethyl-β-cyclodextrin inhibits reactions such as oxidation and accelerates dechlorination to promazine (Uekama, 1985). β-Cyclodextrin improved the photostability of some 1,4-dihydropyridine derivatives up to 200-fold, and accelerated the photodegradation of others by a fraction of four (Mielcarek, 1997). Cyclodextrin increased the photochemical stability of nimodipine by forming liquid inclusion complexes (Mielcarek, 1998).

The (*E*)-(*Z*) photoisomerization of the antiulcer agent, CBC (Utsuki *et al.*, 1993) was accelerated in 1:2 drug to cyclodextrin complex and decelerated in 1:1 complex because of microsolvent effect of the cyclodextrins and steric effect (inclusion may hinder rotation in the transition state causing deceleration), respectively.

4.4. Methods

Forced degradation studies were carried out to investigate effect of RAMEB on the photodegradation kinetics of midazolam, and for determining the photodegradation pathways. For forced degradation studies the concentrations of the samples was decreased to 0.5mg/ml in order to accelerate the rate of degradation. Confirmatory studies were carried out with 10 mg/ml solutions of midazolam containing 30% m/v RAMEB, studies at this concentration in the absence of RAMEB were obviously not carried out due to solubility problems. Thermal stability studies were carried out to investigate the effect of temperature on the solutions, since the irradiation unit was maintained at 40 ^{0}C.

4.4.1. Irradiation of Samples

An Atlas SUNTEST CPS+ (Atlas Material Testing Technology B.V, Germany), fitted with a Xenon lamp and Solar ID65 filter was used for irradiation of samples. The Suntest was calibrated by Premier Technologies, the Agent of Atlas in South Africa. The certificate is attached as annexure A. ICH guidelines (1996) require that samples be exposed for not less than 1.2 million lux hours, with an integrated near-ultraviolet energy of not less than 200 W h/m.^{2} The samples were irradiated at 550 W/m^{2} for 12 hrs in excess of the ICH requirements in order to degrade the drug to approximately 10 % of its original concentration. The temperature in the Suntest cabinet was maintained at 40 ^{0}C.

4.4.2. Photostability (Kinetics)

Solutions of midazolam (0.5 mg/ml) were prepared as in section 3.2.3. 2 ml aliquots in clear ampoules (USP standard type 1 glass) were irradiated, removed at 1 hr intervals over a 12 hr

period and analyzed by the validated HPLC method, the parameters of which are listed in section 3.2.6. The whole 2 ml was used and the ampoule was rinsed with the dilution solvent (50:50 methanol/water) in order to dissolve any degradants that may have precipitated and diluted to 10 ml. Control samples were covered in foil and treated in the same way as the exposed samples. Degradation was calculated as a percentage of the height of the drug peak, with respect to the peak height obtained from analysis of the original solution. The appearance of the major degradation products was also monitored and calculated as percentage of the highest concentration achieved. Samples for the confirmatory studies (10 mg/ml) were prepared and analyzed as above.

4.4.3. Thermal Stability

To investigate temperature dependence on the degradation of midazolam, 2 ml aliquots of midazolam solution (0.5 mg/ml, with and without 30% m/v RAMEB) were sealed in ampoules, covered in foil and placed in ovens at temperatures 25, 30, 40, 50, 60 and 70 ^{0}C. The samples were removed after 12 hrs and analyzed as above.

4.4.4. Identification of Photoproducts by LC-MS/MS

The chomatograms obtained in section 4.4.2 were examined and a representative sample of drug and photoproducts was chosen for analysis by LC-MS to obtain the molecular masses of the degradation products. LC-MS/MS was used to identify the photodegradants.

The Finnigan LCQ system was equipped with an Atmospheric Pressure Chemical Ionisation (APCI) source, which was tuned to give maximum sensitivity, and a Waters Spherisorb 5μ ODS2 column (250 mm x 4.6 mm, i.d.) used. The mobile phase composition was 70:30 methanol/ 30 mM ammonium acetate at a flow rate of 1.0 ml/min and injector was a Rheodyne Model 7125 (Cotati, California) equipped with a 20 μL loop. The capillary temperature was set at 150 ^{0}C, vaporiser temperature 270 ^{0}C, capillary voltage 37 V and, tube lense offset 21 V, discharge current 5μA, sheath gas flow rate was 20 arbitrary units and the auxiliary gas flow rate was 0.

4.5. Results and Discussion

In the samples without RAMEB, degradation products started appearing after 2 hrs irradiation. A slight yellowish-brown colour developed at the same time which intensified on continued irradiation. After 6 hrs, a precipitate was formed which was readily soluble in the dilution solvent used for analysis (methanol/water). The control samples, with and without RAMEB, were clear and colourless until the end of the experiment. Analysis of the exposed samples containing RAMEB showed that degradation products had begun to form during the first hour of exposure. No suspended particles were observed throughout the experiment, but the slight yellowish-brown colour was observed.

Figure 4.1 shows the HPLC chromatograms of the degraded samples, in the presence and in the absence of RAMEB. The midazolam peak appears at 10.5 min. From the chromatograms it is evident that some degradants were formed in the absence of RAMEB, but not in its presence. These degradants, appearing at retention time 5.5 and 6.5 min, were observed in small concentrations throughout the 12 hrs of exposure. Two other degradation products, with retention times 8.3 min and 8.7 min were unique to the RAMEB environment, the latter being prominent. The major degradants in both environments appeared at retention times 6.0 min and 17.2 min. The retention times of all the degradants, except the one eluting at 17.2 min, indicate that they are more polar than the drug. Peaks of major degradants are marked as A, B and C (C only in the sample with RAMEB) on the HPLC chromatograms.

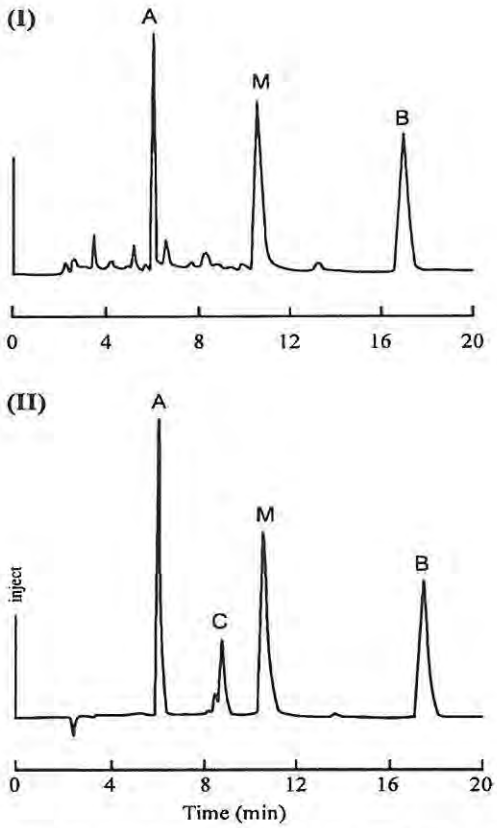


Figure 4.1. HPLC chromatograms showing the peak of midazolam, M, after 8 hrs photodegradation **(I)** in the absence and **(II)** in the presence of RAMEB and the peaks of degradants A, B and C.

4.5.1. Photostability (Kinetics)

The peak height (mm) of the degraded midazolam is divided by the peak height of the drug at t = 0 to give the fraction of the degraded midazolam, α, which is plotted against time in Figure 4.2, to compare the photodegradation of midazolam in the presence and absence of RAMEB. The control values have been subtracted from the experimental to account for possible thermal degradation at 40^{0}C. The behaviour in the presence and in the absence of RAMEB is similar, with an indication of slightly decreased photostability in the presence of RAMEB. This difference in behaviour may be seen more clearly in Figures 4.3 and 4.4. In both instances two degradants, labelled A and B with retention times 6.0 and 17.2 min, respectively, were noted, while in the presence of RAMEB, two further degradants are evident at 8-9 min. For the purpose of this study, only identification of the major component, C was considered. In the absence of absolute amounts, the curves for these degradants have been scaled to fractions, α of the maximum values reached.

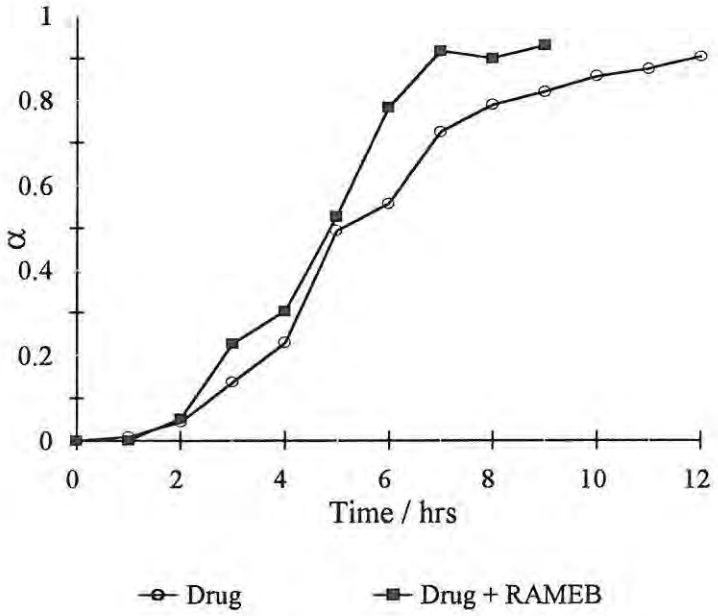


Figure 4.2. Curves for the fraction, α of drug degraded against time, in the presence and absence of RAMEB.

