A COMPARATIVE PHOTOSTABILITY STUDY OF FOUR PROPYL PIPERAZINE-SUBSTITUTED PHENOTHIAZINES

THESIS

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"... Don't believe what your eyes are telling you. All they show is limitation. Look with your understanding, find out what you already know, and you'll see the way..."

Richard Bach, Jonathan Livingston Seagull a story

ABSTRACT

Four structurally related phenothiazines available in South Africa in a variety of dosage forms and as fine chemicals were investigated to ascertain whether their structural differences in terms of the 2-chloro-/ trifluoromethyl-substituents on the phenothiazine nucleus and the methyl-/ β-hydroxyethyl groups on the piperazine ring accounting for differences in pharmacological activity can be correlated with their photostability[2]. The four propyl piperazine-substituted derivatives are ranked in the following decreasing order of neuroleptic activity: fluphenazine > trifluoperazine > perphenazine > prochlorperazine. In order to assess their photostability an HPLC method was developed and validated for linearity, accuracy and precision, selectivity, limit of detection and quantitation and ruggedness. Preliminary solution photostudies under controlled light conditions (UV, sunlight, fluorescent light) indicated that the rate of degradation followed first-order kinetics with perphenazine the most susceptible to photodegradation under all light conditions studied. *In vitro* and *in vivo* metabolism yielding the 5-sulphoxide and its reported presence on decomposition of the phenothiazines[25] led to the development of a synthetic procedure suitable for the sulphoxides of all four derivatives based on the method proposed by Owens et al. in order to provide standards for comparison in the photostudies.[7] Since ICH regulations require that impurities > 0.1% are examined and identified[74] and semi-preparative isolation of photoproducts proved unsuccessful, LC-MS having been well documented for structural elucidation[74, 75, 76, 77] was used to characterize solution (UV, sunlight, fluorescent light) and preliminary solid (UV) photostudies. The chloro-derivatives underwent dechlorination and sulphoxidation with subsequent photosubstitution in the case of prochlorperazine to yield the 2-hydroxy derivative and sulphoxidation of the dechloro-derivative of perphenazine. The sulphoxides of both trifluoperazine and fluphenazine were formed with further oxidation to the respective sulphones occurring. Preliminary solid state (UV) photostudies showed fluphenazine to be the least stable with 30.71% degradation as opposed to 7.57% for prochlorperazine, 4.28% for perphenazine and 7.10% for trifluoperazine with sulphoxidation observed to be the major degradation pathway. Since *in vitro* metabolism of perazine derivatives is reported to occur *via* *N*-oxidation, *N*-demethylation, sulphoxidation and aromatic hydroxylation[18] it does appear that there is some correlation between metabolic and photoproducts. However the fact that solution (UV) photostudies indicates trifluoperazine to be the most and perphenazine the least stable does not concur with the proposed order of pharmacological activity.

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LIST OF ABBREVIATIONS

The four propyl piperazine-substituted derivatives used in the study are abbreviated as follows:

Prochlorperazine	Prochlorperazine dimaleate
Perphenazine	Perphenazine
Trifluoperazine	Trifluoperazine dihydrochloride
Fluphenazine	Fluphenazine dihydrochloride

BP	British Pharmacopoeia
$CDCl_3$	Chloroform-d_1 (99.5%)
CNS	Central Nervous System
D_2O	Deuterium oxide (99.9%)
frit-FAB LC-MS	frit-Fast Atom Bombardment Liquid Chromatography-Mass Spectrometry
GLC	Gas Liquid Chromatography
HCl	Hydrochloric acid
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation Forum
IR	Infrared Spectroscopy
KBr	Potassium bromide
LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
MeOH-d_4	Methanol-d_4 (99.8%)
MS	Mass Spectrometry

NF	National Formulary
NMR	Nuclear Magnetic Resonance Spectroscopy
TIC	Total Ion Current
RSD	Relative Standard Deviation
USP	United States Pharmacopoeia
UV	Ultraviolet
W	Watts

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CHAPTER 1: INTRODUCTION

The phenothiazines, a class of drugs widely used as neuroleptics in the therapy of schizophrenia, organic psychoses, the manic phase of manic-depressive illness and other acute or chronic idiopathic psychotic illnesses[1] are divided into the aliphatic, piperazine and piperidine subclasses, based on differences in their chemical structure and exhibit different pharmacological actions and potency. The piperazine subclass possesses potent antipsychotic activity with more pronounced extrapyramidal but fewer anticholinergic and sedative effects than the other groups.[2] Four structurally similar, piperazine-substituted phenothiazine derivatives: prochlorperazine, perphenazine, trifluoperazine and fluphenazine are the focus of this investigation. These four derivatives are currently available in South Africa as fine chemicals and in a variety of dosage forms, Table 1.1.[2]

Table 1.1 - Dosage Forms of the Four Piperazine-Substituted Phenothiazine Derivatives.[2]

Phenothiazine Derivative	Tablet	Syrup	Injectable	Spansule	Suppository
Prochlorperazine	✓	✓	✓	✓	✓
Perphenazine	✓		✓		
Trifluoperazine	✓			✓	
Fluphenazine	✓		✓		

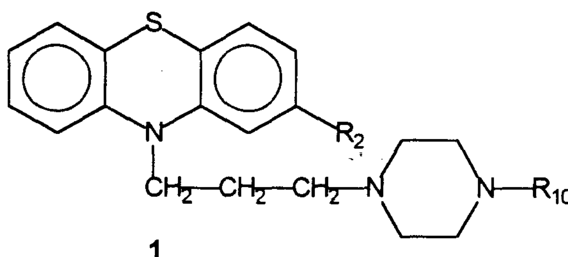

Figure 1.1 - General structure of a piperazine-substituted phenothiazine derivative.

Table 1.2 - Four Piperazine-Substituted Phenothiazine Derivatives.

Phenothiazine Derivative	R_2	R_{10}
Prochlorperazine	-Cl	$-CH_3$
Perphenazine	-Cl	$-CH_2CH_2OH$
Trifluoperazine	$-CF_3$	$-CH_3$
Fluphenazine	$-CF_3$	$-CH_2CH_2OH$

It has been reported that the therapeutic action of the phenothiazine drugs involves blockade of dopamine receptors in the brain[3, 4] with the interaction of the side chain and the R_2 substituent(s) enhancing the ability of these phenothiazines to mimic the preferred *trans alpha* conformation of dopamine. Structure activity relationships have indicated that addition of a piperazine ring onto the three carbon side chain increases neuroleptic activity over that of the aliphatic subclass. This is due to the fact that the additional nitrogen atom in the side chain provides a further point of interaction with the R_2 substituent(s).

When the R_{10} substituent is a β-hydroxyethyl group interaction with the R_2 substituent is enhanced as compared to a methyl group in this position. Thus while the chloro-derivatives, prochlorperazine and perphenazine allow for a single point of interaction, this is not the case with the trifluoromethyl-derivatives, trifluoperazine and fluphenazine, explaining the increased neuroleptic potency of these latter two derivatives. Based on all the above information, fluphenazine can thus be assumed to display the greatest neuroleptic potency of all four derivatives under discussion.

While chlorpromazine is considered the prototype for many neuroleptic drugs in clinical use today,[1] patients frequently suffer adverse effects. Low dosages may cause mild photosensitivity with the development of a form of dermatitis on parts of the skin that are exposed to sunlight, while a higher dosage and more prolonged treatment can produce severe dermatitis. Such patients may also suffer damage to the retina, ocular opacity and loss of

vision.[5, 6]

On irradiation of chlorpromazine with light, toxic photoproducts, which have the ability to react with cell constituents and thus cause these adverse effects, are formed.[6] The chlorine group in the 2-position is proposed to cause these photosensitivity effects with resulting implications for the two chloro-derivatives, prochlorperazine and perphenazine under investigation.

The photoionization of chlorpromazine leads to the formation of the cation radical and electron, and subsequent reaction of the cation radical with oxygen to result in sulphoxidation.[8] These phenothiazine sulphoxides have demonstrated negligible dopamine receptor binding and therefore do not contribute to the antipsychotic activity[7] but only to the adverse effects of these drugs. Oxidation of the phenothiazines occurs readily, but is dependent on the nature of the R_{10} substituent, and therefore is facilitated by the presence of a β-hydroxyethyl group. This in turn, has implications for the stability of perphenazine and fluphenazine.

While structural modifications of the prototype drug molecule, chlorpromazine, have introduced compounds with greater neuroleptic potency, it is important to ascertain whether these modifications have had an impact on the stability of the resultant compounds.

In spite of the structural similarities of these four piperazine-substituted derivatives in terms of the R_2 and R_{10} substituents, no evidence exists in the literature of their comparative photostability. Between 1980 and 1985, the 10-substituted phenothiazine derivatives constituted one of the largest drug classes in the official monographs in the *British Pharmacopoeia* (*BP*) and the *United States Pharmacopoeia* (*USP*).[8] Although these statistics may not apply in 1997, the requirements of regulatory authorities for more detailed information on stability profiles and the nature of degradants, together with the movement of the International Conference on Harmonisation forum (ICH) towards standardizing light stability

studies, renders the phenothiazines an important drug class. This information has motivated the study which is to investigate the comparative photostability of these four phenothiazines and to make mention of a relationship, if any, to their activity.

In order to achieve this aim the introduction provides a brief overview of the structural similarities of these derivatives related to their neuroleptic potency, stability and resulting photosensitizing ability. To investigate their relative stability, a high performance liquid chromatography (HPLC) method has been developed and validated. This method is required to be accurate and precise, provide a suitable range over which accurate and precise measurements may be taken, allow for day-to-day variability, and reflect the system's ability to resolve degradation products from the drugs. This detailed report on the development and validation of the method is provided in chapter 2. A complete profile of the derivatives under investigation is given in chapter 3, together with some preliminary photodegradation studies. The poor stability of the phenothiazine series results in the degradation of these compounds *in vitro* to similar *in vivo* metabolites, with a major degradant or metabolite identified in both cases to be the 5-sulphoxide. This is significant not only due to the lack of activity of this compound in terms of antipsychotic effects, but also due to its role in the adverse effects associated with these drugs, although this effect does appear to be concentration dependent. This, together with the frequent occurrence of the sulphoxides in degraded samples, led to the need to develop a synthetic method in order to provide standards for comparison in the photostudies. The synthetic procedure and subsequent structural elucidation of the resultant sulphoxides is contained in chapter 4. The availability of these derivatives in a variety of liquid dosage forms, tablets, suppositories, and as fine chemicals, prompted an investigation into the photostability of both the liquid and solid state, under normal and accelerated storage conditions. Chapter 5 contains information on the degradants present in the samples studied, which, together with results from chapter 3, allows some conclusions to be made in chapter 6 on their comparative photostability.

1.1 Structure Activity Relationships (SAR)

Structure activity describes the relationships between molecular structure or physical properties and activity and the effect of this structure on the interaction of the drug with the relevant receptor(s). Approaches to structure activity relationships fall into two broad categories. The first encompasses theoretical methods in which the actual structure or structural characteristics are examined and quantified leading to a true structure activity relationship when coupled with biological evidence. The second category attempts to find parallel trends between the biological activities and physicochemical properties of a series of molecules. Although this may not strictly correspond to a quantitative structure activity relationship (QSAR) method, it facilitates predictions for further drug synthesis.[9, 10] Quantitative structure activity relationships involve the ability to differentiate between and quantify the contributions of drug structure or property to drug activity. If such a differentiation can be accomplished, a novel drug structure can then be proposed and by a process of integration, its properties and activity can be predicted. However, several limitations arise in the prediction of the activity of a new drug on the basis of a single quantititative structure activity relationship. These limitations result from the fact that a drug structure has to be optimized for so many facets of its action. Thus a quantitative structure activity relationship can provide a correlation to the structure activity information, which is a useful tool in drug design.[10]

A widely used theoretical method based on the work of Hansch considers relationships between biological activity and common physical properties namely lipid solubility, degree of ionization or molecular size. Hansch proposed that these properties can be measured, correlations derived, and hence biological activity explained in terms of a physical model.[9] In an attempt to explain correlations of physical properties of compounds with biological activity, an octanol-water system was developed to allow the measurement of the preference of drugs for the hydrophilic or lipophilic phase.

This preference is described by Hansch as the partition coefficient, P. The octanol-water system, however, has certain limitations for the accurate measurement of partition coefficients of ionizable compounds, molecules with extreme values of log P and compounds with poor ultraviolet absorption. Hansch went on to standardize the parameter for the phase distribution relationship, as log P or π.[10]

In equation 1.1, π represents the substituent constant for a group X replacing the hydrogen, H. The measured quantities are partition coefficients of the derivative P_X and parent P_H, thus π is a measure of the contribution of the substituent to the lipid solubility of the molecule, with π values greater than zero indicating that the substituent contributes to an increase in the lipophilicity of the molecule with the reverse applying for values less than zero.[10]

$$\log (P_X/P_H) = \pi \qquad \text{Equation (1.1)}$$

It must be appreciated that a drug molecule, to be effective, needs to possess certain physical and chemical characteristics which will enable it to reach the relevant receptor. Hansch explains that molecules which are highly hydrophilic will not readily partition from water into the lipid of a membrane. If the receptor is within or beyond that membrane, such a molecule will have a low probability of reaching it in the time interval under study. Conversely, molecules which are highly hydrophobic will readily partition into the first of a series of lipid membranes but will be held there and thus slowed down in any journey to a remote site of action, with such a journey being termed the "random walk".[10] Thus the idea developed that for any particular receptor, some optimum value of log P would be found to correspond to the maximum probability of reaching the receptor in a given time with this optimum log P value for a compound being largely a function of the biological system.

It has been shown that the penetration of neutral compounds into the central nervous system (CNS) is most favourable for those compounds having a log *P* of about 2. This needs to be taken into account in the design of drugs acting on the CNS.[9]

Once at the site of action, the shape of the molecule becomes a critical factor in determining the biological activity of the compound. In order for a compound to evoke a maximal response, or indeed any response at all, an interaction with the drug molecule and relevant biological receptor is essential. For "flexible" drugs, however, the emphasis is on the determination of the preferred conformation of the drug molecules. Since the therapeutic action of the phenothiazine derivatives involves blockade of dopamine receptors in the brain,[3,4] lipid solubility is the initial important consideration to allow passage through the blood brain barrier and precludes the facile penetration of ionized molecules. A further consideration is thus the ability of the molecules to mimic the shape of the dopamine receptor.

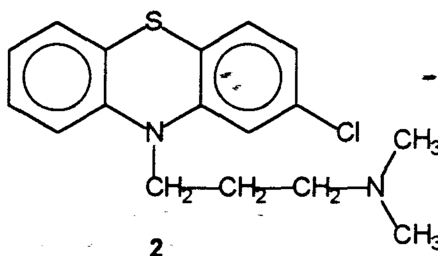

Figure 1.2 - Structure of phenothiazine prototype, chlorpromazine.

Chlorpromazine is recognised as the prototype for the many neuroleptic drugs in clinical use today, because of the two benzene rings can be predicted to be sufficiently lipid soluble to penetrate the brain. Chlorpromazine is a weak base (pK_a 9.2) and at physiological pH 7.4 is 98.4% ionized. The high lipid solubility of the unionized form of the drug (1.6%), however, causes the preferential partitioning into lipids rather than aqueous media, with the presence of the chlorine substituent on the benzene ring further enhancing lipid solubility.[1]

A characteristic feature of the phenothiazines is that they have a non-polar tricyclic nucleus that is bridged by a hetero atom and a substituent at the 2-position.[4] The two benzene rings contained in the phenothiazine nucleus of prochlorperazine, perphenazine, trifluoperazine and fluphenazine contribute significantly to the lipophilic properties of these molecules. It is evident from the pK_a values, Table 1.3, that these derivatives are extensively ionized at physiological pH. Comparisons of the pK_a values of these derivatives with that of chlorpromazine, suggest them to be stronger bases, with a lower percentage of drug in the ionized form, thus displaying a greater partitioning into lipid media.

Table 1.3 - pK_a Values.[11, 12, 61]

Phenothiazine Derivative	pK_a
Prochlorperazine	3.7, 8.1
Perphenazine	3.7, 7.8
Trifluoperazine	3.9, 8.1
Fluphenazine	3.9, 7.8

pK_a Values obtained for the piperazine derivatives are as a result of the dibasic nature of the piperazine ring. While the propyl side chain connecting the nitrogen of the phenothiazine ring and the more basic side chain nitrogen has been reported to contribute to neuroleptic potency, Green reports the nature of the side chain to be a major factor affecting the ionization constants of these compounds. The electron-withdrawing properties of the substituent on the terminal nitrogen atom further affects the ionization constants of these compounds where the stronger the electron-withdrawing character of the substituent, the lower the respective pK_a value. The effect of the 2-substituent on the pK_a of the compound has been reported to be minimal.[11]

The fact that the substituent at the 2-position on the phenothiazine nucleus has been implicated in affecting the lipophilicity of the molecules is supported by the physical model proposed by Hansch, where the substituent constant, π, is a measure of the contribution of

the substituent to the lipophilicity of the molecule.[10] Table 1.4 contains π values for substituents attached on the aromatic ring system. These values may be applied to the four derivatives under investigation, due to the presence of the chloro or trifluoromethyl group at the 2-position on the phenothiazine nucleus.

Table 1.4 - π Values for Aromatic Ring Substituents.

Substituent	π
- CF_3	1.07
- Cl	0.76
- OH	-0.67

The data in Table 1.4 confirm that the trifluoromethyl group at the 2-position on the phenothiazine ring in trifluoperazine and fluphenazine, has a greater contribution to the lipophilicity of the compounds, as compared to that of the chloro group at this position.

The phenothiazines constitute a class of drugs exhibiting action at biological membranes and this has resulted in an investigation into whether this surface activity has an influence on biological activity.

The surface activity, although not solely responsible for pharmacological activity, does reflect the hydrophobicity of the molecule. In a study by Zographi et al. to investigate surface tension as a means of observing hydrophobic behaviour, chlorpromazine was again used as the standard, and the ratio of drug concentration required to produce a required surface pressure to chlorpromazine concentration required to do this was calculated. Prochlorperazine and trifluoperazine were two derivatives considered in this investigation. Since values less than 1 indicate greater surface activity than chlorpromazine, values of 0.58 for prochlorperazine and 0.12 for trifluoperazine confirmed greater surface activity for these two compounds. These values highlight the significant hydrophobic effect of the trifluoromethyl group over that of the chloro group, where no other structural modifications have been made. Comparisons

of chlorpromazine which has a value of 1, with prochlorperazine, 0.58, illustrates the influence of adding a piperazine ring onto the propyl side chain, presumably due to the presence of additional carbons.

It is thus evident that surface activity of the phenothiazine derivatives is influenced by the nature of the substituent at the 2-position on the phenothiazine nucleus, with such activity being proportional to the electron-withdrawing character of the substituent, provided it has no ionic character. The trifluoromethyl group has thus been observed to have a greater activity as compared to a chloro group due to the substituent hydrophobic effects.[12]

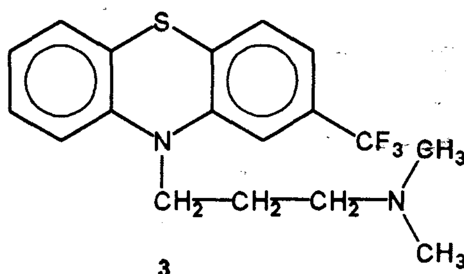

Figure 1.3 - Phenothiazine derivative, triflupromazine.

Based on the relationship of hydrophobicity and activity of the phenothiazines, Nightindale et al. in a simple and inexpensive biological test system (death measurement of gold fish) demonstrated the correlation of pharmacological effect with the physicochemical properties of phenothiazines and report the role of the absorptive process in modifying such a response.

Apparent partition coefficients between water and dodecane for various phenothiazine derivatives were determined at pH 5.3 due to complete ionization at this value. These values for triflupromazine **3** and chlorpromazine show the same rank order relationship as in the surface pressure determinations for these compounds i.e. triflupromazine > chlorpromazine. While time of death determinations indicate that the greater the surface activity or partition

coefficient, the greater the toxicity, only the free base of the phenothiazines partitions into dodecane, while both the free base and the ionic species partition into octanol. Since dodecane partition coefficients correlate with the times of death of the goldfish it appears that the free base is being absorbed. It would appear, therefore, that these partition coefficients are more effective in predicting phenothiazine absorption efficiency than π values or octanol partition coefficients. These data suggest a relationship between phenothiazine absorption and hydrophobicity.[13]

The idea of "dissecting" drug activity into physical contributions (hydrophobic, electronic, steric) is the central theme of the Hansch approach, and emphasises the importance of the relationship of hydrophobicity to activity. It can therefore be concluded that the trifluoromethyl-derivatives, trifluoperazine and fluphenazine are more active than the chloro-derivatives, prochlorperazine and perphenazine, based on their structure activity relationships.

1.2 Mechanism of Action

The mechanism of action of the phenothiazine drugs involving blockade of dopamine receptors in the brain is by virtue of their ability to mimic the *trans alpha* conformation of dopamine. The use of a dopamine sensitive adenylate cyclase enzyme system in the rat corpus striatum and other dopamine rich areas of the brain made it possible to examine the effects of various dopaminergic agonists and antagonists on the system, thus providing a useful *in vitro* model of the dopamine receptor. A competitive interaction was found in the studies on the influence of the dopamine sensitive adenylate cyclase enzyme system and in direct receptor binding studies.[4]

The X-ray structures of chlorpromazine and dopamine have been studied as the key to explaining the phenothiazine blockade of the dopamine receptor. Dopamine is a flexible molecule with the *trans* and the two *gauche* conformers being the most important

preferred form in the solid state, solution and at the receptor, Figure 1.4. [4]

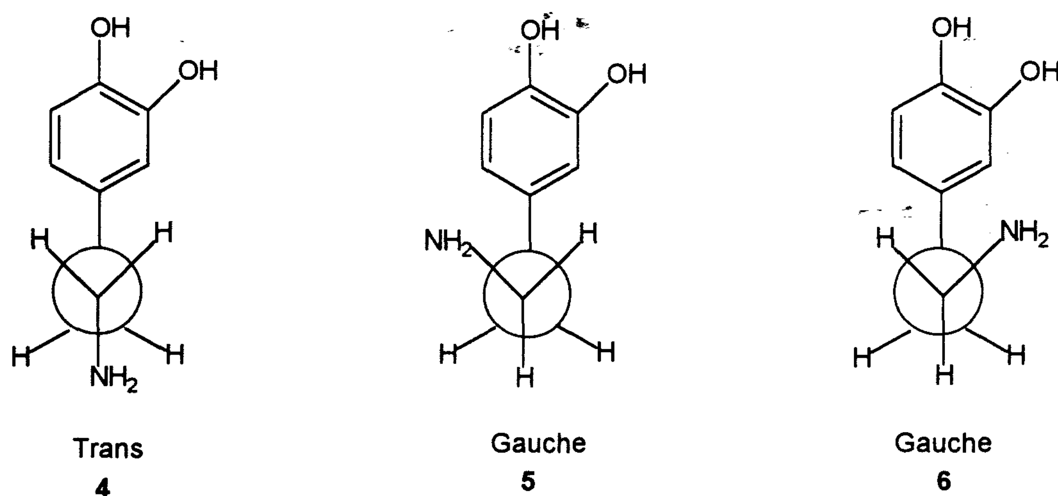

Figure 1.4 - Newman projections of the conformations of dopamine.[4]

In the preferred conformation of dopamine, the distance of the nitrogen atom from the centre of the catechol ring was determined by Horn et al. to be 5.14 Å. It is thus suggested that the binding site for dopamine's amine group at its receptor is about 5.1 Å distant from the centre of the aromatic ring.

Examination of the X-ray structures of compounds provided information about structural and conformational requirements for effective dopamine antagonism. Data for chlorpromazine illustrate values for distances from each aromatic ring to the nitrogen atom in the side chain where A - and B - N_1 are 6.70, and 6.18 Å respectively. Values obtained for triflupromazine are 6.38, and 6.42 Å for A - and B - N_1 respectively, Figure 1.5. In the case of the piperazine derivatives, it is reported that the distances of the nitrogen atoms of the piperazine ring from the centres of the aromatic rings, are more or less constant, and correspond closely to those occurring at the dopamine receptor.[4]

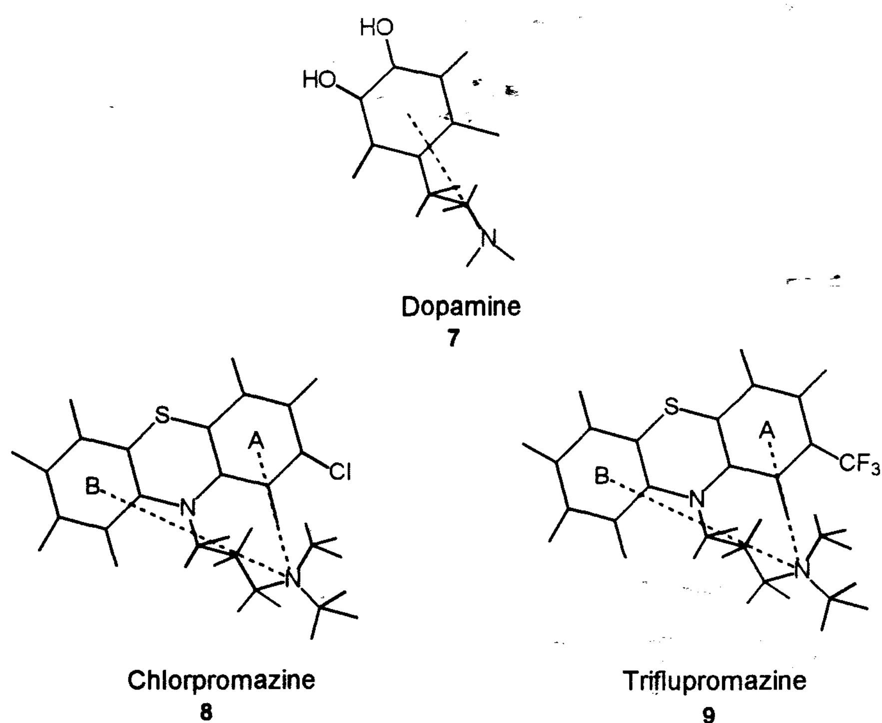

Figure 1.5 - Conformations of dopamine, chlorpromazine and triflupromazine.

The slight differences observed in the A - and B - N_1 dopamine distance for chlorpromazine, indicate that either the difference is compatible with effective binding or that chlorpromazine undergoes a conformational change on binding to the receptor, due to its flexible nature. This is illustrated by the partial superimposition of chlorpromazine and the preferred conformation of dopamine in Figure 1.6.[9]

Horn et al. report that a more or less correct interatomic distance of about 6 Å for the amine nitrogen atom from the centre of one aromatic ring is not sufficient for activity and that in addition to this requirement, the compound must also possess a non-planar ring system.[4]

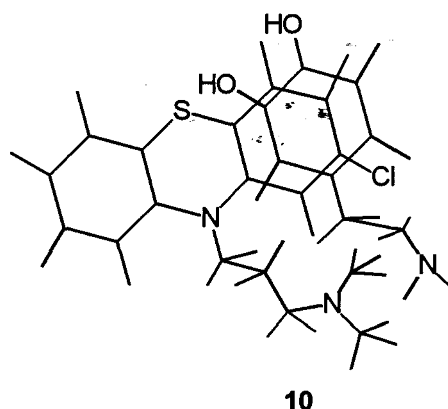

Figure 1.6 - The superimposition of chlorpromazine and dopamine.[9]

It has been postulated that the phenothiazines interact with the dopamine receptor at three distinct sites, A, B, and C, to produce a neuroleptic response, Figure 1.7. The highest degree of structural specificity is required at site B, followed by sites C and A. As a certain degree of free rotation is necessary for the side chain, the structural specificity of the second carbon atom in the side chain is important. The introduction of a methyl group or ring system at this point results in a loss or decrease in neuroleptic activity, due to the bulky nature of these groups. The presence of the amino side chain and the inclusion of substituents at the 2-position on the phenothiazine nucleus increase potency further, possibly due to the receptor effects of these groups, rather than any strictly conformational effects on the drug itself.[9]

The folding of the tricyclic ring system along the S-N axis has been indicated in the phenothiazines, to be responsible for the conformation of the side chain, which suggests that this may be indirectly affected by the 2-substituent on the phenothiazine nucleus.[9] This folding results in the 2-substituent being too far from the receptor to exert any steric influence. The increased neuroleptic potency as a result of the 2-substituent is proportionally related to its electron-withdrawing property, provided it has no ionic character. Where a phenothiazine derivative has been reported without a substituent at this position, a weak dopamine antagonistic effect has been observed. This is demonstrated by the activity of chlorpromazine

which is 20 times more potent than that of promazine where the 2-chloro substituent is absent.[3]

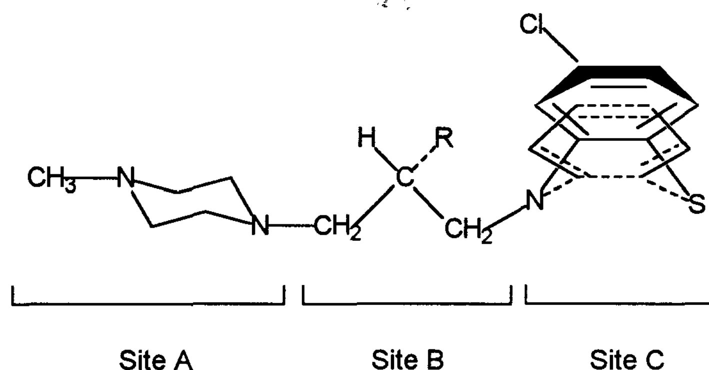

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Figure 1.7 - Preferred conformation of prochlorperazine at the neuroleptic receptor surface.[9]

As previously mentioned, the trifluoromethyl group of trifluoperazine and fluphenazine, provides a greater potential for Van der Waals interaction than the single point interaction resulting from the chloro group in prochlorperazine and perphenazine. In Figure 1.5, the distance of the side chain nitrogen atom from the geometric centre of ring A in the tricyclic system supports this statement, as a smaller distance between ring A and the side chain nitrogen is evident for triflupromazine as opposed to that observed for chlorpromazine. Based on this observation, the trifluoromethyl-derivatives display greater neuroleptic potency than the chloro-derivatives.