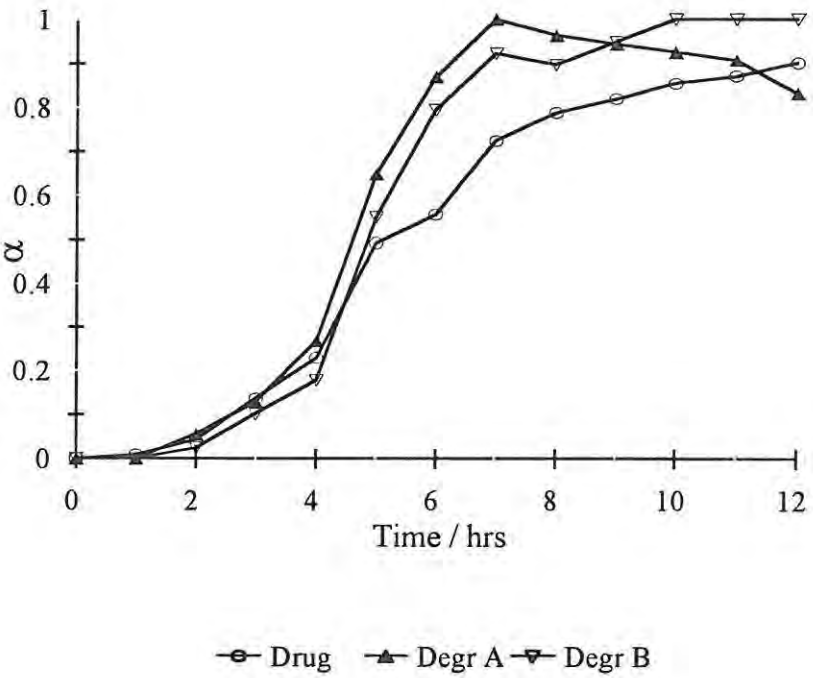


Figure 4.3. Curves for the disappearance of midazolam in the absence of RAMEB and the formation of degradants A and B.

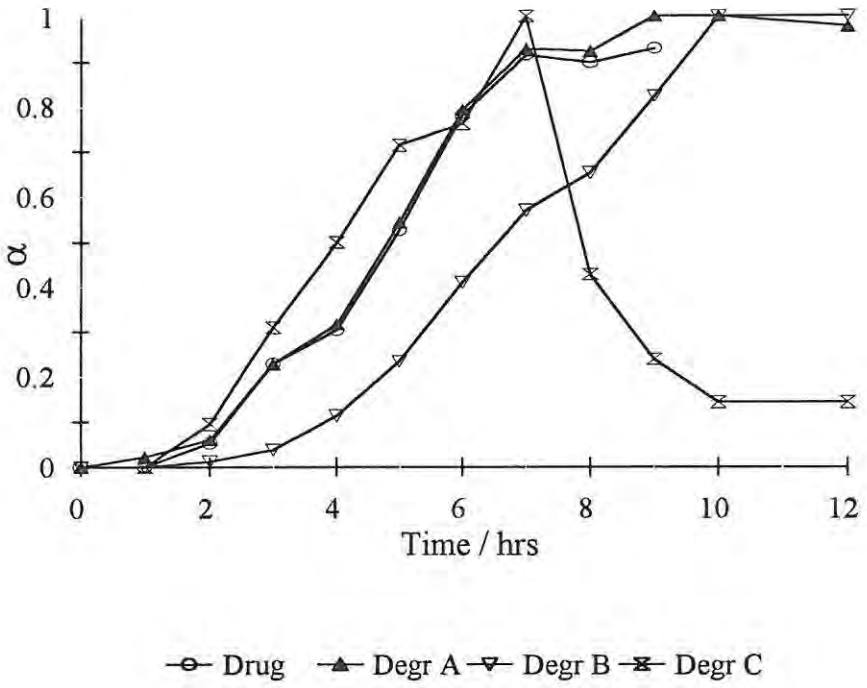


Figure 4.4. Curves for the disappearance of midazolam and formation of degradants A, B and C, in the presence of RAMEB.

In Figure 4.3, the three (α,t) curves for the disappearance of midazolam without RAMEB and formation of degradants A and B coincide until about 25% degradation. In the latter stages, the curves for the degradants lie above that for the drug, but are similar to each other. The formation of the degradants A and B can be represented by the equation:

$$\mathrm{M} \xrightarrow{k_1} \mathrm{A} \qquad \mathrm{M} \xrightarrow{k_2} \mathrm{B} \tag{4.1}$$

where M is midazolam, k_1 and k_2 are the rate constants for the formation of A and B, respectively.

The initial stages of degradation are acceleratory which suggests an autocatalytic effect of the degradants on the rate of degradation of the drug (Laidler, 1965; Stevens, 1961). For this region, a power law (Galwey and Brown, 1999) could be applied to give a rate equation of the form:

$$\alpha = (kt)^n \tag{4.2}$$

where k is the rate constant for the degradation of the drug, and n represents the accelerating effect of the degradants on the rate of degradation. Plots of ln α (ln Alpha) vs ln t are shown in Figure 4.5. Calculation of the slopes from the straight lines obtained gave the values of n, and the intercepts represented n ln k. The values of k and n which gave the best fit to the experimental results are shown in Table 4.1.

Table 4.1. The Power Law Parameters, n and k.

	k / hr^{-1}	n
Without RAMEB	0.137	2.33 ± 0.09
With RAMEB	0.154	2.34 ± 0.26

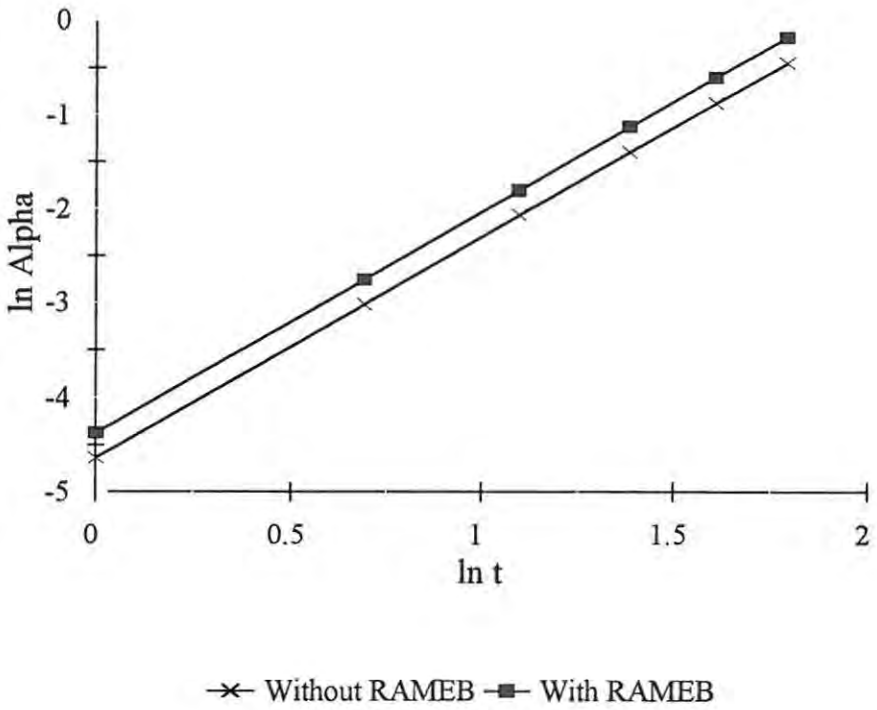


Figure 4.5. ln Alpha vs ln t curve for the degradation of midazolam in the absence and in the presence of RAMEB.

The calculated plots of $\alpha = (kt)^n$ using the values of n and k from Table 4.1 are compared with the experimental results in Figure 4.6, for degradation in the absence of RAMEB, and Figure 4.7 in the presence of RAMEB. In Figure 4.6, the calculated curve coincides approximately with the experimental results for the first 7 hrs. In Figure 4.7, a close fit between the calculated and the experimental curves is observed until t = 6 hrs.

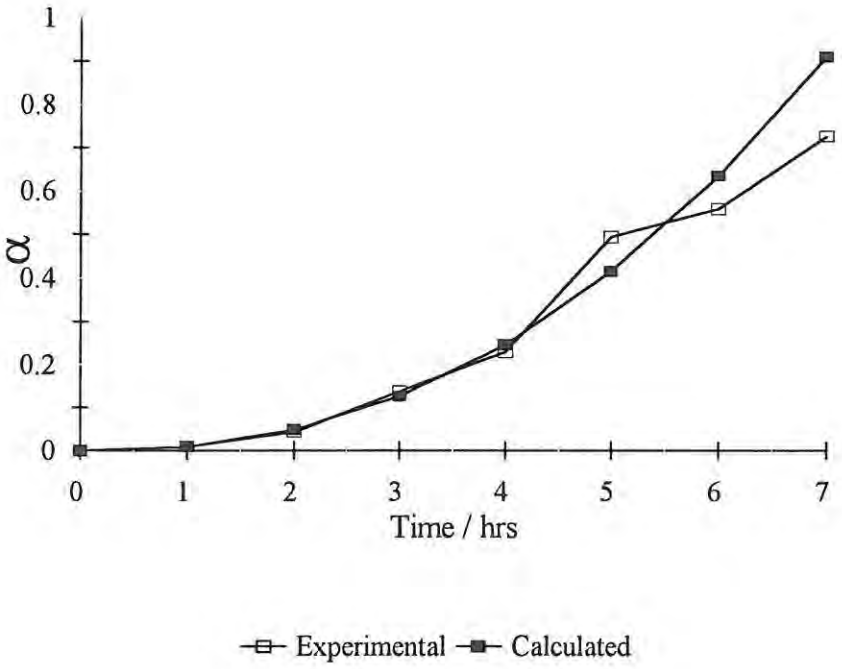


Figure 4.6. Comparison between the calculated and the experimental curves for the degradation of midazolam in the absence of RAMEB using the power law parameters n = 2.33 and k = 0.137 (Table 4.1).