Structural specificity at site A is important in the transverse direction only as the molecule must fit into a "narrow slot". Due to the free rotation of bulky groups and resulting steric interference, the size of the *N*-substituents must be considered. The piperazine ring of prochlorperazine for example, allows a better fit into the "narrow slot" than the two methyl groups attached to the propylamino side chain of chlorpromazine further explaining its increased neuroleptic potency. In addition to the contribution of the piperazine ring to

interaction with the 2-substituent, the presence of a β-hydroxyethyl group in fluphenazine as opposed to a methyl group in trifluoperazine has led to a four fold increase in potency as previously mentioned in the structure activity relationship studies.[3] The presence of the side chain and R_2 substituents allow the phenothiazine molecule to mimic the preferred conformation of dopamine by interaction with each other.[9]

Blockade of the dopamine receptor by the phenothiazine derivatives, although necessary for their therapeutic activity, results in the development of the prominent extrapyramidal side effects associated with this group of drugs. Thus the mechanism of action can be explained not only in terms of interaction with the dopamine receptor and the relative activity but by also taking into consideration basic structure activity relationship principles and hydrophobicity values. This results in the phenothiazine derivatives under investigation being ranked in decreasing order of activity as follows: fluphenazine, trifluoperazine, perphenazine and prochlorperazine.

1.3 General Pharmacokinetics

The absorption and fate of the phenothiazines can be explained in terms of the prototype, chlorpromazine, which is readily absorbed from the gastrointestinal tract even though considerable first-pass metabolism occurs in the gut wall. Chlorpromazine is widely distributed in the body and across the blood brain barrier to achieve higher concentrations in the brain than in plasma, extensively metabolised in the liver and excreted in the urine and faeces.[14]

Doluisio et al. report a method for studying gastrointestinal prochlorperazine absorption from isolated gut segments of the anaesthetised rat *in situ*. The principal advantage of this technique is that it provides absorption rates which are comparable to those calculated from blood concentration data following oral drug administration to humans and intact animals.

The average half-life for prochlorperazine absorption from the rat gut lumen (pH 6) is 23 minutes and from the rat gastric lumen (pH 3), greater than 350 minutes. These findings can be explained in terms of a greater fraction of the weakly basic drug present in the lipid insoluble ionized form at the lower pH. However, a shift in the pH of the gastric lumen to 6 resulted in a half-life for prochlorperazine of 111 minutes, reflecting the contribution of the relative surface areas and physiological organisations of the two sites as well as the relative surface to volume ratio. This method is advantageous in that it allows the determination of absorption rates from various segments of the gastrointestinal tract and the opportunity to study various realistic aspects of prochlorperazine absorption.[15]

Prochlorperazine, in common with other phenothiazines, is available for intramuscular and oral administration but exhibits low oral bioavailability. In an attempt to avoid these problems, Hessell et al. report a comparative study undertaken of the bioavailability of prochlorperazine following oral and buccal administration. Because of the low concentrations involved, the definitive assay of prochlorperazine plasma levels following a single oral dose was difficult, however, it allowed predictions of multi-dose steady-state plasma levels. Results indicated that 3 mg buccal prochlorperazine maleate in a twice daily dosage regimen would be equivalent to the standard 5 mg oral dose given three times daily.[16] In the case of fluphenazine, although elimination half-lives of intramuscular and oral administrations were similar, plasma concentrations after oral fluphenazine were less than those after intramuscular injections. A higher peak concentration at an earlier time after oral administration indicated the lower fluphenazine plasma concentrations to be due to "first pass" metabolism rather than poor or slow absorption.[17]

1.3.1 Metabolism

In a study on the *in vitro* metabolism of perazine, liver microsomes from male rats were primarily used as an enzyme source and the metabolites formed were the products of *N*-

oxidation, *N*-demethylation, sulphoxidation and aromatic hydroxylation of the perazine molecule. These metabolites corresponded to those found in human urine. The *N*-oxidation proceeds linearly over a time interval of at least 15 minutes, whereas the rate of demethylation, sulphoxidation and aromatic hydroxylation decreases already after 5 minutes.[18]

Scheme 1.1[19]

Although the metabolite pattern found corresponds qualitatively to that of chlorpromazine, quantitatively however, there are certain differences in that the high capacity for *N*-oxide formation from perazine seems remarkable. When comparing these *in vitro* studies with those obtained by analysing urinary excretion patterns in humans and rats it appears that aromatic hydroxylation and sulphoxidation play a minor role in microsomal systems and give rise to

major urinary metabolites in the living organism. This could be explained by the fact that phenolic metabolites *in vivo* are rapidly conjugated with glucuronic acid and the resulting conjugates and sulphoxides are excreted. Desmethylperazine on the other hand is lipophilic enough to be adsorbed and is subject to further metabolism. Whether perazine *N*-oxide is reduced *in vivo* is unsure. There are various mechanisms proposed for this reduction similar to that of nicotine *N*-oxide by intestinal flora. Such reaction cycles would explain the amount of *N*-oxide available for excretion decreasing.[18]

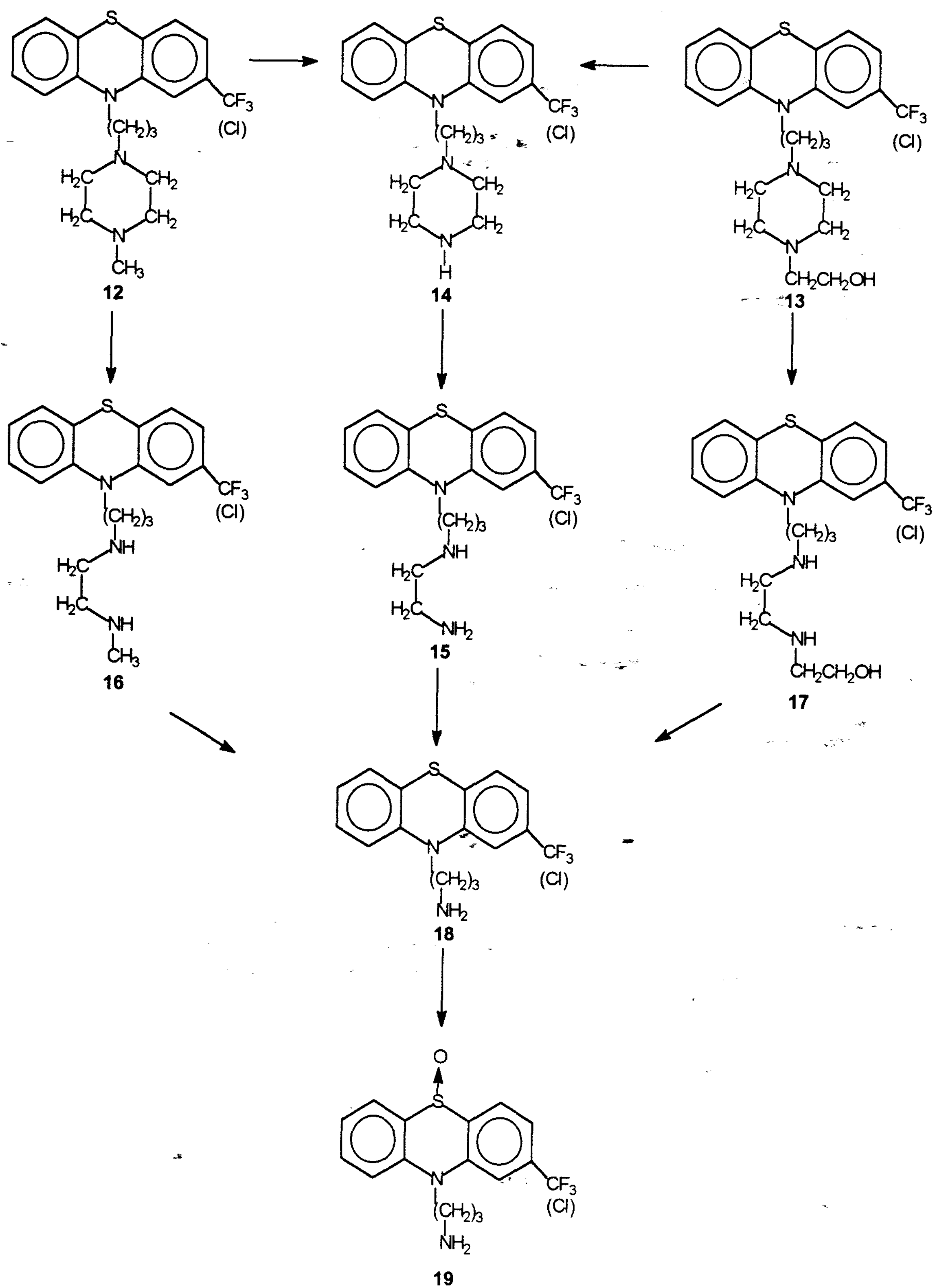
The fact that until recently, biotransformation reactions on the piperazine ring were not known, is not surprising, since as this ring does not occur in nature and thus it is unlikely that the body would break down or use such a ring structure. However, when considering piperazine-substituted phenothiazines, in addition to the sulphoxides, *N*-oxides and phenols formed, a major biotransformation reaction is the removal of the methyl or β-hydroxyethyl group from the terminal nitrogen atom as seen in Scheme 1.1.[19]

However, this ring cleavage can also occur with retention of the methyl or β-hydroxyethyl group on the terminal nitrogen atom. *In vivo* studies show that the piperazine ring is further degraded to yield ethylene-diamine derivatives. Complete removal of the alkyl bridges connecting the two nitrogen atoms of the ring will result in the formation of γ-(phenothiazinyl-10)-propylamine, Scheme 1.2, and its 2-substituted analogues **18**. These compounds have been described as *in vivo* metabolites of the dimethylamino-substituted phenothiazine drugs. In all four relevant piperazine-substituted phenothiazine drugs, degradation of the piperazine ring gave rise to **15**, which was excreted in the urine as the sulphoxide **19**. Also following administration of intermediates **16** and **17**, the sulphoxide **19** was obtained as a urinary metabolite. Identical products resulted from demethylation and sulphoxidation of the corresponding dimethylamino-substituted phenothiazines. These results show that *in vivo* common metabolites occur from two major classes of phenothiazine drugs i.e. those carrying

a dimethylamino group and those containing a piperazine ring in the side chain. Thus the reaction sequence by which the piperazine ring is degraded occurs irrespective of whether the substituent on the ring is methyl (perazine, prochlorperazine, and trifluoperazine) or β-hydroxyethyl (fluphenazine and perphenazine), and it is observed in all three series with different substituents in the 2-position on the phenothiazine nucleus (H, CF_3, and Cl). This piperazine ring cleavage assumed to proceed *via* two consecutive *N*-dealkylations is a minor pathway in the total biotransformation in the rat due to rapid aromatic hydroxylation which could not be demonstrated *in vitro.* These ethylene-diamine derivatives need to be taken into account in long-term therapy due to their tendency to accumulate and their presence in a concentration equal to or higher than that of the parent drug and dealkylation products. Even though their pharmacological activity has not been proven at this stage it is possible that these metabolites can serve as precursors for biodegradation of phenothiazinyl propylamines, which as previously mentioned are common to the dimethylamino- and piperazine-substituted phenothiazine drugs.[19]

The concept of rapid aromatic hydroxylation is supported in the results achieved in a study in dogs and rhesus monkeys where on administration of fluphenazine, sulphoxidation and hydroxylation occurred. Similar results were achieved in the case of chlorpromazine, with hydroxylation occurring in the dog and man primarily at the 7- and to a lesser extent at the 3-position on the phenothiazine nucleus.[20]

In a study of fluphenazine kinetics in man, fluphenazine, 7-hydroxy fluphenazine and fluphenazine sulphoxide in the urine were mainly conjugated. This conversion of fluphenazine to a conjugate(s) allows an indirect assessment of its kinetics, and arises by combination of the alcoholic group with the conjugating molecule(s). This approach has application to perphenazine due to its ability to be transformed directly into a water soluble conjugate.[21] Various analytical methods have been developed to quantify trifluoperazine and its



Scheme 1.2[22]

metabolites in humans and rats.[21]

In a study undertaken by Breyer et al. a thin layer chromatographic separation showed that the "parent drug" fraction consisted to a large percentage of the demethylated metabolite, particularly when obtained from the liver. The sulphoxide is only present in very small quantities in the extract from the lung and kidney and represents a minor part of the total biotransformation products in tissues. Preliminary data from rats sacrificed between 0.5 and 6 hours after an intraperitoneal injection points to a steady decline of drug levels in orally treated rats confirming the idea of first-pass elimination being predominantly hepatic.[22, 23]

In a study conducted by Huang et al. on albino rats injected intraperitoneally with trifluoperazine, the sulphoxide was found to be the major non-polar metabolite in the urine with only trace amounts of the unchanged drug detected. An investigation into the other metabolites indicated a considerable presence of polar metabolites speculated to be hydroxides and / or glucuronides of trifluoperazine.[24] In another study the 24-hour and timed interval urinary excretion of trifluoperazine and its metabolites from mental patients was determined. Results concurred with those found by Huang et al. in that unchanged trifluoperazine accounted for less than 1% of the dose indicating extensive metabolism of the drug with the sulphoxide as 3.6% of the total dose, and the ratio of sulphoxide to drug, being 7.

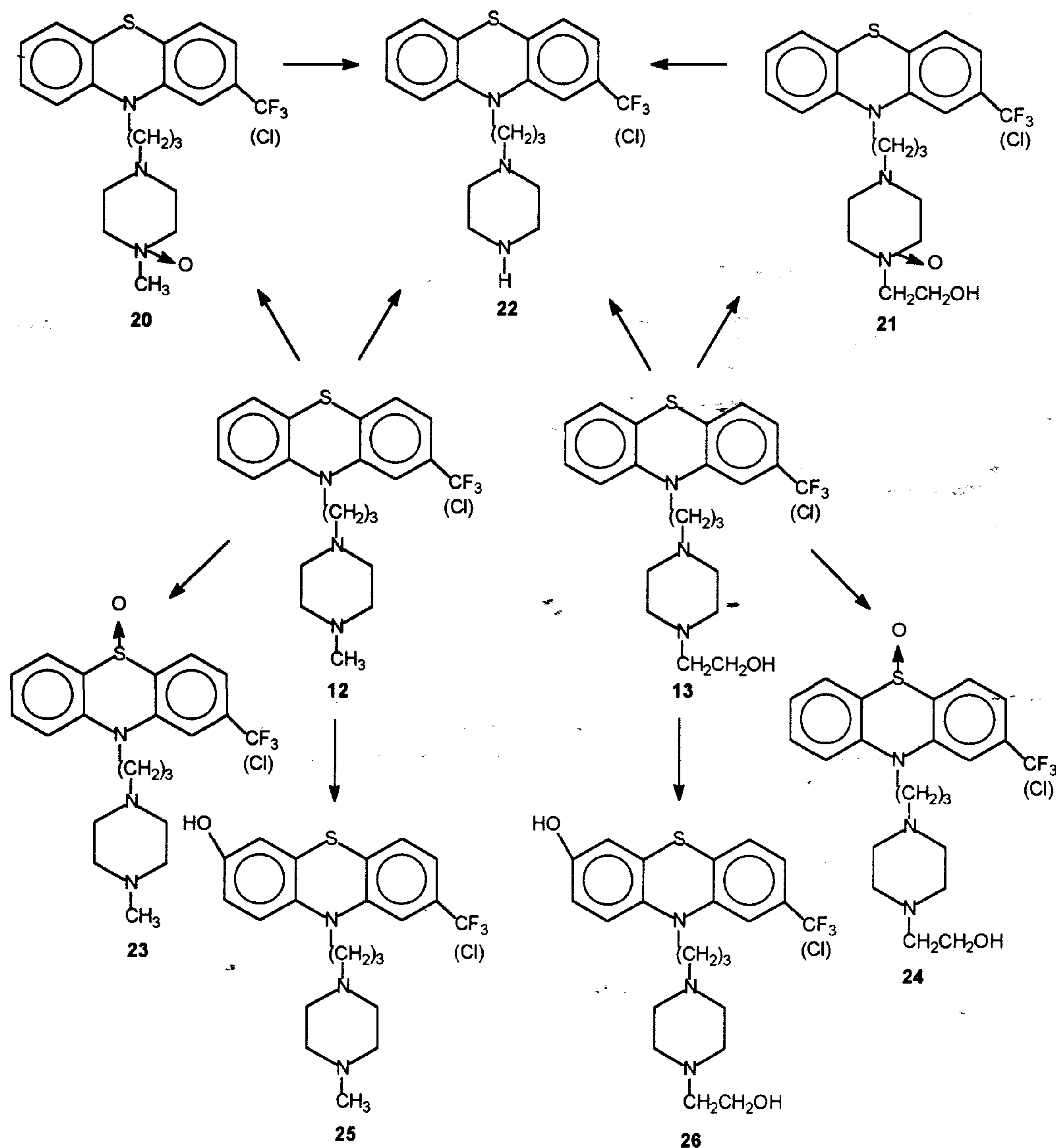
The biotransformations undergone when a wide range of medicinally used phenothiazines, including trifluoperazine, prochlorperazine and fluphenazine were incubated with a "microsomal and soluble" fraction of livers from albino Wistar rats have been described. The purpose was to determine whether the predominant pharmacological action of a given compound might be related to observed biotransformations. Prochlorperazine and trifluoperazine gave a positive reaction for the presence of a secondary amine which could arise from demethylation of the methyl piperazinyl group or by opening of the piperazine ring.

Since the sulphoxides were not detected, it is not likely that sulphoxidation precedes hydroxylation. These results confirmed the same hepatic biotransformation pathway for all these phenothiazines, with no correlation between chemical structure and pharmacological activity on this basis.[25]

Gaertner, Breyer and Liomin have reported the major *in vitro* pathways of phenothiazine metabolism to be *N*-dealkylation, *N*-oxidation, sulphoxidation and hydroxylation of the aromatic ring system. In a study undertaken on piperazine-substituted phenothiazine drugs, Scheme 1.3 represents the structures of phenothiazine drugs investigated and their *in vitro* metabolites. The sulphoxides of the drugs were identified by comparison of their UV spectra with those of known standards and the structures of the *N*-dealkylated products, the dealkylated sulphoxide and *N*-oxides confirmed. The phenolic metabolites could only be tentatively identified as the 7-hydroxy derivatives of the drugs.[25] In the case of 7-hydroxy fluphenazine the structure could be assigned based on work done by Dreyfuss et al.[20] When considering the rate of these *in vitro* transformations, it appears that the concentration of the substrate is important, with *N*-oxidation enhanced and demethylation and sulphoxidation retarded with an increase in the prochlorperazine concentration above 0.5 mM. Demethylation of trifluoperazine and prochlorperazine, was faster than removal of the β-hydroxyethyl group from fluphenazine and perphenazine. Formation of the sulphoxides and aromatic hydroxylation proved to be slow reactions *in vitro*.[25]

In vivo and *in vitro* findings indicate that there is good agreement in regard of the dealkylated fraction. *In vivo* findings for trifluoperazine and prochlorperazine yielded urinary sulphoxides in the form of secondary amines as opposed to the sulphoxides of the undegraded drugs of fluphenazine and perphenazine which correlated with the *in vitro* findings that the *N*-methyl group is cleaved faster than the β-hydroxyethyl group. An increase in the dose of prochlorperazine leading to an increased percentage excreted in the form of the *N*-oxide gave

further evidence of the correlation of *in vivo* and *in vitro* findings.[25] An in depth knowledge of the piperazine-substituted phenothiazine metabolites may provide insight into the identification and characterization of the degradants formed in *in vitro* photostability studies to be performed on these four compounds, chapter 5, since there is often some similarity in metabolic and degradation products.



Scheme 1.3[25]

1.4 Clinical Uses

The clinical activity of the four phenothiazines, prochlorperazine, trifluoperazine, perphenazine and fluphenazine is similar to those of the prototype, chlorpromazine. Chlorpromazine possesses sedative and tranquillising properties with anti-emetic, antipruritic, serotonin-blocking, weak antihistaminic properties and slight ganglion blocking activity. Chlorpromazine, an analgesic and skeletal muscle relaxant, is widely used in the management of psychotic conditions, to control excitement, agitation, reduce the manic phase of manic-depressive conditions, and in schizophrenic patients.[26] The piperazines are potent antipsychotics with more pronounced extrapyramidal effects but fewer anticholinergic effects and are less sedative than the other groups of phenothiazines: aliphatic and piperidine.[2] The clinical uses of the four phenothiazines are tabulated in Table 1.5 below.[26]

Table 1.5 - Clinical Uses of the Four Piperazine-Substituted Phenothiazine Derivatives.[26]

Symptom	Prochlorperazine	Perphenazine	Trifluoperazine	Fluphenazine
Psychoses	✓	✓	✓	✓
Nausea and vomiting	✓	✓	✓	
Vertigo, including that due to Meniere's disease	✓			
Non-psychotic emotional disturbances	✓		✓	✓
Schizophrenia	✓	✓	✓	✓
Acute migraine	✓			

1.5 Adverse Effects

The phenothiazines, a class of drugs known to cause a variety of adverse effects in individuals[27] with high doses producing ocular opacity, tremors and deposition of melanin in the skin,[5, 6] have also been associated with photosensitivity effects in individuals exposed to light. Photosensitivity may be defined as an adverse dermatological reaction that can occur as a result of an interaction between a photosensitizing agent (e.g. phenothiazine drug) in the

skin and light energy.[28] Only the ultraviolet portion (200 - 400 nm) of natural sunlight contains sufficient energy to induce a photochemical reaction in human skin. The ultraviolet spectrum is arbitrarily sub-divided into three sections: UV-A (320 - 400 nm); UV-B (290 - 320 nm) and UV-C (200 - 290 nm), with only UV-A and UV-B radiations reaching the Earth's surface to cause a biological effect in human skin. Exposure to UV-A radiation is the major cause of photosensitizing reactions.[27] The drug may induce a photosensitizing response if administered by the oral, topical or parenteral route.

Of the many drugs reported to cause adverse photosensitivity effects, a significant number contain one or more chlorine atoms as substituent(s), such as chlorpromazine, prochlorperazine and perphenazine. Adverse photosensitivity action can occur by a Type I, free radical chain process, i.e. autoxidation, and / or by a Type II mechanism with the involvement of excited singlet molecular oxygen, i.e. oxygenation.[29]

On irradiation, chlorpromazine has been reported to produce free radicals as well as singlet molecular oxygen, thereby implying both Type I and II mechanisms of photooxidation. It was reported that two substrates can be employed to enable classification of the mechanism of a drug's participation in photooxidation. These substrates are 2,5-dimethylfuran, an efficient acceptor for singlet oxygen, and acrylamide, an effective free radical scavenger thereby undergoing polymerization.[28]

The phenothiazine derivatives are well known for the generation of free radicals which may be scavenged by oxygen molecules. The polymerization reaction is evident by the appearance of turbidity in a reaction mixture due to the insolubility of polyacrylamide, as well as by the contraction in volume of the reaction mixture observed in the dilatometer capillary, Table 1.6.[28]

Table 1.6 - Photoinitiated Polymerization of Acrylamide Solutions.[29]

Phenothiazine Derivative	Dilatometer Contraction Rate, mm/hr
Chlorpromazine	26
Prochlorperazine	38
Trifluoperazine	48

Moore reported that the photolability of the chlorine atom in chlorpromazine is a correlation of its ability to photoinitiate the polymerization of acrylamide.[29]

Oxygen uptake rates for some phenothiazine solutions i.e. chlorpromazine, prochlorperazine and trifluoperazine irradiated in the presence of UV light have been measured. The oxygen uptake is reported to be zero order, indicating that the absorption of light is the rate-limiting step. In order to assess the relative photosensitizing ability of the derivatives, a drug concentration was selected which resulted in an absorbance of approximately 0.5 at the wavelength of maximum absorption, λ_{max}, in the 300-400 nm region. Comparisons were based on the absorbance values of the compounds at 365 nm, the major irradiating wavelength. The phenothiazine derivatives were reported to react approximately in accordance with their absorbance at 365 nm. UV characteristics of the phenothiazines are based primarily on the phenothiazine tricyclic nucleus, with only minor effects as a result of the substituents. Trifluoperazine and prochlorperazine displayed λ_{max} values of 313 and 312 nm respectively, while a marked difference was observed in their respective UV absorptions at 365 nm, 0.072, for trifluoperazine and 0.030 for prochlorperazine. Although the absorption for trifluoperazine was more than double that for prochlorperazine, an 8 fold increase in the rate of oxygen uptake for both solutions was observed in the presence of 2,5-dimethylfuran. Chlorpromazine, however, demonstrated a 4 fold increase in the rate of oxygen uptake for the solution irradiated in the presence of 2,5-dimethylfuran. It is thus evident that these phenothiazine derivatives are capable of photosensitizing oxygenation of 2,5-dimethylfuran, an efficient acceptor molecule for singlet oxygen.[29]

Chlorpromazine has been demonstrated to act as a sensitizer for its own oxidation by the singlet oxygen mechanism. In the presence of oxygen, the chlorpromazine triplet state energy is efficiently transferred with the formation of singlet molecular oxygen, and this has been supported by the measurement of degradation rates following the addition of 2,5-dimethylfuran. Reactions of the chlorpromazine cation radical with oxygen have been reported to lead to sulphoxide formation.[29]

Davies et al. suggested that the Type I and II mechanisms may relate to the photoallergic and phototoxic effects of a photosensitizing drug, respectively.[30]

Photosensitivity Reactions

The photosensitivity reactions which occur in human skin follow the Grotthus-Draper law of photochemistry, which states that non-ionizing radiation must be absorbed by a drug or metabolic product before a reaction can proceed. The transmission of radiant energy through the skin varies with wavelength and the area of skin exposed. UV-A radiations and visible wavelengths penetrate deeply into the dermis. A systemic drug can thus induce a photosensitivity reaction in susceptible individuals by virtue of the fact that the entire blood volume of a resting adult can circulate through the skin approximately once every 11 minutes, thus permitting exposure of the drug to the penetrating UV-A rays which impinge on the network of capillaries and small vessels present in the dermis. Photosensitivity reactions may be classified as either photoallergic or phototoxic.[27]

Photoallergic Reactions

A photoallergic reaction can result in acute or chronic dermatitis. An acute reaction usually appears as a typical allergic contact dermatitis, the chronic reaction however, is morphologically characterized by abrasive, lichen planus lesions.[28]

An immunological mechanism of action plays a primary role in the photoallergic reaction. It has been suggested that the photosensitizing drug in the skin absorbs radiant energy, visible light or UV rays, and is converted to a hapten, a stable photoproduct, which then binds to the skin protein to form a complete antigen that stimulates antibody production. After an incubation period, a cell-mediated immune response occurs. A photoallergic reaction can thus occur at a distant site that was not originally exposed to sunlight, and occurs within 24 - 48 hours following exposure to the photosensitizing drug and light.[28]

Phototoxic Reactions

A phototoxic or non-allergic response, depends upon sufficient exposure to light energy of the appropriate wavelength and photosensitizing drug present in the subject's dermis or epidermis. When the photosensitizing drug absorbs light energy of the appropriate wavelength, the molecule becomes excited, returns to ground state, and releases its excess energy in a variety of forms into the surrounding dermal tissue. Oxygen may or may not be involved in the phototoxic reaction.[28]

Drug-induced phototoxic reactions can occur in one hundred percent of individuals receiving systemic or topical preparations containing photosensitizing drugs. It is characterized by erythema and oedema followed by hyperpigmentation and eventual desquamation.[28]

Although the mechanism by which the phenothiazine derivatives cause photosensitivity reactions has not been entirely resolved, the photolability of the aromatic chlorine substituent and the accompanying generation of free radicals *in situ* appear to be important precursors. Moore et al. reported that when the drug is located in a region where the concentration of oxygen is significant, the polarity of that environment would appear to determine whether the photoreaction occurs predominantly *via* free radical formation or molecular oxygenation of a suitable oxidisable species.[31]

Two physical models were developed to assess the photosensitizing properties of the phenothiazine drugs. The phospholipid spherule model developed by Bangham et al. was selected to assess photosensitizing agents by measurement of their influence on membrane permeability,[32] while the lecithin monolayer model developed by Nejmeh et al. and Felmeister et al. is a convenient *in vitro* method for studying the reaction between the phenothiazine drugs and body tissues under the influence of light.[6, 33, 34]

Researchers[34] have shown that phospholipids, when permitted to swell in an aqueous salt solution form salt-containing compartments bounded by bimolecular membranes. These spherules exhibit permeability characteristics similar to those of biological membranes. Furthermore, these spherules have been shown to be useful tools for studying membrane-drug interactions, particularly when changes in permeability are involved. These changes in permeability of the spherules induced by light-irradiated drugs are a measure of the photosensitizing properties of these drugs. The phospholipid spherules used in the model by Bangham et al. were modified, by initially saturating the lipid material with chromate ions, then injecting into each cell with a solution of chromate and drug. The model considers the effect of both the irradiated and non-irradiated phenothiazine derivatives on the release of chromate ion from the spherule. Chlorpromazine and prochlorperazine, both chlorine-containing derivatives, displayed a 86 and 82% increase in the release of chromate from the phospholipid spherules on irradiation, while triflupromazine and fluphenazine, two trifluoromethyl-derivatives, showed no release of chromate ions as a result of irradiation.[32]

The large increase in leakage induced by the ultraviolet irradiation of chlorpromazine and prochlorperazine suggests the formation of species on irradiation which are considerably more membrane active than the parent compounds. It has been reported that the photopolymerization of the chlorine derivatives occurs *via* a free radical formed by the elimination of the chlorine from the 2-position. The resultant photopolymer has been

suggested as being the membrane-active species. The results suggest, that if either prochlorperazine, or chlorpromazine accumulate in cell membranes or cell organelles, even at levels below that required to produce lysis, irradiation could convert them to species with significant lytic activity. Such lytic species in turn could lead to increased cell membrane permeability and subsequent oedema and inflammation. This suggests that the photoactivated sensitizing phenothiazine drugs do not react directly with cellular components. Instead, they form a new, stable, more surface-activated compound capable of inducing a cutaneous reaction, probably *via* changes in cell membrane permeability, which has been related to phototoxicity.[34]