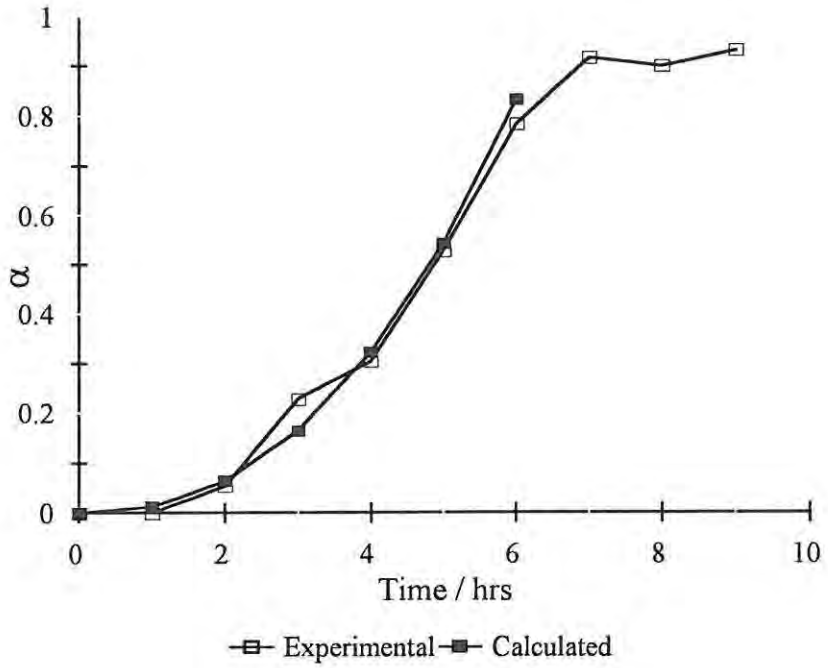
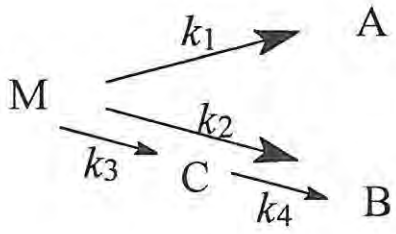


Figure 4.7. Comparison between the calculated and the experimental curves for the degradation of midazolam in the presence of RAMEB using the power law parameters n = 2.34 and $k = 0.154$ (Table 4.1).

In Figure 4.4, which shows the degradation of midazolam in the presence of RAMEB, the curve for the production of degradant A is very similar to that for the degradation of the drug. Production of the third degradant, C, reaches a maximum amount after about 7 hrs, which corresponds to about 90% degradation of the drug. The amount of C then decreases over about 3 hrs to about 15% of its maximum value. At this total time of 10 hrs, the amount of degradant B reaches a maximum. These results suggest:



(4.3)

where k_3 and k_4 are the rate constants for the conversion of M to C and C to B respectively.

4.5.2. Thermal Stability

Results of the thermal stability studies (Table 4.2) show that up to 1.5% degradation occurred in all the samples subjected to heat for 24 hrs, both in the presence and in the absence of RAMEB. At 40 ^{0}C, no degradation was observed in the absence of RAMEB and 1% degradation occurred in the presence of RAMEB. No degradation products were detected on the HPLC chromatograms. These results suggest that any significant degradation observed in the photostabibilty studies samples was due to light and not heat.

Table 4.2. Thermal Degradation of Midazolam

Temperature (^{0}C)	Peak Height (mm)		% Degradation	
	Midazolam Only	Midazolam + RAMEB	Midazolam Only	Midazolam + RAMEB
Initial	207	200	0	0
25	204	200	1.4	0
30	204	198	1.4	1
40	207	198	0	1
50	207	198	0	1
60	204	197	1.4	1.5
70	204	200	1.4	0

4.5.3. Confirmatory Studies

The results of the kinetics of the confirmatory photodegradation studies are shown in Table 4.3 as peak heights and the fraction, α, of the drug degraded and the fractions of the degradants A, B and C formed with time. In Figure 4.8 which shows the curves for the disappearance of midazolam and the appearance of the photodegradants A, B and C, the production of degradants A and B is acceleratory throughout the duration of the expriment. The maximum production of

C is reached after 6 hrs and stays there until 7 hrs. In the next hour the amount of C drops to about 60%, and then to zero. This pattern of increasing amount of C until 7 hrs followed by a sudden decrease was also observed in the results of the forced deradation studies. This further confirms that C is an intermediate.

Table 4.3. Photodegradation of 10mg/ml Midazolam in Aqueous Solution (30% m/v RAMEB) at pH 5.0

Time (mm)	Midazolam		Degradant A		Degradant B		Degradant C	
	Peak Height (mm)	Fraction α	Peak Height (mm)	Fraction α	Peak Height (mm)	Fraction α	Peak Height (mm)	Fraction α
0	183.5	0	0	0	0	0	0	0
1	174	0.052	1	0	0	0	0	0
2	164	0.106	2.5	0.056	1	0.045	1	0.667
3	161	0.123	4	0.139	2.5	0.09	1	0.667
4	151	0.177	5	0.277	4	0.09	1	0.667
5	146	0.204	6	0.333	6	0.136	1	0.667
6	136.5	0.256	8	0.444	8	0.227	1.5	1
7	125.5	0.316	11	0.611	11	0.364	1.5	1
8	134	0.27	10	0.583	10.5	0.409	1	0.667
9	115	0.373	14.5	0.806	14.5	0.682	0	0
10	113	0.384	16	0.889	16	0.818	0	0
11	109	0.406	18	1	18	1	0	0

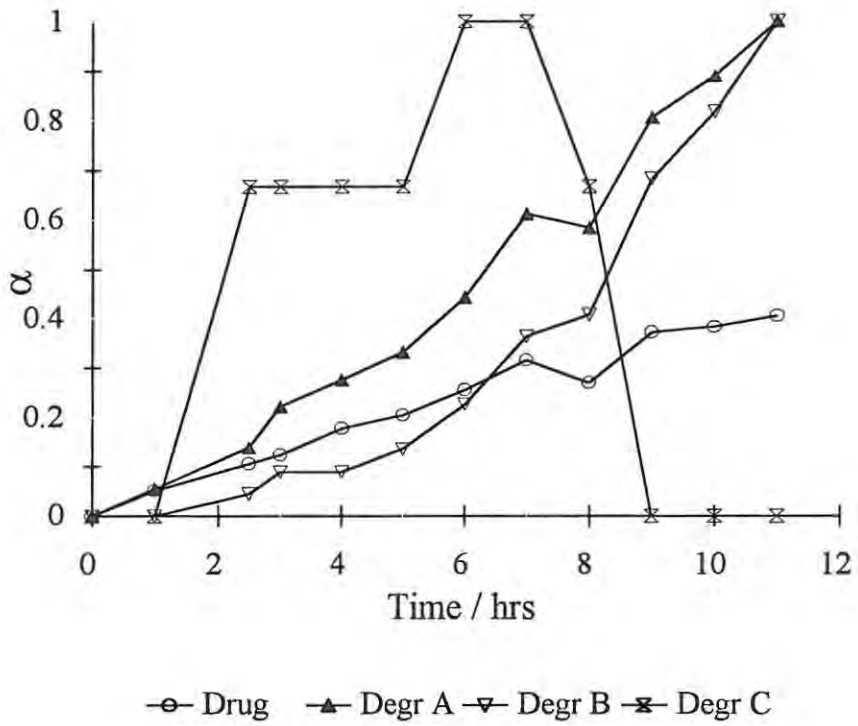


Figure 4.8. Curves for the disappearance of midazolam and formation of degradants A, B and C, in the presence of 30 % m/v RAMEB at midazolam concentration of 10 mg/ml.

A plot of the fraction degraded, α vs time for the photodegradation of midazolam in the presence of 30% m/v RAMEB (Figure 4.9) gives a straight line, showing that the kintics of the degradation of midazolam in the presence of RAMEB at high concentration follows zero-order kinetics. The zero-order rate constant for the reaction is 0.037 $hr.^{-1}$ The amount of midazolam in Figure 4.8 reaches 60 % after 11 hrs, whereas 90% degradation was already reached after 7 hrs in the forced studies. Thus as expected the rate of degradation is increased in the less concentrated solutions.

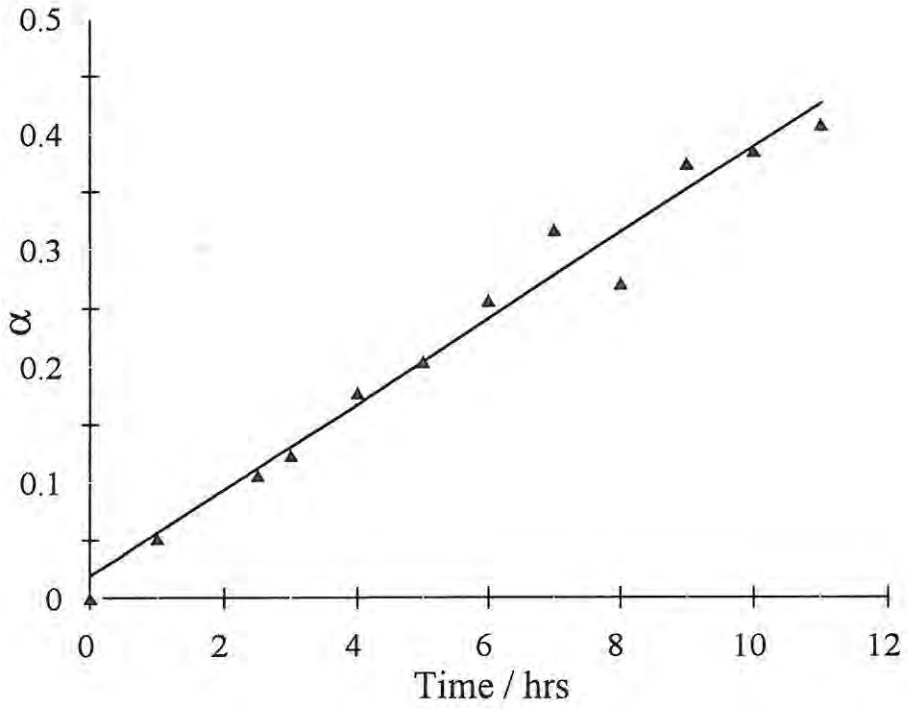


Figure 4.9 A curve of α vs time for the degradation of 10 mg/ml midazolam in the presence of 30% m/v RAMEB.

4.5.3. Identification of Degradants

The results of LC-MS analysis including the M+1 peaks for the degradants, their retention times and proposed structures are listed in Table 4.4. The degradation products appearing only in the absence of RAMEB at retention times 5.5 and 6.5 min exhibited peaks at $m/z = 342$ (14%, relative abundance - RA) and 343 (27%, RA), respectively. Structures proposed for the photoproduct with m/z = 342 are the hydrolysis products 1-hydroxymethylmidazolam **(10)** or 4-hydroxymidazolam **(11)**, which are also the primary metabolites of midazolam. The photoproduct with m/z = 343, with proposed stucture **(13)** results from contraction of the diazepine ring, followed by contraction of the imidazole ring and subsequent substitution of the alkylamino group by a carbonyl in solution.

Table 4.4. Retention Times, M + 1 Peak Values and Proposed Structures of Photodegradants of Midazolam.

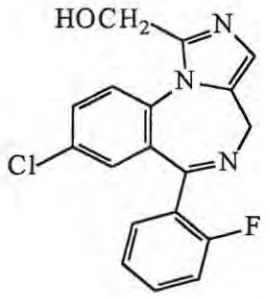
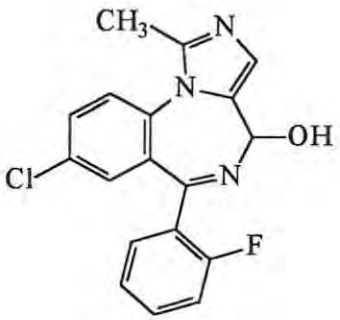
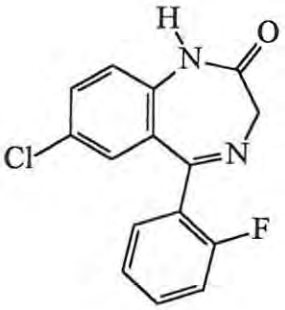
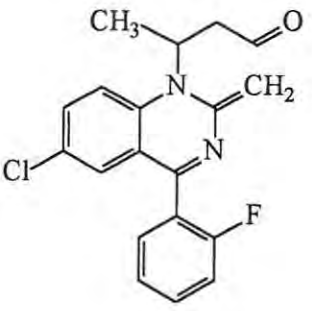
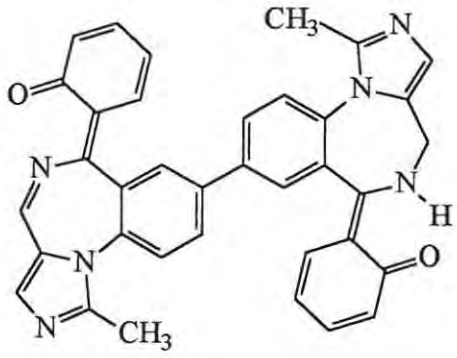
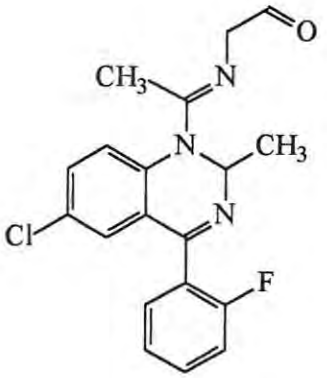
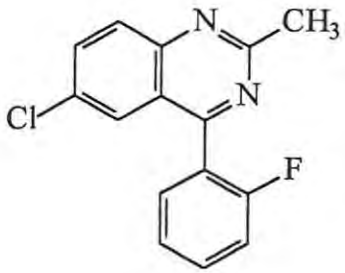
Retention Time	M + 1	Proposed Structure
5.5	342	 **(10)**
	or	 **(11)**
6.0	289	 **(3)**
6.5	343	 **(13)**

Table 4.4. *Continued.*

Retention Time	M + 1	Proposed Structure
8.3	575	 **(14)**
8.7	358	 **(15)**
17.2	273	 **(16)**

Mass spectra of degradants with m/z 289, 273, 358 and 575 obtained by MS-MS analysis are shown in Figures 4.10, 4.11, 4.12 and 4.13, respectively. In Figure 4.10, the molecular-ion peak (m/z 289, 23 % RA) is still visible, and an NH_4 adduct (m/z 306, 11 % RA) is observed. The molecular mass of this compound is the same as that reported by (Andersin *et al.*, 1994) and, on this basis, degradant A is proposed to be N-desalkylflurazepam, or 7-chloro-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one. The base peak at m/z 261 (100 % RA, M - 27) could be due to loss of HCN, and other peaks observed were at 270 (35 % RA, M - H_2O), 260 (16 % RA, M - CO), also observed by Selkämaa and Tammilehto (1989), 226 (57 % RA), 208 (36 % RA) and 140 (56 % RA). Degradant A was thus identified as one of the starting compound in the synthesis of midazolam, namely compound **(3)** in Figure 1.2. Photodegradation of midazolam to degradant A follows opening of the imidazole ring with subsequent sustitution to form the benzodiazepinone.

In Figure 4.11, the M + 1 peak m/z 273 is not detected, which means this species is very unstable. From the molecular mass of this compound, degradant B, it can be suggested that it is 6-chloro-2-methyl-4-(2′-fluorophenyl)-quinazoline (Andersin *et al.*, 1994). The base peak is at m/z 177 (100 % RA, M - C_6H_4F) and other peaks were m/z 237 (44 % RA, M - Cl); 245 (21 % RA) and 253 (14 % RA, M - F). A peak at m/z 237 was reported by Selkämaa and Tammilehto (1989) as being due to loss of Cl.

Andersin *et al.* (1994) also reported a precipitate in solutions of midazolam exposed to daylight. Their analysis of the purified precipitate revealed that the precipitate was 6-chloro-2-methyl-4-(2′-fluorophenyl)-quinazoline. In this study, the precipitate appeared after 6 hrs of exposure and, as already shown in Figure 4.3, at this time the amount of B was approaching a maximum value. The proposed identification of B as compound **(16)**, is consistent in that the compound is more hydrophobic than degradant A, compound **(3)**. The retention time of B was 17.2 min, while every other degradant appeared before the drug (R_t = 10.5 min), i.e., B is very non-polar. It is, therefore, not surprising that as the concentration of B increased, it precipitated out of the aqueous solution. The absence of a precipitate in the RAMEB solutions, in spite of the evidence of the presence of B as a major degradation product, can be explained by the ability of RAMEB to improve the solubility of hydrophobic compounds.