The study of the photodecomposition of chlorpromazine with lecithin monolayers used lecithin as a model cell membrane or monolayer on the surface of an aqueous solution of the drug. The system was then irradiated with light of the appropriate wavelength and measurements of the changes that occurred in the force area curves of the monolayer were taken. It was found in all systems containing chlorpromazine that irradiation caused expansion of the films. A thin layer chromatographic method used to analyze the results revealed 3 spots: unchanged chlorpromazine, chlorpromazine sulphoxide and hydroxypromazine.[6] Felmeister et al. on investigation of the effect of ultraviolet irradiation on the surface activity of phenothiazine drugs, report an additional photodegradation product for chlorpromazine, the *N*-oxide.[33]

In addition to the degradants produced on irradiation of the system with ultraviolet light, a decrease in pH is observed. The sulphoxide which was produced was reported to be less surface active than that of the hydrochloride salt, and in amounts formed would not contribute to the expansion that was observed when the lecithin films of chlorpromazine were irradiated. Since in the absence of chlorpromazine, the lecithin films appeared to be unaffected by ultraviolet light, it was concluded that the expansion was due 2-hydroxypromazine, the formation of which has been proposed as follows by Nejmeh et al, Figure 1.8.[6]

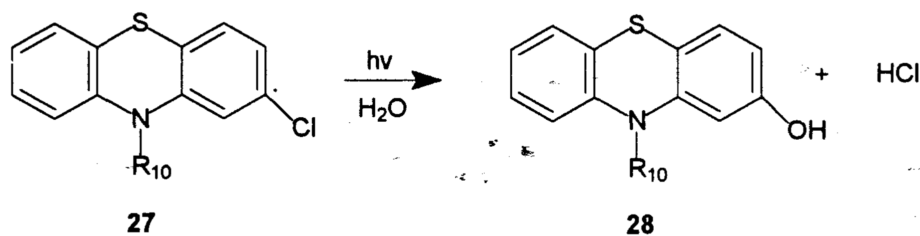


Figure 1.8 - The liberation of HCl from 2-chloro-derivatives under the influence of UV irradiation.[6]

This liberation of HCl on formation of the 2-hydroxy derivatives, and hydrogen ions on the formation of the sulphoxide, is reported to contribute to the decrease observed in the pH of the system. These findings explain the sensitivity, inflammation or dermatitis that usually precedes the deposition of melanin in the skin of patients treated with large doses of this drug.[6]

This model suggests that the ability of the UV irradiated phenothiazine drugs to interact with the lecithin monolayer may be a measure of their *in vivo* membrane-penetrating and phototoxic properties. A study was therefore undertaken to determine whether the irradiation-induced changes were related to changes in surface activity of the drugs themselves. This was done by measurement of the effect of UV irradiation on the surface pressure of solutions of several phenothiazine derivatives and the known photooxidation products of chlorpromazine. Determinations of surface pressure of irradiated solutions of chlorpromazine and prochlorperazine, showed an initial rapid increase, with a slower secondary change, which indicates the formation of more surface active compounds as a result of irradiation of the chloro-derivatives. In contrast, irradiation of the trifluoromethyl-derivatives resulted in a rapid initial decrease in surface pressure followed by a slower secondary decrease. However, fluphenazine after about 13 minutes of irradiation reversed its trend and showed a marked increase in surface pressure that persisted for about 25 minutes even though the irradiation had ceased during part of that time. The observed effect has been reported to be a result of

the presence of the β-hydroxyethyl group in the side chain of fluphenazine.[34] In an attempt to determine whether the photoproducts resulting from irradiation of solutions of chlorpromazine are responsible for the observed increase in surface activity, solutions of each of these compounds were prepared and surface pressure determined before, during and after irradiation. The chlorpromazine solution exhibited no surface pressure either before or after irradiation. The *N*-oxide, in contrast, exhibited a surface pressure of 5.1 dynes/cm. as opposed to less than 1 dyne/cm. for an equal concentration of chlorpromazine. However, since the irradiation of chlorpromazine resulted in an increase in surface pressure of 9.7 dynes/cm., the formation of the *N*-oxide *via* a photooxidation could not account in itself for the total observed increase in surface pressure. Further irradiation of the *N*-oxide resulted in a further increase in surface pressure. A solution of a representative hydroxy derivative prior to irradiation exhibited no significant surface pressure. However, on standing the surface pressure of this material increased without irradiation, reaching a maximum value of almost 15 dynes/ cm in about 10 minutes. The formation of the hydroxy derivative *via* photooxidation and its subsequent change in surface activity could be in part responsible for the changes observed during the irradiation of chlorpromazine.[33] It can thus be concluded that the irradiation-induced changes in surface activity of these drugs are directly related to, and possibly responsible for, the irradiation-induced changes in reactivity toward a lecithin film, which supports the suggestion of the formation of a new, stable, more surface-active compound as a result of irradiation.

It is evident from the study, that the photoproduct most responsible for the change in surface activity, in the case of chlorpromazine, is the *N*-oxide. Formation of the hydroxy derivative of chlorpromazine *via* irradiation, could also account for some change observed following irradiation of chlorpromazine. The sulphoxide, however, at low concentration levels, can not be expected to contribute to this change. It would thus suggest that the accumulation of the metabolites of chlorpromazine in the skin may be responsible for the reported phototoxic

reactions.[33] Pilpel et al. explain the adverse effects associated with the phenothiazines on exposure to sunlight, to be due to the formation of the hydroxy derivatives only by the action of UV irradiation, while the sulphoxides which are natural metabolites of the phenothiazines in the body and therefore, in low doses are unlikely to be responsible for the adverse effects of these drugs associated with sunlight.[5]

In contrast to the increased surface activity observed with the chloro-derivatives, the trifluoromethyl-containing phenothiazine derivatives in this study trifluoperazine and fluphenazine, are essentially non-photosensitizing.[33]

1.6 Stability

As a result of the reported phototoxicity of chlorpromazine over the years, its photostability has been investigated and decomposition products including the *N*-oxide, the sulphoxide, promazine, 2-hydroxypromazine and a dimer and polymer of chlorpromazine isolated and identified.[36] In order to explain the mechanism of action of chlorpromazine, its effect on various enzyme systems *in vitro* has been investigated where chlorpromazine's inhibition of the enzyme uridine diphosphate: NAD^+ oxidoreductase was not effective until incubation with an enzyme in daylight, suggesting that the presence of a free radical may be required for *in vitro* activity.[37] Since phenothiazine and its derivatives are sensitive to oxidation it has been proposed that the cation radical formed on oxidation of chlorpromazine for example could be an intermediate of the metabolism of the drug and thus the active pharmacological entity. The stability of the 10-alkylphenothiazine free radicals in this study has been shown to depend on the nature of the substituent at the 2-position on the phenothiazine nucleus and the nature of the 10-alkyl substituent. Scheme 1.4 proposes a mechanism of oxidation for phenothiazine derivatives.[38]

The oxidation proceeds by two steps: **29** produces an uncoloured solution, **30** a coloured

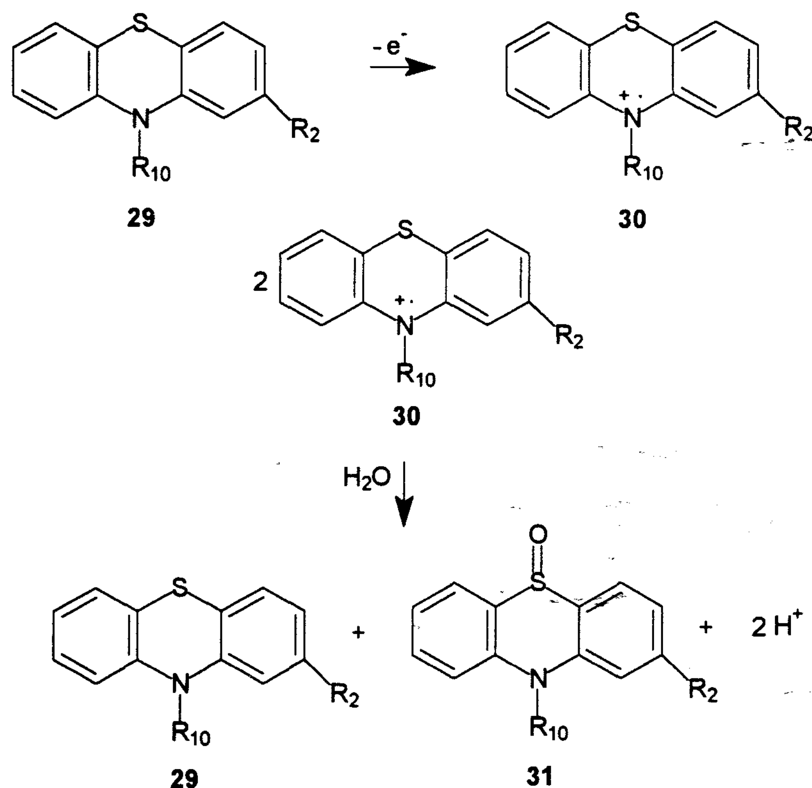
solution and in the case of the substituted R_{10} derivatives the reaction is expressed in Scheme 1.4. The resulting sulphoxide solution **31** is decolourised.[37]

The perazine cation radicals decayed slowly enough to ascertain that in the UV spectrum a Cl substituent shows a bathochromic shift with the CF_3 producing no shift in the bands. There is a relationship between the intensity of the bands of each radical cation and the substituents, however, the R_{10} substituent appears to have little effect while the R_2, Cl shows a hypochromic effect and the CF_3 due to its affect on the π electronic structure of the tricyclic nucleus produces hyperchromic shifts of its maxima. Although the R_{10} substituent with a long chain is capable of producing a more stable radical the influence of R_2 on the decay constant of the radicals is clear with the R_2 substituent CF_3 resulting in the strongest increase in the decay constant. Although the decay proceeds by second-order kinetics, hydrolysis of the phenazothionium ion proceeds by first-order kinetics with the decay of the free radical resulting from the hydrolysis of the phenazothionium ion followed by disproportionation.[37]

As is illustrated by Scheme 1.4, two equivalents of radicals disappear for each equivalent hydrolysed. Since it has been suggested that the phenothiazine radical cation has some relationship to pharmacological activity / stability and structure activity relationship studies confirm greater neuroleptic activity of compounds with R_2 substituents as CF_3 as opposed to Cl, Ortiz et al. confirm that the stability of these radicals varies inversely with the activity of neuroleptics.[38] According to Levy, Tozer and Tuck, the design of a radical as an enzyme inhibitor would include a 10-(4-dimethylaminobutyl), R_{10} substituent since it gives rise to a slow decaying radical which appears suitable for further study.[37]

Since sulphoxide formation is an important degradation pathway in aqueous preparations in contact with air, Davidson developed a method to determine sulphoxides in degraded phenothiazine formulations by difference spectrophotometry. The presence of a shoulder

around 345 nm in the absorption spectrum of an aqueous solution characteristic of the presence of the sulphoxides is utilised in this study.



Scheme 1.4[38]

These results were used to consider the percentage intact phenothiazine and sulphoxide present in various formulations and while tablet preparations contained low percentages of sulphoxide even after several years, aqueous formulations were found to contain up to 29% sulphoxide after 18 months storage. This concentration of sulphoxide is however dependent upon the storage duration and the relative volume of air in the container.[39] Owens et al. prepared prochlorperazine and perphenazine sulphoxide using aqueous nitrous acid because of their importance as metabolites in the common biotransformation pathways for all phenothiazines.[7] This together with the work by Anderson et al. has been devoted to the

development of analytical techniques to detect these compounds. The possible presence of the *N*, *S*-dioxide, the sulphone and the sulphoxide di-oxide **32** emphasises the importance of these analytical techniques in stability studies.[40]

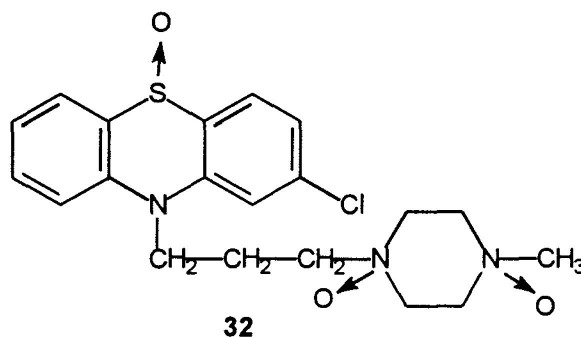


Figure 1.9 - 2-chloro-10[3-(1-methyl-*N,N'*-bisoxido-4-piperazinyl)propyl]phenothiazine 5-oxide.[40]

Structural elucidation of **32** was accomplished by ^{13}C-NMR, IR, and CHN analysis.[40]

Because of the photostability studies conducted in this investigation previous relevant stability studies are summarised. Pawelczyk et al. report the rate and type of photodegradation of aqueous solutions of various perazine derivatives to be dependant upon the nature of the R_2 substituent. The methodology used involved irradiation by UV light of 254 nm (low pressure TUV 30 Philips lamp) of an aqueous solution of the salts of perazine derivatives in a phosphate buffer at pH 3.00 in the presence of air or nitrogen. Results indicate the absence of sulphoxides in the presence of nitrogen and for prochlorperazine and trifluoperazine the degradation proceeds due to reversible first-order photooxidation. The photochemical degradation of perazine and thiethylperazine is complex consisting of parallel reactions of photolysis and photooxidation while substituents Cl and CF_3 prevent photolysis such that the rate of degradation is determined by the rate of the photooxidation process. Based on these findings, this group continued the investigation on some physicochemical parameters in order to explain the chemical reactivity of these compounds. Many of the observations can be

explained in terms of the volume of the R_2 substituent (Å) including the fact that the basic properties increase with an increase in the substituent volume. In a Hammett type plot showing the relationship between the basicity of the compound and the rate of degradation, prochlorperazine (Å = 29) exhibits a higher degradation rate under the influence of light as compared to trifluoperazine (Å = 88). The double-lined Hammett plot due to different R_2 substituents suggests two different mechanisms of reaction confirming degradation by photooxidation for those derivatives with Cl and CF_3 substituents and photooxidation and photolysis for the H and SC_2H_5 containing derivatives.[41, 42]

The fact that both Moore et al. and Sharples report on the photolabile nature of chlorine in chloro- aromatic compounds has implications for prochlorperazine and perphenazine in this study. Although these compounds are capable of undergoing both Type I (free radical) and Type II (singlet molecular oxygen) reactions, prochlorperazine yielded chloride and hydrogen ions at half the rate observed for chlorpromazine and thus appears to be a more effective photosensitizer of 2,5-dimethylfuran oxidation. In both studies methanolic solutions of the phenothiazines were irradiated over a period of time under nitrogen or oxygen as desired using in the case of Moore and co-workers, a medium pressure mercury lamp (Hanovia, 125 W) and in the other study an Allen type A 409 fixed wavelength (365 nm) UV lamp.[31]

Sharples reports that the 2-chlorophenothiazines give rise to a dechlorinated product, a dimer and the corresponding sulphoxide.[36]

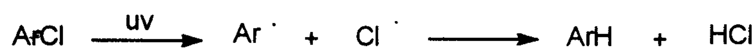


Figure 1.10 - Formation of free radicals from chloro-substituted phenothiazines.[36]