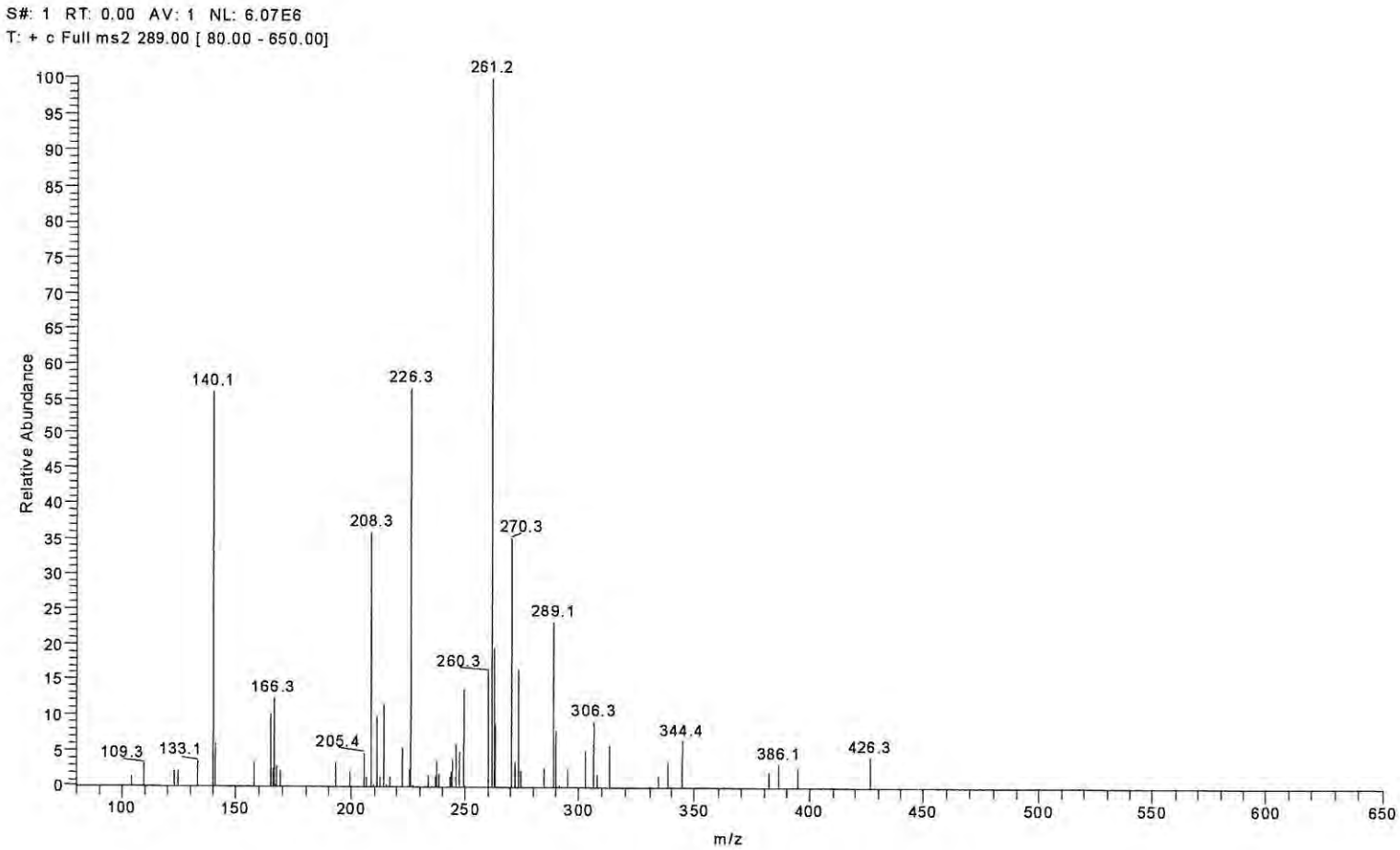


Figure 4.10. Mass spectrum of degradant A, m/z 289

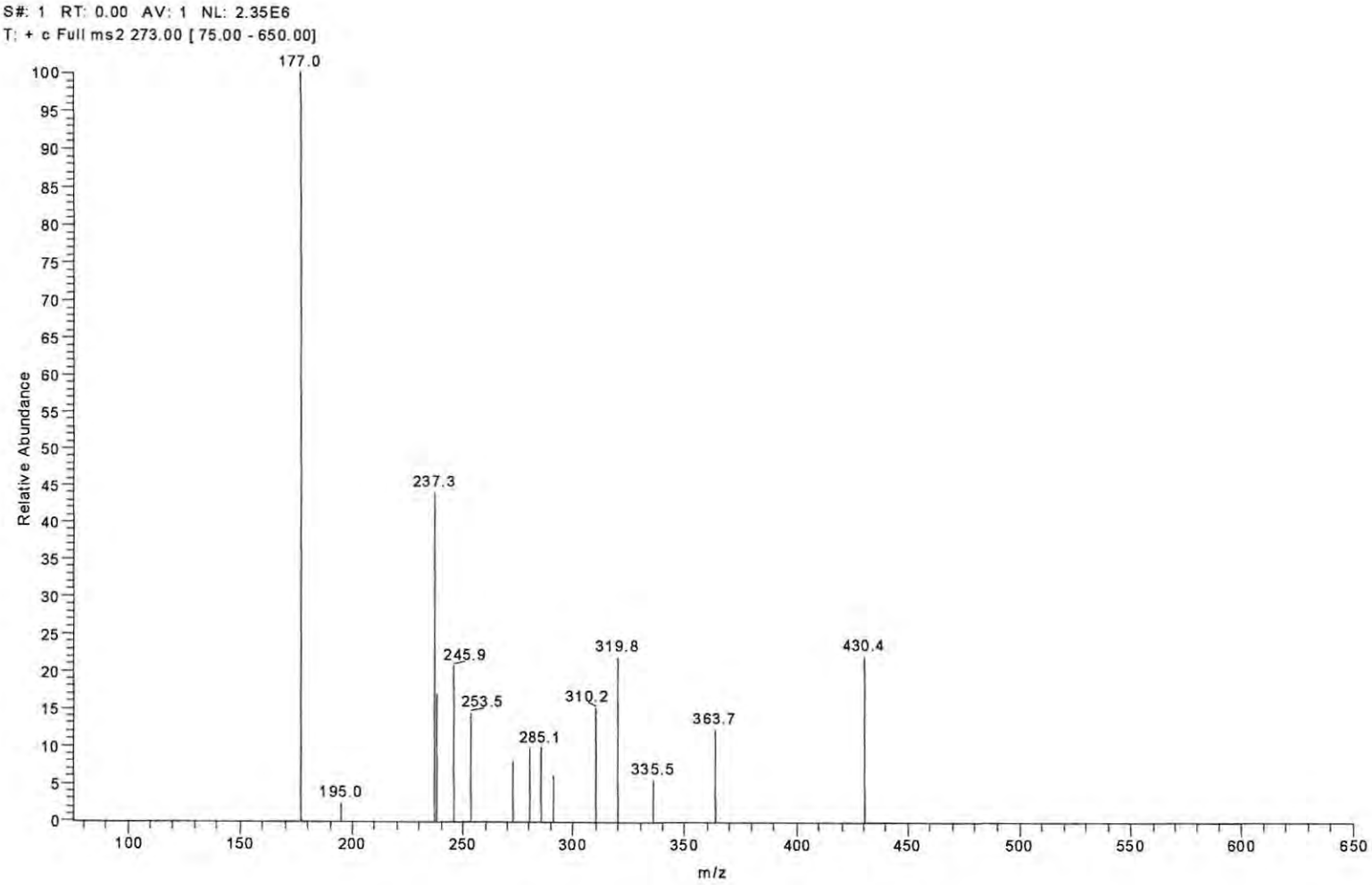


Figure 4.11. Mass spectrum of degradant B, m/z 273.

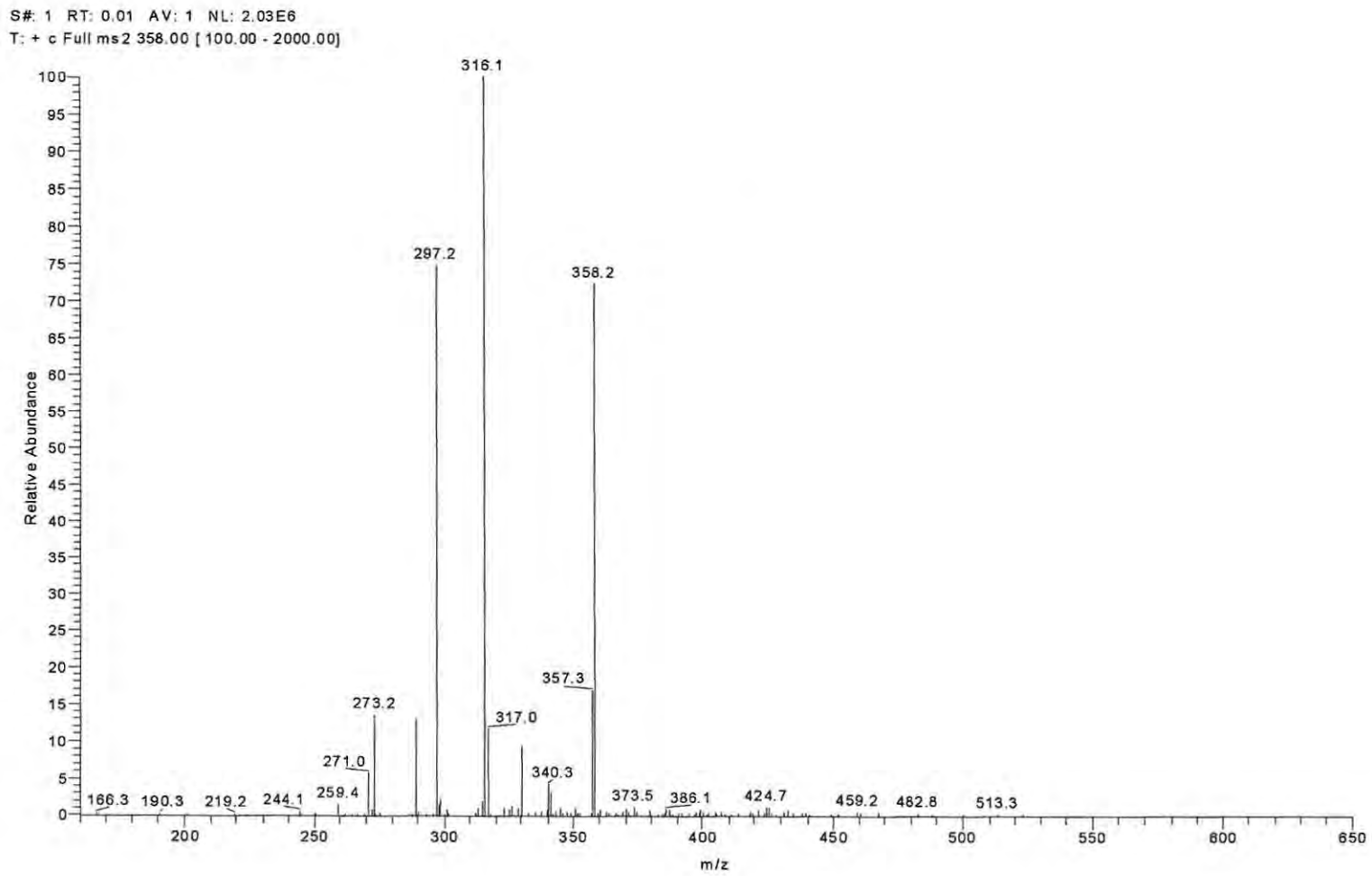


Figure 4.12. Mass spectrum of degradant C, m/z 358.

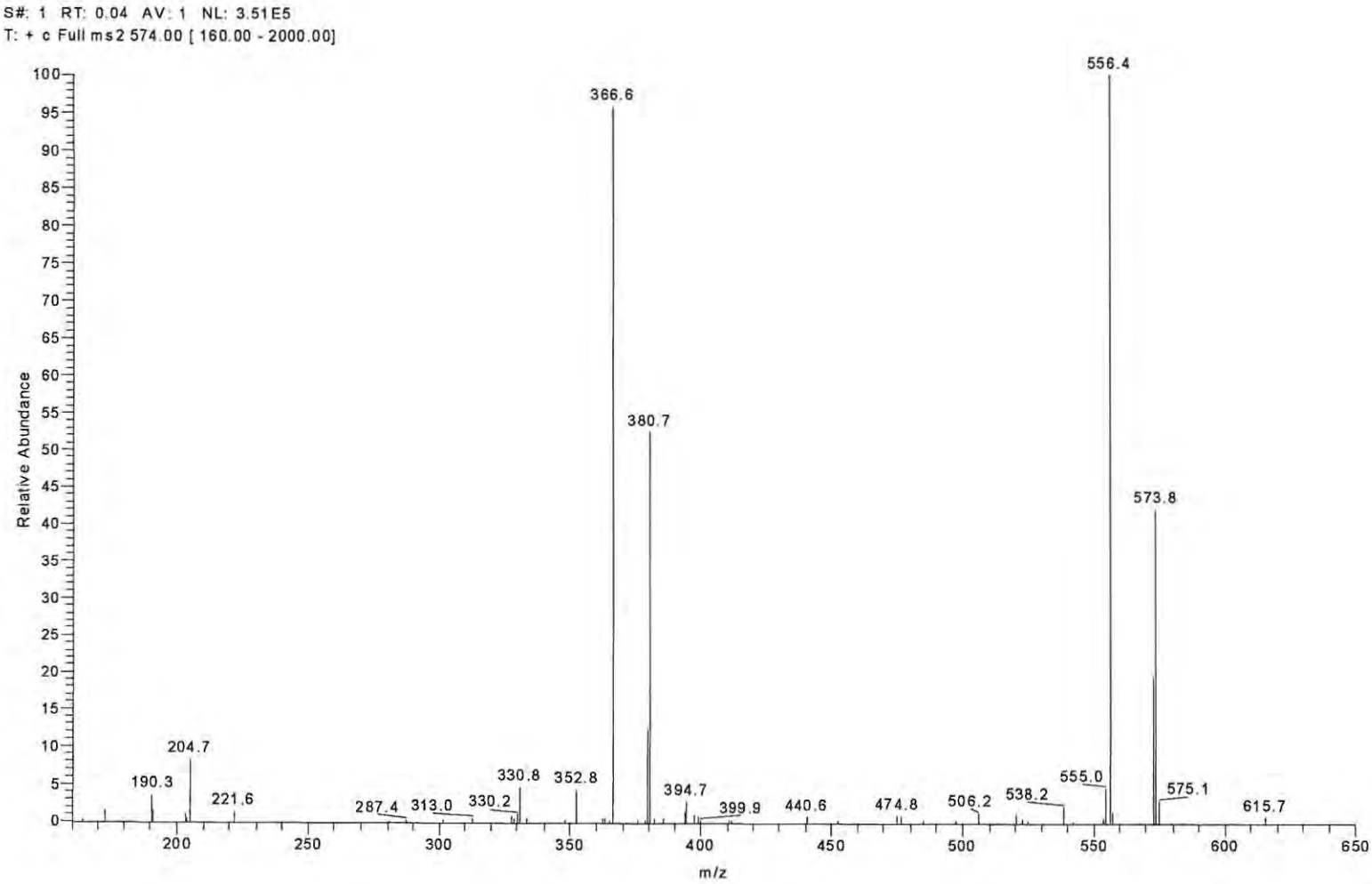


Figure 4.13. Mass spectrum of degradant at m/z 574.9.