These free radicals formed as previously mentioned are believed to explain the high phototoxicity of the chloro-substituted phenothiazines, Figure 1.10. The results concur with those reported by Moore et al. who also found that the HCl yield is independent of the solvent

used. When considering the mechanism whereby chlorpromazine photoinitiates the polymerization of acrylamide, it is possible that either the promazine radical arising from direct homolysis of the triplet chlorpromazine or the chlorpromazine cation radical may be implicated. This cation radical on reaction with oxygen gives rise to the sulphoxide. This is verified by the UV spectrum after irradiation in either an air-saturated buffer or methanol.[31] Irradiation of 2-chlorophenothiazine in methanol may give rise to phenothiazine and 2-methoxyphenothiazine. Bunce et al. reported on the photodechlorination of the same compound in acetonitrile-water implicating the *N*-alkyl substituent in the acceleration of chlorine removal by an intramolecular electron transfer mechanism.[43]

The work published by Underberg et al. and Abdel-Moety et al. represents important findings in respect of the R_2 trifluoromethyl-substituted phenothiazines.[44, 45] Although the report by Underberg considers the thermal degradation of selected phenothiazines, it is an attempt to ascertain whether there is a relationship between oxidative degradation and the nature of the R_2 substituent. To this end they included 2-methyl-, trifluoro-, and cyanopromazine in the study. In the case of the trifluoromethyl-derivatives it was found that degradation was pH dependent, yielding the sulphoxide at a pH $\leq$ 3, while at pH 6.3 an additional product, *N*-monomethylnortrifluopromazine, was isolated. This latter product occurs in the degradation profile due to the cleavage of the side chain of these molecules only if the R_2 substituent is electron-withdrawing and if the dimethylamino group is unprotonated.[44] Because of the inclusion of the trifluoromethyl-derivatives, trifluoperazine and fluphenazine (solution and solid studies) in this study, mention of a photochemical stability study was made where solutions of trifluoperazine HCl in water were subject to irradiation in a chromato UVE light cabinet, Model CC60, with a 60 W lamp Model Assy 00-60-SL at 254 nm in an open pyrex container. In addition to this, an aqueous drug solution of trifluoperazine in a closed 1 cm quartz cell was also irradiated. Solid state photostability studies were also carried out on powdered tablets exposed to fluorescent and daylight for 5 days and stressed irradiation with 60 W short UV

light for 2 hours. Results from the accelerated drug photolysis carried out on the aqueous solution of the drug using a 60 W UV lamp (254 nm) indicate the development of a coloured solution (yellowish red → reddish brown) and a new photoproduct, 3-trifluoromethyl(biphenylthiophen)sulphoxide, characterized by mass spectrometry. The solid state photostudy gives rise to the same degradation products for all light sources but in relatively appreciable amounts in the case of the short UV irradiation. In the UV spectrum of the irradiated aqueous solutions an increase in the light absorption in the visible region at about 523 nm caused a red colouration. The observed red colour in the case of trifluoperazine can be attributed to the stable red radical. Because of the distribution of π electrons in the trifluoperazine molecule, radical forms may develop at the S atom, the N_{10} and between the S and N atom on the phenothiazine ring. Due to the effect of short-lasting UV irradiation a radical at the S atom is expected. Decomposition of the resulting sulphoxide dimer results to give rise to the sulphoxide, which is confirmed by the presence of relevant absorption bands in the UV spectrum.[45]

In the initial structure activity relationship studies, the proposed mechanism of interaction with the receptor and consideration of various physicochemical models it was concluded that the order of decreasing activity is fluphenazine, trifluoperazine, perphenazine and prochlorperazine. While the metabolism of these four piperazine-substituted phenothiazine derivatives is proposed to occur *via* *N*-oxidation, *N*-demethylation, sulphoxidation and hydroxylation, the degradation of the piperazine ring is claimed to be independent of the presence of a methyl or β-hydroxyethyl substituent and the R_2 substituent. There is no other mention of the role of these substituents in the metabolism of these compounds. However, there certainly is a relationship between phototoxicity and the R_2 substituent where the chloro-derivatives are photosensitizing with the trifluoromethyl-derivatives showing few adverse effects. In the stability studies reported, the degradation of those compounds with Cl and CF_3 R_2 substituents is proposed to occur *via* photooxidation. It can thus be seen that these R_2 and

R_{10} substituents which contribute to activity also play a role in the metabolism, development of adverse effects and the stability of these compounds.

CHAPTER 2: METHOD DEVELOPMENT AND VALIDATION

2.1 Introduction

Traditionally, analytical methods used to monitor the stability of dosage forms have involved non-specific spectrophotometric or titrimetric procedures for the assay of the active ingredient, and thin layer chromatographic methods for the determination of related substances. However, in the last 5 years, the technique of choice for the quantitation of the active and degradation product(s) is reported to be high performance liquid chromatography (HPLC). HPLC is advantageous in that it both separates, measures and lends itself well to automation, however, in the absence of automation, the technique may be time consuming and relatively expensive.[46]

Apart from HPLC, other techniques such as gas chromatography, spectrofluorimetry and nuclear magnetic resonance spectroscopy (NMR) have some application in the assay of the drug component and its related substances. The choice of the appropriate method of quantitation depends on both a scientific and practical evaluation of the drug(s) of choice and dosage form.[46] Al-Obaid et al. report on the simultaneous quantitation of some phenothiazine drug substances and their monosulphoxide degradants by HPLC. Previous analytical techniques used for this purpose were spectrophotometry, second- and third-derivative ultraviolet (UV)-spectrometry and gas liquid chromatography (GLC). Although the derivative UV- spectrometric methods are more selective than ordinary spectrophotometry the sensitivity is not improved, while the high temperatures required for GLC render this method ineffective in the determination of the phenothiazines and their degradants. Thus HPLC separation of selected phenothiazines and their degradants was performed on a Novapak-phenyl-4 (150 x 3.9 mm i.d.) column and a mobile phase of methanol: 1.5 x 10^{-1} M sodium acetate, pH 6.5 (81:19 v/v) with isocratic elution and UV detection. Results indicate that the HPLC method measures the phenothiazine (perphenazine and trifluoperazine) and its sulphoxide to 0.25%

m/m[47] as opposed to the *BP* 1988 spectrometric method which quantitates only the total of parent compound and its sulphoxide.[48] Thus based on the needs of this study to quantitate the phenothiazines, prochlorperazine, perphenazine, trifluoperazine and fluphenazine separate from their degradants, HPLC is considered to be the most effective technique for providing a stability-indicating assay.

The analysis of drug substances and finished products within the pharmaceutical industry is carried out to satisfy both manufacturer and regulatory authority, about the quality, integrity and stability of the medicine administered to the patient.[49] Regulatory Authorities are becoming increasingly more aware of the necessity of ensuring that the data provided to them in applications for marketing and clinical authorizations have been acquired using validated analytical methodology. According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the *USP* and the *NF* constitute legal standards.[50] Standards set by the *USP* for the quality or purity of the four derivatives under investigation are listed as specifications which lie between 90.0 and 110.0% purity.[51]

Edwardson et al. report that the most important area of method development is the validation of the method which is done to ensure that the performance and reliability of the method meet the requirements for the intended analytical applications.[52, 53] Analytical method validation must be approached as a process to provide maximum confidence in the reliability of the test procedures and not just as a regulatory requirement, and thus the procedure is viewed by Hokanson as dynamic with the ability to expand as additional information becomes available.[54]

It is important to differentiate method validation from method selection and development. Method selection involves consideration of what is to be measured and with what accuracy and precision, while method development in part involves why such specified conditions were chosen.[55]

Method validation should not be confused with system suitability testing which evaluates the components of the analytical system to show that performance meets the standards required by the method.[52] Since qualitative and quantitative methods are intended to serve different purposes, different guidelines are recommended for validation. A quantitative method for example is required to be accurate and produce an expected response within the concentration range described by the method, however the concentration boundaries are determined by the expected scope of the method.[55]

Method validation should demonstrate that the method is free of systematic errors and that the method detects the substance that it purports to detect. When designing a chromatographic system for the analysis of an active component of a pharmaceutical product it is essential to have a good knowledge of the susceptibility of the drug to degradation and its degradation pathway, assay interference by possible degradants or synthesis precursors and assay interference by chemicals employed in sample preparation and excipients present in the formulation.[55] As the current investigation focuses on the stability of the four phenothiazines: prochlorperazine, perphenazine, trifluoperazine and fluphenazine and attempts at structural elucidation of light degradants and prediction of possible degradation pathways, validation of the method is thus limited to that of the active components with emphasis on the importance of the selectivity / specificity parameter involving the use of photodiode array detection to ensure peak purity and thus the integrity of the active component. In the environment in which the investigation is to be conducted, the ruggedness parameter has particular significance due to the use of different detectors, pumps, integrators/ recorders and columns during the research programme.

The performance characteristics for the validation of a method are expressed in terms of analytical parameters according to the *USP*: precision, accuracy, linearity, limit of detection and quantitation, ruggedness, and selectivity or specificity.[56]

Different validation parameters are required for the various analytical procedures. Selectivity, linearity and limit of detection and quantitation are a measure of equipment suitability. The accuracy assessment measures the effectiveness of the sample preparation process, while precision and ruggedness cover both equipment and test preparation aspects.[54]

The method which was used for the HPLC analysis of the four piperazine-substituted phenothiazine derivatives under investigation supplied by Lennon Ltd, is based on the *USP* method used for the assay of prochlorperazine maleate tablets.[51]

2.2 Method Development

Method of Analysis of Prochlorperazine Maleate Tablets[51]

Chromatographic Parameters: *USP*

Mobile Phase:	Acetonitrile: methanol (MeOH): water (H_2O), (40: 15: 45 v/v), 5 x 10^{-3}M sodium 1-octane sulphonate.
Internal Standard:	Trifluoperazine hydrochloride (0.014% m/v).
Solvent:	Methanol: 2 x 10^{-1} M Hydrochloric acid (HCl) (1:1 v/v).

Liquid chromatographic separations were performed using a μBondapak, C18 (4.0 x 300 mm i.d.), reverse-phase column, and a variable wavelength detector set at 254 nm. The flow rate was 2.0 mL per minute.[51]

Method of Analysis of Trifluoperazine Hydrochloride

Chromatographic Parameters: Lennon Ltd - Trifluoperazine HCl

Mobile Phase:	Methanol: water: glacial acetic acid (70: 30: 1 v/v), 5 x 10^{-3} M sodium-pentane sulphonate adjusted to pH 3.0 with orthophosphoric acid.
Solvent:	Methanol: water (70:30 v/v).

Liquid chromatographic separations were performed using a μBondapak, C18 (4.0 x 300 mm i.d.), reverse-phase column, and a variable wavelength detector set at 254 nm. The flow rate was 1.0 mL per minute.

Although the *USP* method for the HPLC assay of prochlorperazine maleate tablets makes use of an internal standard, trifluoperazine hydrochloride, its stability and the fact that it is one of the piperazine-substituted derivatives in this study precludes it as an internal standard. As an alternative, acenaphthene was considered, however its retention time resulted in an increase of the total run time by approximately 8 minutes. The reproducibility of peak heights of the selected phenothiazines was considered and relative standard deviations over a range of concentrations assessed using a rheodyne injector equipped with a 20 μL fixed loop. Acceptable limits (≤ 2% relative standard deviation, RSD) led to the method being developed in the absence of an internal standard. In order to assess whether the composition of the mobile phase (Lennon Ltd) is optimum for the four phenothiazines under investigation, pH and polarity of the mobile phase were adjusted and the presence / absence of the ion-pairing reagent was evaluated. Since orthophosphoric acid is used in the adjustment of the pH of the mobile phase to 3.00, its absence in the mobile phase was assessed resulting in an increase in the retention times of the derivatives by 2 minutes, with accompanying decrease in peak height due to broadening. Clearly pH adjustment to 3.00 contributes to optimum chromatographic conditions.

Since the HPLC method is used in the quantitation of the phenothiazine and its degradants in stability studies, the retention times of the phenothiazines need to be such to allow separation from its more polar degradants. This emphasises the importance of the specificity parameter in this method validation study. Thus adjustment of the polarity of the mobile phase in terms of an increase in the methanol: water ratio although giving rise to a decrease in retention time of the active is not favourable due to potential lack of resolution of the parent

compounds and the degradants. However an increase in the polarity by a decrease in the percentage of methanol by 10% increases the retention times of the derivatives with any further increase in mobile phase polarity ie 50:50 v/v resulting in infinite retention times for the four derivatives.

In the absence of the ion-pairing reagent, a decrease of between 4.0 and 2.5 minutes in retention times for the derivatives was evident, with prochlorperazine being most significantly affected. Figure 2.1 illustrates the effect of pH, polarity, and the ion-pairing reagent on the retention times / peak shape in comparison to those obtained using the final chromatographic parameters. This confirms the mobile phase (Lennon Ltd) to be optimum and thus the method of analysis is used without further adjustment for the four phenothiazines under investigation.

2.3 HPLC Method

Reagents

All chemicals were of at least analytical grade. Potassium dihydrogen phosphate and sodium hydroxide were obtained from UNILAB, SaarChem (Pty) Ltd, and glacial acetic acid from UNIVAR, SaarChem (Pty) Ltd. Orthophosphoric acid (Analar) was purchased from BDH Chemicals Ltd, and methanol (HPLC grade) from Romil Ltd, Cambridge. Water for chromatography was obtained using a Milli-Q Plus ® water purification system, filtered with a Q Pak, purification pack suitable for water pretreated by reverse osmosis, consisting of an initial 0.5 µm prefilter, activated carbon, nuclear grade ion-exchange resin, and an Organex-Q organic scavenger mixture.

Prochlorperazine, perphenazine, trifluoperazine and fluphenazine were kindly donated by Lennon Ltd (Port Elizabeth, South Africa) and Fine Chemicals Ltd (Cape Town, South Africa).

Buffer

6.805 g of Potassium dihydrogen phosphate (KH_2PO_4) was accurately weighed, transferred to a 250 mL volumetric flask and made up to volume with water (2 x 10^{-1} M) and 0.8 g of sodium hydroxide (NaOH) was accurately weighed, transferred to a 100 mL volumetric flask and made up to volume with water (2 x 10^{-1} M). 250 ML of the KH_2PO_4 solution and 58 mL of the NaOH solution were accurately measured, transferred to a 1000-mL volumetric flask and this was made up to volume with water. The pH of the buffer solution is approximately 6.4.