Photodiode array analysis (Performed by Pharmaceutical Research Laboratories, Port Elizabeth, RSA) was used to determine the UV λ_{max} values of degradants A and B, and these were compared with the literature values (Andersin *et al.*, 1994). As shown in Table 4.4 the experimental values are consistent with the literature values. The experimental λ_{max} values of degradant C are also included.

Table 4.5. UV λ_{max} Values of Degradants A and B and C.

Degradant	λ_{max} (nm)	
	Experimental	Literature Values (Andersin *et al.*, 1994)
A	229	230
	318	318
B		206
	230	230
	327	326
C	215	
	333	

RAMEB also caused formation of degradant C, the retention time of which (8.7 min in the HPLC chromatogram) suggests that it is more polar than the drug. Formation of this compound follows addition of a polar molecule present in solution to the drug, possibly containing oxygen, resulting in a higher molecular mass. The mass spectrum of degradant C is shown in Figure 4.12 with the M + 1 peak at m/z 358 (72 % RA). A compound of this molecular mass has not been reported in the photodegradation studies of midazolam. The base peak at m/z 316 (100 % RA), and the peak at m/z 297 (75 % RA) could be due to loss of CH_3, followed by subsequent loss of CH_3CH_2O. A fragment equivalent to the M + 1 peak of degradant B, m/z 273 is observed. From the kinetic studies it was postulated that degradant C is an intermediate in the formation of B. This means that C may have the structure of B as part of its structure. The degraded solutions were covered in foil, stored in a refrigerator ($\pm 4^0$C) and analyzed by HPLC after a few weeks.

After this time the peak due to the drug had slightly decreased, that of degradant C had almost disappeared, and the peak of B had grown remarkably, further indication that C may be an intermediate of B.

The structure of C was, therefore, proposed to be compound **(15)**. Degradation of midazolam through C to B is an additional pathway that was observed in the presence of RAMEB, explaining the slight increase in the rate of degradation. Because the pathways that RAMEB inhibits lead to formation of low-level products **(10)** or **(11)** and **(13)**, the effect of inhibition did not balance acceleration and, therefore the overall rate of degradation is increased.

Another degradant caused by the presence of RAMEB could be a dimer, because it has an M + 1 peak at m/z 575. The monomer would have a molecular mass of 288 amu, if the two molecules are joined together through loss of an H atom. The mass spectrum (Figure 4.13) shows a base peak at m/z 556 (100 % RA, M - H_2O), and other peaks at 381 (52 % RA) and 367 (95 % RA). The proposed structure, compound **(14)** is a dimer of a compound found as the major degradation product in the studies carried out by Andersin *et al.* (1994). The dimer is formed through loss of Cl atom from each molecule, resulting in free radicals which then combine with each other. Formation of this compound and degradant C is unique to the cyclodextrin environment. The reactions are made possible by the ability of cyclodextrin to stabilize free radicals (Rao *et al.*, 1987), and by conformational control which results in stabilization of certain reaction intermediates.

The effect of the light source on the nature of the degradation products was also noted. The major degradation product (molecular mass, 323.1), obtained in the solutions exposed to high pressure mercury lamp (Andersin *et al.*, 1994), was not observed in this study. Even that product which formed the dimer (m/z 575) was in small quantities. Degradant B, one of the major degradation products in this study, occurred in the solutions exposed to daylight, where A was present in small amounts. This is because photodegrdation is dependent upon the wavelength of irradiation (Carstensen, 1995).

The proposed mechanisms for the degradation of midazolam in the absence and in the presence of RAMEB are shown in Figures 4.14 and 4.15, respectively.

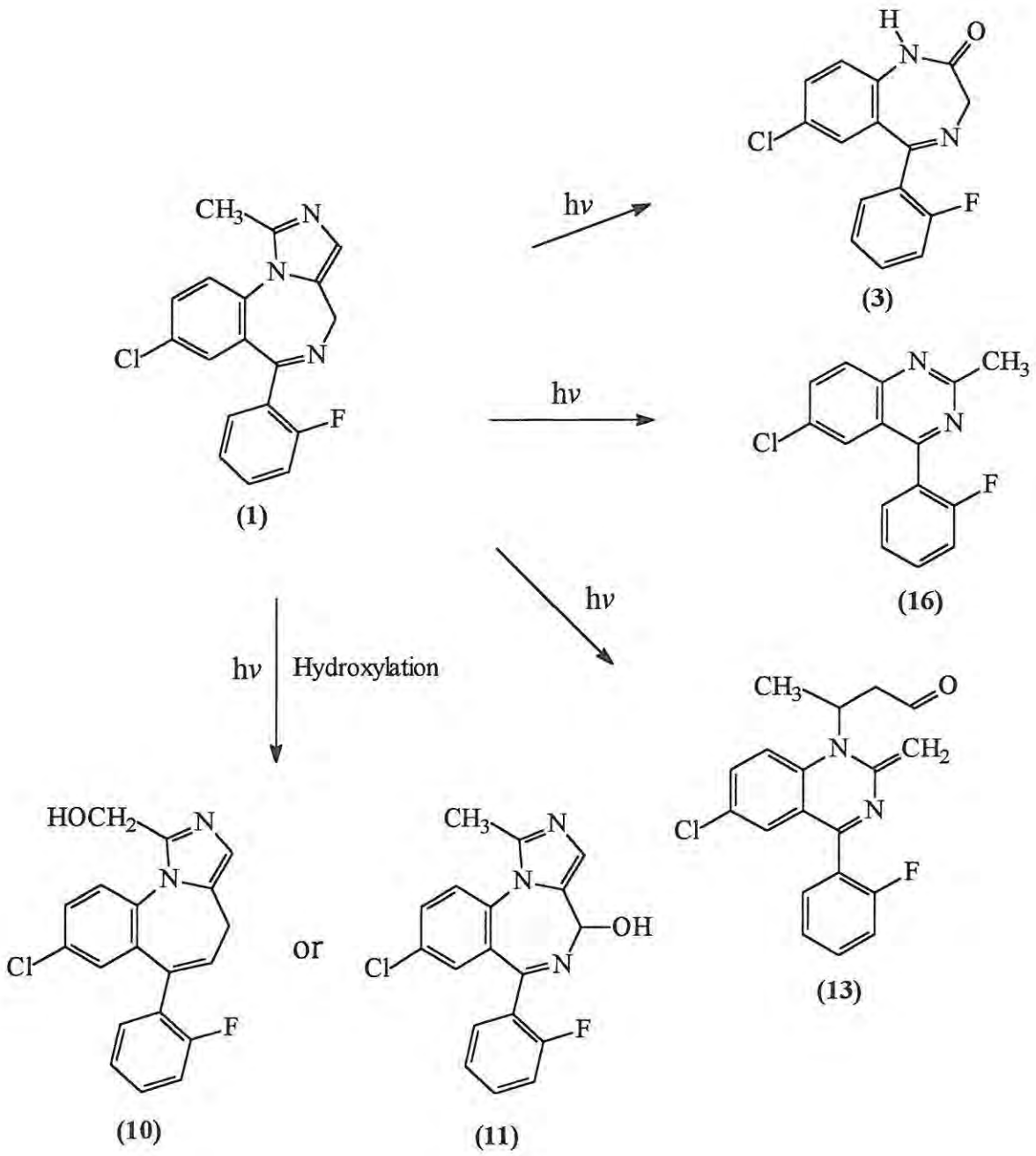


Figure 4.14. Proposed photodegradation pathway of midazolam in aqueous solution at pH 5.0.