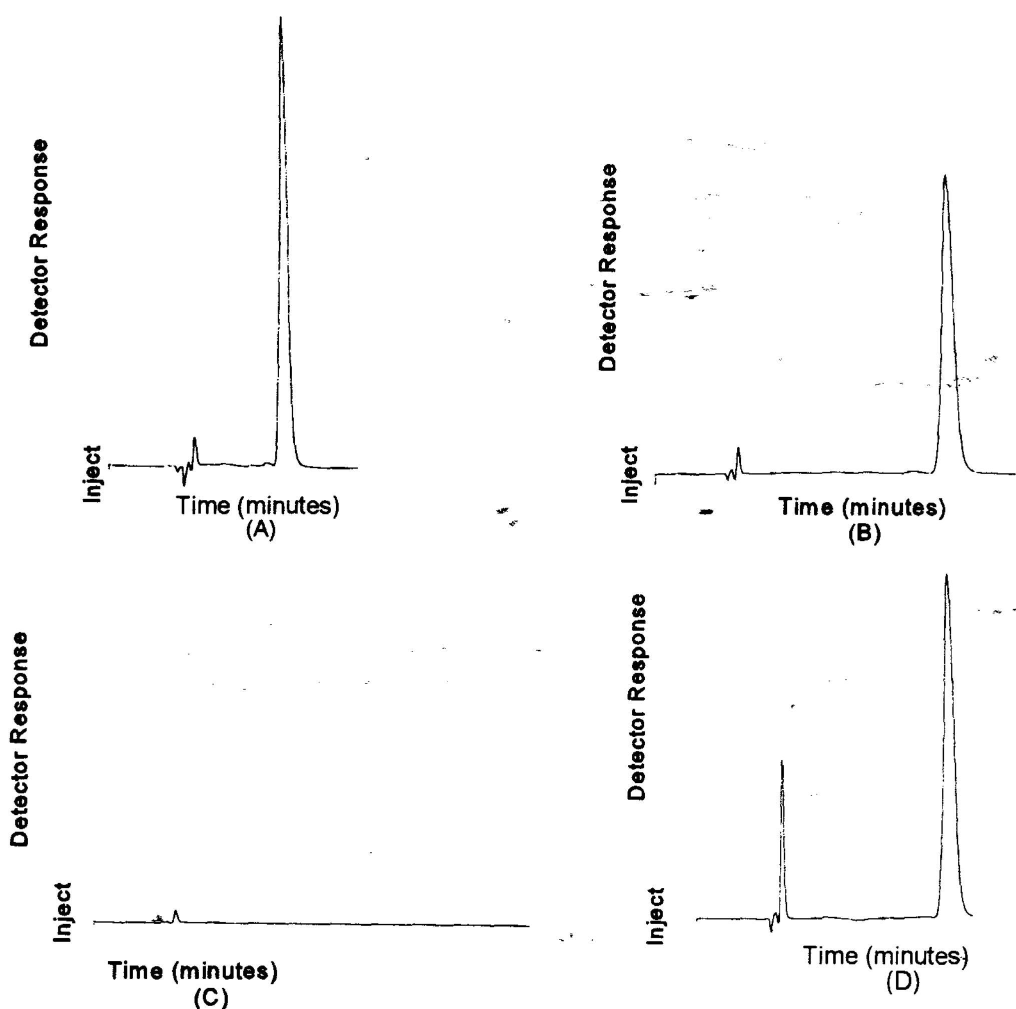


Figure 2.1 - Effects of mobile phase adjustments on chromatograms of prochlorperazine. (a) Without the ion-pairing reagent, < R_t. (b) Without pH adjustment, > R_t. (c) Polarity adjustment, infinite R_t. (d) Mobile phase (final), R_t ca. 6.5 minutes.

Stock Preparation

500 mg of each phenothiazine derivative was accurately weighed and transferred to a 500 mL volumetric flask and made up to volume with buffer resulting in a 1 mg/mL solution.

The low solubility of perphenazine in the buffer required that 500 mg of perphenazine be accurately weighed and transferred to a 500 mL volumetric flask with the addition of 125 mL of 2 x 10^{-1} M KH_2PO_4 to obtain a solution with subsequent addition of 29 mL of a 2 x 10^{-1} M NaOH solution. The stock solution was made up to volume with water.

Standard Preparation

The transfer of 2.5 mL of the stock preparation to a 50 mL volumetric flask, made up to volume with solvent, is required for the preparation of a 5.0 x 10^{-2} mg/mL standard solution used in the HPLC analysis.

Equipment

Φ 32 pH Meter, Beckman 015-247665-A

Precisa 120 A Mass Balance

Final Chromatographic Parameters

Column:	μBondapak, C18 (3.9 x 300 mm id) 10 μm, 125 Å.
Detector Wavelength:	254 nm
Resultant Pressure:	1500 psi
Flow Rate:	1 mL per minute
Injection Volume:	60 μL
Column Temperature:	Ambient (25 ± 2 °C)

A modular HPLC system consists of an Iso Chrom LC (SP 8700) constant-flow pump

(Spectra-Physics), with a Lambda-Max LC variable wavelength UV detector (Pye-Unicam), a Rikedinki recorder and a rheodyne fixed loop 20 μL injector (Model 7125).

Retention Times:	Prochlorperazine	ca. 6.5 minutes
	Perphenazine	ca. 7.5 minutes
	Fluphenazine	ca. 9.0 minutes
	Trifluoperazine	ca. 13.0 minutes.

2.4 System Suitability

Validation of an analytical method confirms its suitability for use on a particular occasion with a particular system. Confirmation of the method's continuing suitability for use with different systems, checks or system suitability tests (SSTs) however are also necessary when the method is in use.

The essence of a system suitability test is that the electronics, equipment, specimens and analytical operations constitute a single analytical system, which is amendable to an overall test of the systems functions. The system suitability requirements include the following:

Relative Standard Deviation (RSD)	$\leq$ 2% (5 injections)
Tailing factor (T)	< 2.0
Number of theoretical plates (N)	> 2000
Resolution (R)	> 5.0.

Method

Five replicate injections of standard preparations for each phenothiazine derivative: prochlorperazine, perphenazine, trifluoperazine and fluphenazine were performed under the following conditions:

Column:	µBondapak, C18 (3.9 x 300 mm i.d.) 10 µm, 125Å
Detector Wavelength:	254 nm
Flow Rate:	1.5 mL per minute

Results and Discussion

The results for the system suitability study are summarised in Table 2.1.

Table 2.1 - System Suitability Test Results.

	Prochlorperazine	Perphenazine	Trifluoperazine	Fluphenazine
% RSD	0.56	0.51	0.56	0.51
Tailing Factor	0.45	0.41	0.36	0.45
Theoretical Plates	2553	2390	2374	2381
Resolution	14.96	11.67	13.14	11.43

Based on these results it can be seen that the requirements of system suitability have been satisfied.

2.5 Method Validation

Method validation is generally an on-line process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose. There are a number of guidelines currently available for the validation of analytical methods with the *USP* amongst the leading authorities on validation procedures recognised by regulatory authorities.[56]

2.5.1 Linearity and Range

The linearity requirement is that response is directly proportional to the concentration of analyte in samples within a given range (25 - 125% of the nominal analyte concentration). The range is the concentration interval over which an analyte needs to be measured with acceptable precision and accuracy.

Method

The linearity for prochlorperazine, perphenazine, trifluoperazine and fluphenazine was established over a concentration range of 6.0 x 10^{-2} mg/mL to 1.0 x 10^{-3} mg/mL. Calibration standards were prepared for each phenothiazine derivative from the respective stock preparations. The necessary dilutions were made with solvent (methanol: water, 70:30 v/v) to produce standards containing 6.0 x 10^{-2}, 4.0 x 10^{-2}, 2.0 x 10^{-2}, 1.0 x 10^{-2}, 5.0 x 10^{-3}, and 1.0 x 10^{-3} mg/mL of each respective phenothiazine. Each standard was assayed in triplicate and the calibration curve constructed for each phenothiazine derivative by plotting the mean peak height in millimetres (mm) *versus* concentration.

Results and Discussion

The calibration line obtained for each phenothiazine derivative, Figures 2.2 - 2.5, was found to be linear over the calibration range studied, Tables 2.2 - 2.5. Linear regression analyses were performed to obtain correlation coefficients of 0.98, 0.99, 0.99, and 0.99 for prochlorperazine, perphenazine, trifluoperazine and fluphenazine respectively.

Table 2.2 - Range of Linearity for Prochlorperazine.

Concentration (x 10^{-2} mg/mL)	Mean Peak Height (mm)	% RSD
6.0	212.00	0.00
4.0	140.70	0.41
2.0	69.70	0.43
1.0	34.20	0.00
0.5	16.45	0.00
0.1	2.26	0.00

Table 2.3 - Range of Linearity for Perphenazine.

Concentration (x 10^{-2} mg/mL)	Mean Peak Height (mm)	% RSD
6.0	204.10	0.14
4.0	136.07	0.00
2.0	70.41	0.00
1.0	36.99	0.00
0.5	20.28	0.00
0.1	6.91	0.00

Table 2.4 - Range of Linearity for Trifluoperazine.

Concentration (x 10^{-2} mg/mL)	Mean Peak Height (mm)	% RSD
6.0	223.27	0.52
4.0	149.62	0.76
2.0	75.97	0.00
1.0	39.15	0.00
0.5	20.73	0.00
0.1	6.00	0.00

Table 2.5 - Range of Linearity for Fluphenazine.

Concentration (x 10^{-2} mg/mL)	Mean Peak Height (mm)	% RSD
6.0	223.39	0.00
4.0	149.60	0.29
2.0	75.81	0.00
1.0	40.27	1.08
0.5	20.47	0.00
0.1	5.85	0.00

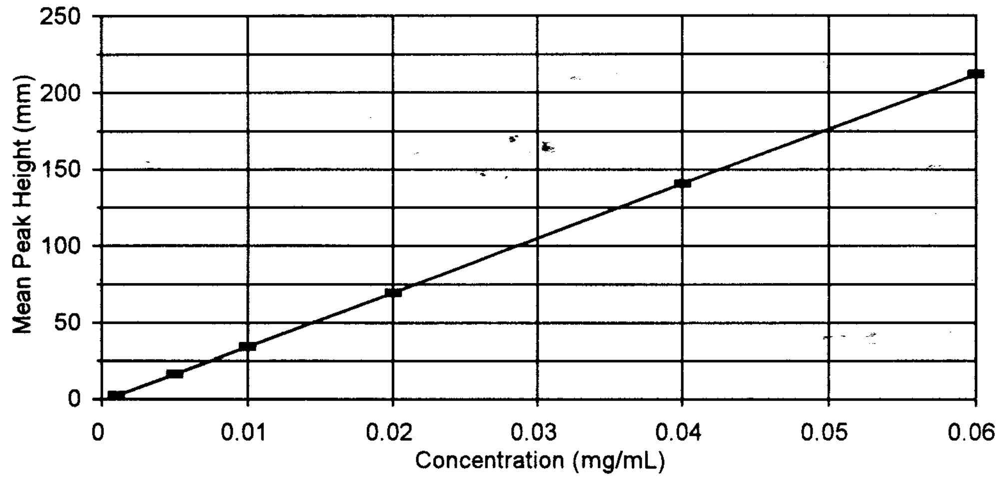


Figure 2.2 - Calibration curve for prochlorperazine, y = 3548.81x - 1.29.

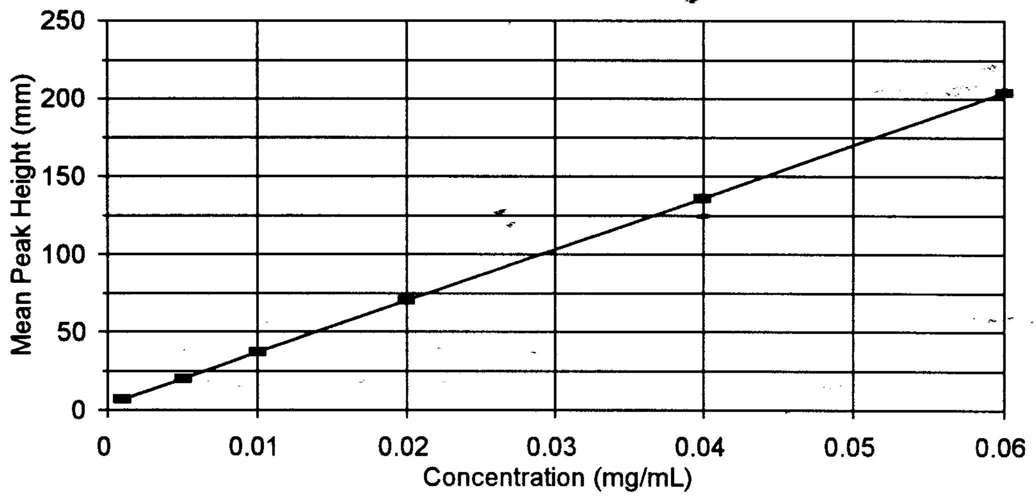


Figure 2.3 - Calibration curve for perphenazine, y = 3342.20x + 3.57.

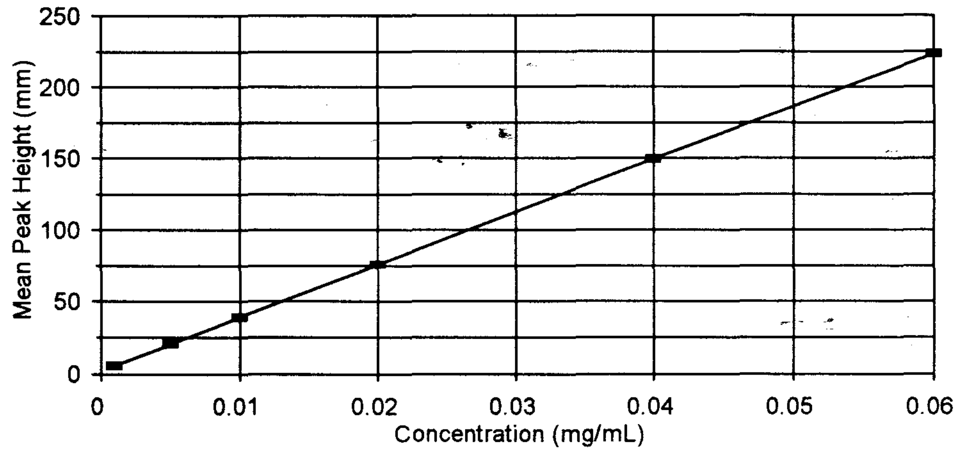


Figure 2.4 - Calibration curve for trifluoperazine, y = 3682.54x + 2.32.

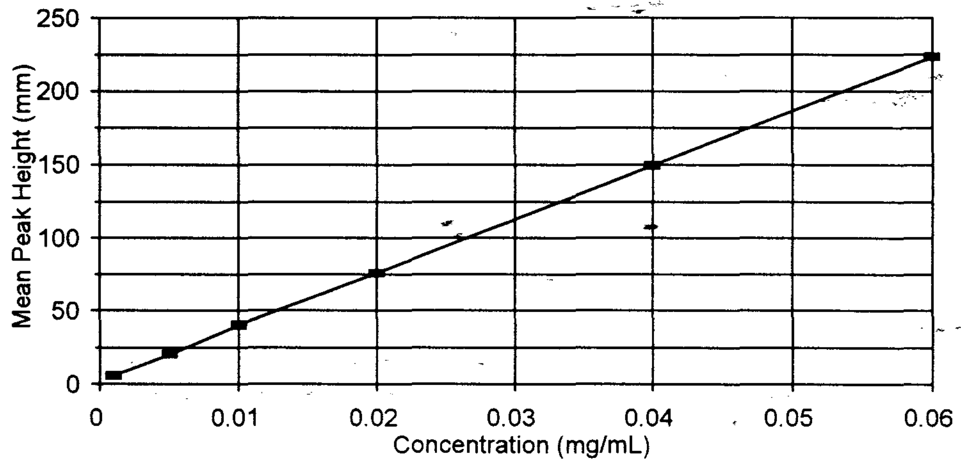


Figure 2.5 - Calibration curve for fluphenazine, y = 3689.4x + 2.03.

These results confirm the linearity of the method over the desired concentration range.

2.5.2 Precision and Accuracy

Method precision gives a measure of the method's reproducibility under normal operating conditions, while accuracy is the closeness of test results to the true value. The mean

concentrations obtained for the replicates are a measure of the accuracy of the method whilst the relative standard deviation of the six samples at any concentration provides a measure of precision.