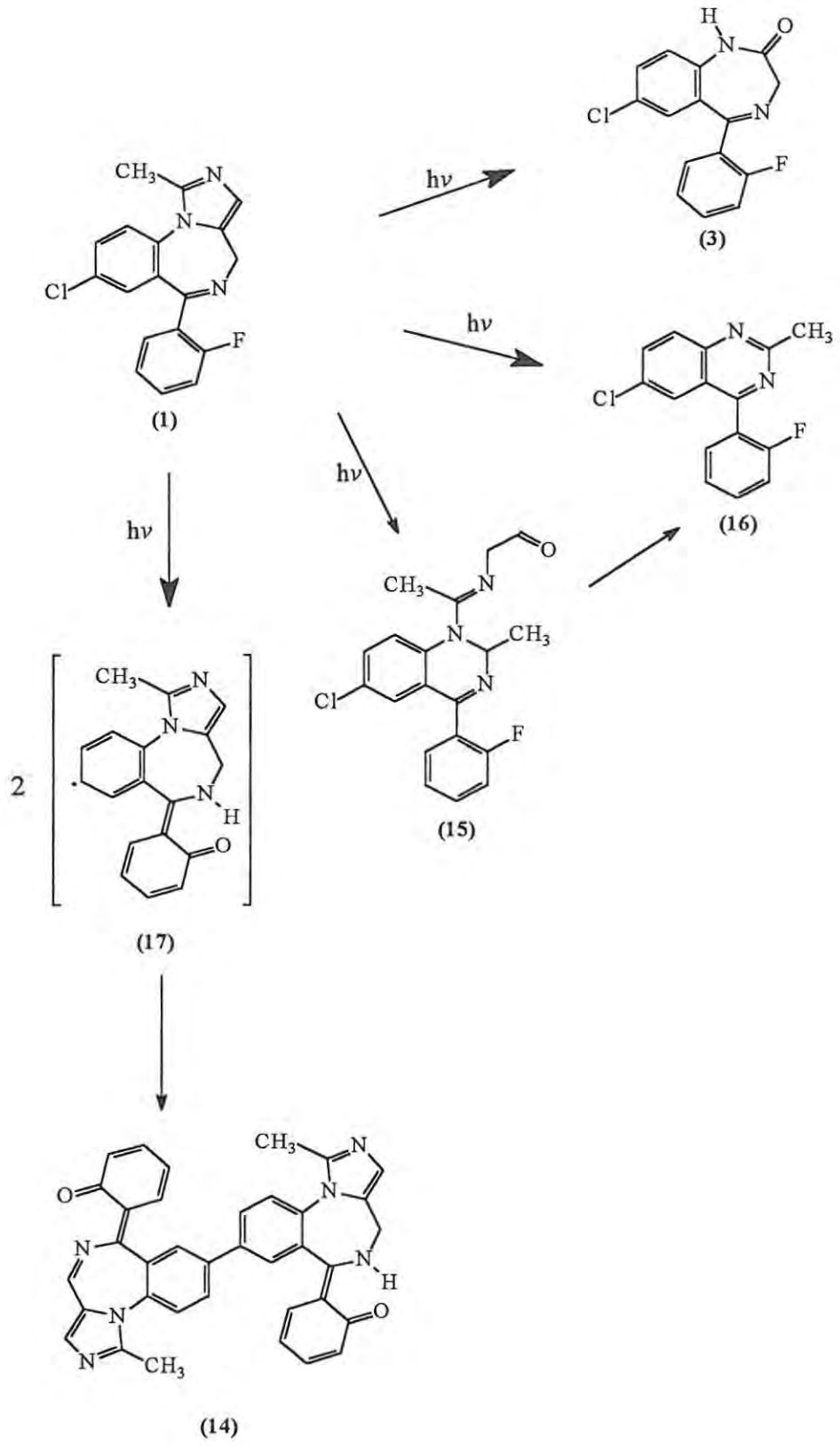


Figure 4.15. Proposed photodegradation of midazolam in aqueous solution containing 30% m/v of RAMEB, at pH 5.

4.6. Conclusion

In the forced degradation studies, the kinetics showed that degradation of midazolam with time at the concentration of 0.5 mg/ml, both in the presence and absence of RAMEB, was not linear. The kinetics of degradation could not be fitted to a specific order as Andersin and Tammilehto (1995) succeeded to do in their study of pH-dependent stability. In this study, the initial stages were acceleratory, suggesting autocatalysis by the degradants. Power law was applied to determine the rate constants for the photodegradation of midazolam. These were found to be 0.137 and 0.154 hr^{-1} in the absence and in the presence of 30% m/v RAMEB, respectively. Thus, RAMEB slightly decreases the photostability of midazolam. The decreased stability was accompanied by the appearance of a new degradation product. The new degradant has been proposed to be an intermediate of one of the degradation products,6-chloro-2-methyl-4-(2'-fluorophenyl)-quinazoline, because the former reached maximum concentration after 7 hrs and then started decreasing to 15% of its maximum concentration while the latter reached a maximum.

Confirmatory studies showed that the photodegradation of midazolam in in the presence of RAMEB in concentrated solutions follows zero-order kinetics. The zero-order rate constant was found to be 0.037 hr^{-1} which is four times less than that obtained in the forced degradation studies. Thus the rate of photodegradation as expected is decreased in the concentrated solution. Photoproduct formation followed the same pattern as in the forced degradation studies, further confirming the presence of an intermediate.

Thermal stability studies which were carried out at temperatures ranging from 25 to 70^{0}C resulted in a maximum of 1.5% degradation. Thus midazolam is stable to heat, therefore it can be concluded that any degradation occurring during the photostability studies is entirely due to light and not affected by heat.

The two major degradation products that occurred in the presence and absence of RAMEB are proposed to be N-desalkylflunazepam and 6-chloro-2-methyl-4-(2'-fluorophenyl)-quinazoline. These compounds have been reported as photodecomposition products of midazolam (Andersin *et al.*, 1994). Two other photoproducts which occurred only in the presence of RAMEB are of

higher molecular mass, 357 and 574. This indicates that the former is possibly an addition product of a carbonyl in solution to an open imidazole ring of midazolam. The formation of this product also involves contraction of the diazepine ring to a quinazoline. The latter is a dimer of a free radical intermediate formed by the removal of chlorine from a compound that was not detected in this study, but was reported as one of midazolam photodegradation products (Andersin *et al.*, 1994). Occurrence of these products in RAMEB solutions is attributed to the ability of cyclodextrin to provide conformational control and stabilize free radicals.

CHAPTER 5: GENERAL CONCLUSIONS

The fast onset and the short duration of action of midazolam has made it favorable over the classical benzodiazepines such as diazepam used in sedation and induction of anaesthesia. It lowers the blood pressure without affecting the cerebral intake of oxygen, and it is referred to as an anaesthetic of good controllability (Hilfiker and Kettler, 1981). Intravenous administration of the drug in the existing formulations causes irritation but to a lesser extent than diazepam (Bardhan *et al.*, 1984). The water soluble nature of midazolam makes possible the preparation of aqueous solution formulations. Gerecke (1983) reported the pKa of midazolam as 6.15 ± 0.1, and further stated that the aqueous solutions prepared form midazolam salts are around pH 3.5 and are (especially the hydrochloride) tolerated intravenously and intramuscularly. For the purpose of this study, however, which was to formulate a midazolam solution for topical application it was necessary to prepare solutions as near as possible to pH 5.8 and that required a solubilizer.

RAMEB has been proved to able to significantly increase the aqueous solubility of some drugs, and to be a good absorption enhancer. The absorption enhancing effect of RAMEB would be an added advantage for the transdermal transport of the drug. However, in addition to improving the solubility, cyclodextrins are known to affect the stability of drugs either positively or negatively. Since midazolam is a light sensitive drug, it would be advantageous if the RAMEB improves its photostability. However the stability may be decreased due to RAMEB and the introduction of new photodegradants and pathways may occur. It is thus imperative that the degradation products be identified, and their nature be established so that the method of analysis can be validated to detect these products in the final formulation. Photosensitivity reactions that have occurred in patients to whom light sensitive drugs have been administered, has been associated with formation of toxic photoproducts. Such drugs decompose to form radical intermediates and highly reactive products, which reacts with the tissue cells resulting in adverse effects, making the detection and identification of photodegradants important.

The drug used for this study was successfully characterized using NMR and FTIR spectroscopy. UV spectrophotometric studies were used to determine the λ_{max} which was 258 nm in 0.1M HCl,

and was used as the detection wavelength in the phase solubility analysis. TLC, melting point determinations and DSC confirmed purity while TG showed decomposition of the drug occurring in one step. Mass spectrum showed the molecular ion peak at m/z 325, confirming the molecular mass, and other fragments which have been reported in the literature (Jones *et al.*, 1989).

A simple UV method in which 0.1 M HCl was a solvent was developed for use in midazolam-RAMEB phase solubility studies. The method was validated and found to show good linearity, with a correlation coefficient of 0.9998, and accurate with percent recovery of 98.9 - 101.6%. It was adequately selective for the determination of midazolam in the presence of cyclodextrin, showing good specificity.

The moisture content of RAMEB was 9.2% as determined by Karl Fischer and these results were used in the solubility studies. For equilibrium solubility studies, three media at pH 4.0, pH 5.0 and pH 5.8 were investigated. In all of them, equilibrium solubility was achieved by 18 hrs, hence 24 hrs was considered enough time for equilibration of phase solubility samples before analysis. Phase solubility studies, which were carried out with phosphate buffers pH 5.0 and pH 5.8 only, revealed that 30% m/v RAMEB improved the solubility of midazolam to achieve the target concentration of 10 mg/ml at pH 5.0, where the concentration of 10.6 mg/ml was obtained. This implies that midazolam solutions for topical application can be formulated at pH 5.0 with RAMEB (30% m/v).

Assuming that RAMEB improved the solubility by some form of association with the drug, possibly complexation (implied by the Ap type phase solubility diagrams obtained), the apparent stability constants for the formation of 1:1 drug to cyclodextrin complex and 1:2 complex were calculated. At pH 5.0, the values obtained for $K_{1:1}$ and $K_{1:2}$ are 46.05 and 18.78, respectively, while at pH 5.8, $K_{1:1} = 96.16$ and $K_{1:2} = 10.03$. The low values of the stability constants suggest poor association of midazolam molecules with RAMEB cavities but does not necessarily mean that the stability will be decreased.

After reaching a potential formulation at pH 5.0, it was necessary to follow the international guidelines which recommended that new drug substances and products be tested for photostability to demonstrate that light exposure does not result in unacceptable change (ICH, 1996). For this

purpose, a stability-indicating method of analysis was required. Some substances related to midazolam cannot be detected by GC without derivatization, therefore LC is preferred especially when the nature of other compounds present is not known. Vasiliades and Sahawneh (1982), attempted to develope an LC method for detection of midazolam and its metabolites, but there was a problem of resolution of midazolam from some metabolites, so they used a different method, GC-MS, for determination of midazolam. The stability-indicating HPLC method used by Steedman *et al.* (1992) was reported to accurately measure midazolam, even in the presence of degradation products. However, since the method was only designed for the quantitation of midazolam, it does not make allowance for identification and quantitation of degradants. The retention time of midazolam was less than 5 min, which implies that there could be lack of resolution between midazolam and its photodegradants.

A method that presented potential for use in this study is that used by Bhatt-Mehta *al et.* (1993). So a column with the same dimensions and an ODS packing material was investigated with varying mobile phase compositions. Finally, a mobile phase containing 70:30 methanol/water with 30 mM ammonium acetate (pH 7 ± 1) was used because it gave good resolution between the drug and degradants.

The method has been successfully validated in terms of linearity, precision, accuracy, range, specificity, limit of quantitation, stability of analytical solutions, robustness and ruggedness. Recovery of the method over the range 80 - 120 % falls between 98% and 102% of the theoretical value, which means that the method is accurate and suitable for quantitation of midazolam in the presence of RAMEB. The coefficient of correlation of 1.00 shows that the method is linear and obeys Beer's Law within the range of concentrations 0.004 and 0.02 mg/ml, injected. This means that the results obtained by the method are directly proportional to the concentration of the drug in the sample. Results obtained with the precision test, %RSD of 0.6 % and 0.5 %, show good agreement between the solutions of same concentrations. Although the LC-MS proved successful in confirming the integrity of the drug peak, in the presence of RAMEB there was some interference due to ionization of RAMEB, therefore to confirm the integrity of the drug peak in the presence of RAMEB, photodiode array analysis was undertaken.

The limit of quantitation was determined and validated as 0.002 mg/ml. A maximum deviation of 2% from the original concentration of the test solution, stored in the dark at room temperature, was observed within 24 hrs. Evaluation of robustness during method development included variation of pH of mobile phase, mobile phase composition and use of different columns. It has been established that the concentration of ammonium acetate can vary between 20mM and 40mM, the pH of the mobile phase can be maintained at pH 7 ± 1 pH unit, while the mobile phase composition has to be 70:30 methanol water when the Spherisorb ODS2 (5μ) is used.

Photostability studies involved degradation kinetics and identification of degradation products. In the kinetic studies it was found that degradation of midazolam with time, both in the presence and absence of RAMEB, was not linear. The kinetics of degradation could not be fitted to a specific order as Andersin and Tammilehto (1995) succeeded to do in their study of pH-dependent stability. In this study, the initial stages were acceleratory, suggesting autocatalysis by the degradants. Application of the power law made possible the determination of rate constants for the degradation of midazolam. These were found to be 0.137 and 0.154 hr^{-1} in the absence and in the presence of RAMEB, respectively. Thus, RAMEB slightly decreases the photostability of midazolam. The decreased stability was accompanied by the appearance of a degradation intermediate **(15)**. The new degradant has been proposed to be an intermediate of photoproduct **(16)** because the former reached maximum concentration after 7 hrs and then started decreasing to 15 % of its maximum concentration while the latter reached a maximum.

Separation and identification of degradation products was achieved by LC-MS/MS. The two major degradation products that occurred in the presence and absence of RAMEB are proposed to be N-desalkylflunazepam and 6-chloro-2-methyl-4-(2′-fluorophenyl)-quinazoline. These compounds have been reported as photodecomposition products of midazolam (Andersin *et al.*, 1994). Two other photoproducts which occurred only in the presence of RAMEB have not been reported. Both products are of higher molecular mass, 357 and 574, indicating that the former is possibly an addition product of a carbonyl in solution, and the latter a dimer. The dimer is believed to have been formed by self reaction of free radicals formed by the removal of chlorine from a product reported as one of midazolam photodegradants (Andersin *et al.*, 1994), but was not detected in this study. Occurrence of these products in RAMEB solutions is attributed to the

ability of cyclodextrin to provide conformational control and stabilize free radicals.

In conclusion, RAMEB improves the aqueous solubility of midazolam as desired but the introduction of new photoproducts under the irradiation conditions used must be taken into account, and the information generated used in further method development and validation. Suitable packaging could be considered to guard against the appearance of these products in the final formulation. Further work could involve the isolation and structural elucidation of the photodegradation products using a variety of techniques to confirm the proposed structures. In addition further studies could be undertaken to determine the phototoxicity of these photodegradants.

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ANNEXURE A

ATLAS
MATERIAL TESTING SOLUTIONS

Messprotokoll
Protocol of Measurement

Zertifikatnummer *Number of Certificate*	Datum der Kalibration *Date of Calibration*
1002	**10-Nov-00** Gültigkeit maximal 1Jahr *Validity maximum 1Year*

Kunde *Customer*	Rhodes
	Grahamstown
Ansprechpartner *Person in charge*	Mr. D. Morley
Abteilung *Department*	

Gegenstand *Object*	Belichtungs- und Bewitterungsgerät
Hersteller *Manufacturer*	XENOTEST GMBH
Gerätetype *Type*	SUNTEST CPS
Seriennummer *Serial number*	
Service Nummer *Service number*	

Stempel *Stamp*	15-11-00 Datum *Date*	Unterschrift Service *Signature Service*

ATLAS Material Testing Technology GmbH
Vogelsbergstraße 22
D-63589 Linsengericht-Altenhasslau

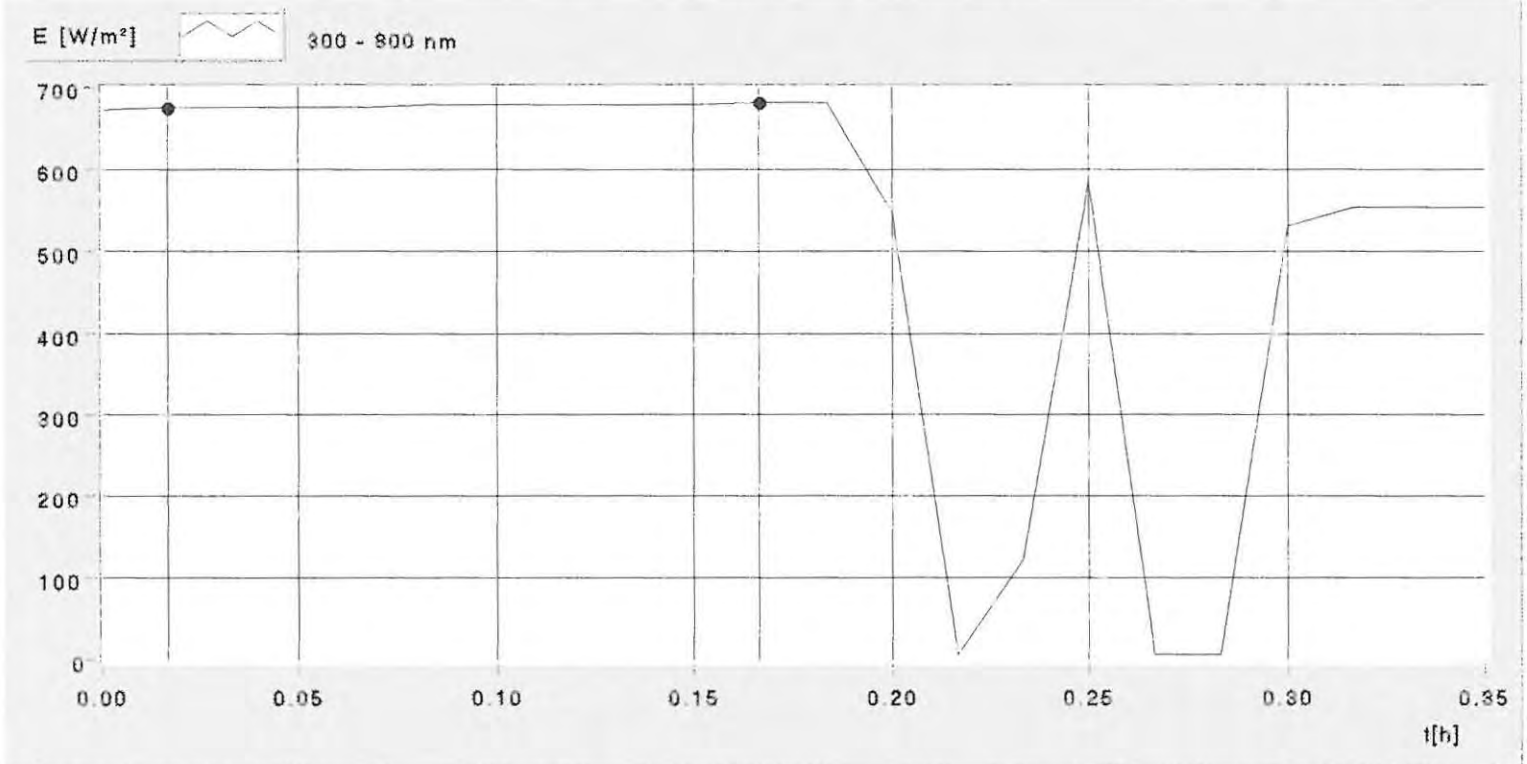
ATLAS
MATERIAL TESTING SOLUTIONS

XenoSoft

Device: Suntest CPS

Filter: G (Solar ID 65)

RHODES00.DAT



Start date: 10.11.00

Start time: 10:25:07

E = 677.19 W/m²

H = 365.5 kJ/m²

t = 0 h 9 min

Geräte Information / *Unit information*

Gerätebetriebsstunden *Operating hours*	
Strahlerbetriebsstunden *Lamp hours*	
Filtersystem *Filter system*	**SOLAR ID 65**
Software-Version *Software*	

1) Labor Werte / *Laboratory Conditions*

Temperatur *Temperature*	Feuchte *Humidity*
21,0 °C	**50,0** %RH

2) Bestrahlungsstärke bei 300-800 nm / *Irradiance at 300-800 nm*

Sollwert *Set value*			Referenzwert aktuell *Reference value current*	Abweichung *Deviation*
550 W/m²			**677** W/m²	**-127** W/m²
W/m²			W/m²	W/m²

3) Probenraumtemperatur / *Chamber Temperature*

Sollwert *Set value*			Referenzwert *Reference value*	Abweichung *Deviation*
°C			°C	°C

4) Schwarzstandardtemperatur / *Black Standard Temperature*

Sollwert *Set value*			Referenzwert aktuell *Reference value current*	Abweichung *Deviation*
40 °C			**44** °C	**-4** °C
°C			°C	°C

Rückführbarkeit / *Traceability*

Nr	Parameter *Parameter*	Sensor Typ *Sensor Type*	Kalibrierschein-Nr. *Number of Certificate*	Gültig *Validity*
2	Bestrahlungsstärke */Irradiance*	Radialux Auswerteeinheit		☐ Ja
		Radialux UV		☐ Ja
		Radialux Global		☐ Ja
		Xenocal 300-400 UV		☐ Ja
		Xenocal 300-800 Global		☒ Ja
4	Schwarzstandardtemp. */BlackStandardTemp.*	SST Sensor		☐ Ja
1+3	Probenraumtemp. */Chamber Temperature*			☐ Ja
1	Probenraumfeuchte */Chamber Relative Humidity*			☐ Ja
3+4	Tauchfühler / *PT 100 for liquids*			☐ Ja

Die Werte wurden gemessen von unserem Servicetechniker Herrn P.Kemp.
The values are measured by our service technician Mr.

Bemerkungen / *Remarks* :

No