Method

The precision of the analytical method was determined by assaying six spiked samples, each at the upper and lower limits of the concentration range studied (6.0×10^{-2} mg/mL and 1.0×10^{-3} mg/mL) for each of the respective phenothiazine derivatives. The accuracy of the method was determined by the mean concentrations obtained for the replicates and the percentage (%) difference. Samples were prepared by dilution of stock preparations with solvent for each phenothiazine derivative.

Results and Discussion

Precision and accuracy results for prochlorperazine, perphenazine, trifluoperazine and fluphenazine are represented in Table 2.6.

Table 2.6 - Precision and Accuracy Results.

Phenothiazine Derivative	Spiked Concentration (mg/mL)	Mean Concentration Found (mg/mL)	% RSD (n=5)	% Difference
Prochlorperazine	0.005	0.005	1.22	0.00
	0.040	0.039	0.91	0.25
Perphenazine	0.005	0.005	1.14	0.00
	0.040	0.039	0.33	0.25
Trifluoperazine	0.005	0.005	0.00	0.00
	0.040	0.040	0.83	0.00
Fluphenazine	0.005	0.005	1.86	0.00
	0.040	0.040	0.32	0.00

With a % RSD not exceeding 1.86 and a % difference of 0.25 only for prochlorperazine and perphenazine at 0.040 mg/mL, the requirements for precision and accuracy are satisfied.

2.5.3 Limit of Detection and Quantitation

Tests to determine the limit of detection (LOD) and quantitation (LOQ) are performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte which can be detected above baseline noise, but not necessarily quantitated, while LOQ is the minimum level at which duplicate samples of the analyte can be detected with acceptable accuracy and precision. The LOD and the LOQ are determined either from the signal to noise ratio (*IUPAC*), or by the sequential dilution and determination of an observable chromatographic peak.

Method

According to *IUPAC* the determination of LOD and LOQ is performed using the following equation:

$$C_1\ (q_1) = ks_B/S, \qquad \text{Equation (2.1)}$$

where C_1, is the smallest concentration or q_1, amount, k is a constant, s_B is the standard deviation of analytical blank signal, and S is the slope of response *versus* concentration curve. Recommended values for k are: for LOD, $k = 3$, and for LOQ, $k = 10$. The LOQ can also be determined as the lowest analyte concentration for which duplicate injections result in a % RSD $\leq$ 2%. s_B is related to baseline noise, and is estimated from the measured noise in a blank injection over an area of 20 times the peak width of the active component. The noise is then determined from the largest peak-to-peak fluctuation, when $s_B = N_{p\text{-}p}/5$, or from the largest deviation from the mean response, when $s_B = N_p/2$, Figure 2.6.[50]

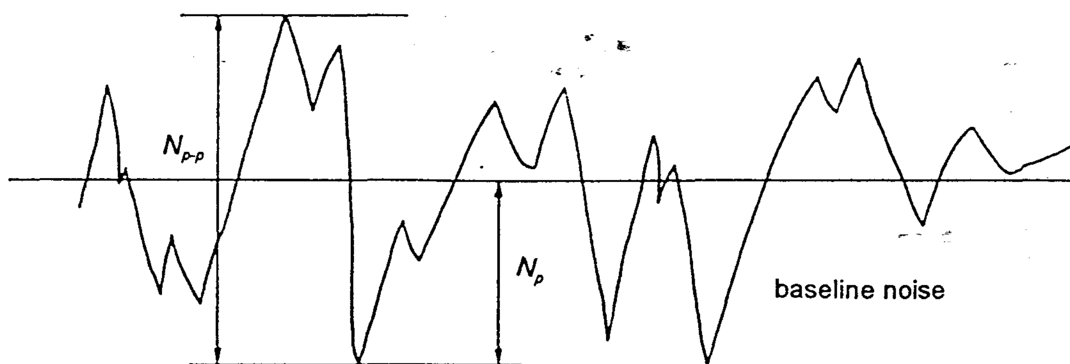


Figure 2.6 - Estimation of chromatographic baseline noise.[50]

Results and Discussion

The calculated results obtained from the *IUPAC* method, depend on predictions made with analytical blank solutions, and estimations of noise intensity based on the heights of noise fluctuations.[50] Jenke recommends that the calculated LOD and LOQ results be confirmed by injecting samples prepared to contain the analyte at or near the LOD and LOQ.[58] On comparison of the results obtained from the sequential dilutions of the respective samples, with the results obtained from the *IUPAC* method, differences in the LOD and LOQ values were evident. The calculated values predicted a lower level of analyte than that which can be accurately quantitated, while differences also existed for LOD values obtained from the two methods and it is therefore recommended that values for the LOD for the four phenothiazine derivatives under investigation should be specified by determination of an observable chromatographic peak in practice. These values are represented in Table 2.7. LOQ is expressed by the concentration of phenothiazine for which duplicate injections resulted in a relative standard deviation of $\leq$ 2%, Table 2.8.

Table 2.7 - Limits of Detection.

Phenothiazine Derivative	Practical LOD ($\times 10^{-4}$ mg/mL)	*IUPAC* LOD ($\times 10^{-4}$ mg/mL)
Prochlorperazine	5.0	8.4
Perphenazine	5.0	9.0
Trifluoperazine	4.0	6.1
Fluphenazine	5.0	6.1

Table 2.8 - Limits of Quantitation.

Phenothiazine Derivative	Practical LOQ ($\times 10^{-3}$ mg/mL)	*IUPAC* LOQ ($\times 10^{-3}$ mg/mL)
Prochlorperazine	3.5	2.8
Perphenazine	3.0	3.0
Trifluoperazine	4.0	2.0
Fluphenazine	4.0	2.0

2.5.4 Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, including different laboratories, analysts, instruments, batches of reagents, assay temperatures, different days, etc. Ruggedness is the lack of influence on test results of operational and environmental variables of the method.

In recent years regulatory authorities have become increasingly interested in analytical methods and their validation, and have stressed the need for an analytical method or methods to be capable of providing a mass balance throughout the stability study. This implies that if the formulation initially contains 0.1% (m/m) of active ingredient, then the value of active plus degradation product(s) (if present) must be maintained at 0.1% (m/m) throughout the period of the stability study.[54]

Method

The ruggedness of an analytical method is demonstrated by running composite samples on each of two days using different analysts and analysing (14 days) in the laboratory (fluorescent light / diffuse daylight) and in the dark.

The ruggedness test was performed on two separate days, instruments and columns. Six separate assay preparations of six individual weighings were performed on each day for each phenothiazine derivative: prochlorperazine, perphenazine, trifluoperazine and fluphenazine. Standard preparations prepared for each phenothiazine derivative, were injected in duplicate and a mean peak height (mm) was calculated for each sample.

Day 1

Column:	µBondapak C18 (3.9 x 300 mm i.d.) 10 µm, 125Å	
Analyst:	Patricia Drummond	
Instrument:	Spectra-Physics Iso Chrom LC Pump	SP8700
	Pye Unicam LC-UV Detector	298823

Day 2

Column:	Bondex C18 (3.9 x 300 mm i.d.) 10 µm, 125 Å	
Analyst:	Sagaran Abboo	
Instrument:	Waters Associates Chromatography Pump	6000A
	Waters Millipore Wisp	710B
	Kratos UV Detector	SF769

In order for the method to assess the stability of samples for injection and to determine whether the principle of mass balance applies, standard preparations of each derivative which were prepared for ruggedness studies were allowed to stand for 14 days, one set was protected from light, while a second set was exposed to normal laboratory light (fluorescent

/ diffuse light). Chromatograms of each sample after 14 days were compared with the initial chromatograms obtained on preparation of the samples.

Results and Discussion

The results for prochlorperazine, perphenazine, trifluoperazine and fluphenazine ruggedness studies are summarised in Tables 2.9 - 2.12.

Table 2.9 - Ruggedness Study for Prochlorperazine.

	Day 1	Day 2
Mean % Claim	100.9	101.3
% RSD	0.72	1.69

RSD between day 1 and day 2 = 0.97

Table 2.10 - Ruggedness Study for Perphenazine.

	Day 1	Day 2
Mean % Claim	101.16	102.4
% RSD	1.19	1.83

RSD between day 1 and day 2 = 0.64

Table 2.11 - Ruggedness Study for Trifluoperazine.

	Day 1	Day 2
Mean % Claim	101.22	102.58
% RSD	0.99	1.19

RSD between day 1 and day 2 = 0.2

Table 2.12 - Ruggedness Study for Fluphenazine.

	Day 1	Day 2
Mean % Claim	101.83	103.6
% RSD	0.99	1.52

RSD between day 1 and day 2 = 0.53

Mass balance investigations of the derivatives gave the following results: the chloro-derivatives were least stable on standing, and their stability was independent of the storage conditions, with approximately 25.58% degradation evident in solutions of prochlorperazine protected from diffuse daylight and 39.68% in solutions exposed to laboratory conditions for 14 days. Degradation of these derivatives gave rise to the development of two unidentified compounds. Samples of the trifluoromethyl-derivatives were stable on standing when protected from light (0% degradation), while samples exposed to laboratory conditions, i.e. diffuse daylight indicated approximately 40% degradation for trifluoperazine resulting in the formation of two unidentified compounds. The formulations initially contained 5% (m/m) of active ingredient and the value of active plus degradants was maintained at 5% (m/m), which is an indication of the ability of the HPLC method to resolve all potential degradants. It can therefore be concluded that samples of the derivatives should be injected immediately after preparation.

2.5.5 Selectivity / Specificity

The selectivity of an analytical method is its ability to measure, with a requisite level of accuracy and precision, the analyte in the presence of components that might be expected to affect the method's sensitivity to potential sample-related interferents reflecting the system's ability to resolve all other sample components that will give a detector response from the peak of interest.

A series of injections were performed to verify or exclude the existence of impurities in the

mobile phase, solvent, unstressed and stressed phenothiazine derivatives. The respective phenothiazine derivatives were stressed under the following conditions: acid and base, oxidation, ultraviolet irradiation, fluorescent / diffuse daylight, sunlight and heat.

Method

Triplicate injections of the mobile phase, solvent, and unstressed standard preparations were undertaken for the respective phenothiazine derivatives providing standards against which the samples stressed under the above conditions could be assessed. Stock preparations of the derivatives were prepared and stressed accordingly.

Acid and Base Degradation

100 mg of each phenothiazine derivative was accurately weighed and dissolved in 4 mL of methanol in a 100 mL volumetric flask and 96 mL of 3 M HCl or NaOH was added to volume. The solutions were refluxed for 2 hours over a water-bath at 83°C. Standard preparations of the respective stressed solutions were assayed in triplicate.

Oxidation

The respective phenothiazines were subject to the nitrous acid method of oxidation reported by Owens et al.[7] which involved the oxidation of an aqueous solution of each derivative by the addition of sodium nitrite and 9 M HCl. This resulted in oxidation of the phenothiazines to their respective sulphoxides.

Photolysis

30 Watt Ultraviolet Philips Lamp (G 30 TH)[41, 42]

Solution

10 mL of the stock preparation of the phenothiazine derivatives was placed in a series of clear glass ampoules which were heat sealed and stressed under a 30 Watt UV Philips Lamp for

a period of 32 days, sampling at 4 day intervals. Standard preparations of the stressed samples were assayed in triplicate.

Solid

Powder samples of the phenothiazine derivatives were placed in clear glass petri dishes and stressed under a 30 Watt UV Philips Lamp for a period of 5 months, sampling at monthly intervals. Standard preparations of the stressed samples were assayed in triplicate.

400 Watt High Pressure Mercury Lamp

Solution

Stock preparations of the respective phenothiazines were stressed under a 400 Watt UV lamp in an immersion-well photoreactor for a duration of approximately 2 hours, sampling at 10 minute intervals for the first 30 minutes, then 15 minute intervals for a further 30 minutes, and finally 30 minute intervals for the remaining time. Standard preparations of the stressed samples were assayed in triplicate.

Sunlight and Fluorescent / Diffuse Daylight (Philips, 55W/33, S72T12)[45]

Solution

10 mL of a stock preparation of the respective phenothiazine was placed in a series of clear glass ampoules which were heat sealed and stressed, either under fluorescent lights for a period of 32 days, sampling at 4 day intervals, or on a windowsill in direct sunlight for a period of 90 minutes, sampling at 15 minute intervals. Standard preparations of the stressed samples were assayed in triplicate.

Heat (Memmert Oven, F-Nr 830 281, 50 Hz, 2800 W, 220°C, Schwaback, W-Germany)

Solution and Solid

Stock preparations in pyrex volumetric flasks and powder samples of the respective

phenothiazine derivatives in glass petri dishes, were stressed at 60°C in a controlled environment for a period of approximately 2 hours, sampling at hourly intervals. Standard preparations of the stressed samples were assayed in triplicate.

Results and Discussion

Acid and Base Degradation

No degradation was apparent for the respective phenothiazine derivatives on exposure to acid.

The solubility of the respective phenothiazine derivatives is influenced by the nature of the substituents at the 2-position on the phenothiazine ring, with the trifluoromethyl-derivatives, trifluoperazine and fluphenazine, being readily soluble in water at neutral pH with a decrease in the pH reducing the solubility of these compounds, while the chloro-derivatives, prochlorperazine and perphenazine, are practically insoluble in water at neutral pH, yet dissolve readily on addition of acid. To obtain a solution of the four derivatives, methanol was added prior to the 3 M NaOH which resulted in turbidity in the case of prochlorperazine and perphenazine with a further solubility problem being experienced in the refluxed solutions for the two phenothiazine derivatives containing β-hydroxyethyl groups attached to the piperazine side-chain i.e. fluphenazine and perphenazine. These solubility problems did not permit an accurate assessment of the stability of the derivatives under basic conditions.

Oxidation

Literature reports suggest that the accepted method for the assessment of the oxidative stability of compounds involves dissolving the respective derivatives in water with the addition of hydrogen peroxide.[7, 24, 45] The solution is then either left to stand overnight with stirring or heated for several hours.

Solubility of the two chloro-derivatives, prochlorperazine and perphenazine in water precludes the use of this method of oxidation in stability assessments for these compounds.

A recent investigation by Al-Obaid et al. reports the preparation of the sulphoxides of three phenothiazine derivatives: perphenazine, trifluoperazine and triflupromazine based on Davidson's procedure involving the dissolution of the derivatives in methanol, prior to the addition of the oxidizing reagent, a mixture of hydrogen peroxide and glacial acetic acid which overcomes the problem of poor water solubility for the chloro-derivatives.[47] However the inclusion of methanol may result in the formation of solvent addition products as has been reported by Sharples in the presence of light.[36]

As sulphoxidation has been reported as a major degradation pathway for the phenothiazine derivatives, oxidative degradation of the solutions to form the respective sulphoxides was a necessary component of the specificity parameter of the method validation.

Owens et al. reported a nitrous acid method of oxidation,[7] which with some adjustments was developed as the method, for the complete oxidation of the four phenothiazine derivatives under investigation (chapter 4). It is however noted that the method for the oxidation of both chloro- and trifluoromethyl-derivatives by Al-Obaid et al. is able to serve as an alternative method for the oxidation of these derivatives.[47]

Ultraviolet Irradiation

Solution

The use of an immersion-well photoreactor (400 Watt UV source) provided an accelerated stress condition and exposure of the phenothiazine derivatives to the UV spectral range of below 310 nm because of the quartz nature of the reaction vessel. This is as opposed to the 30 Watt Philips UV source and the use of clear glass ampoules.

All solutions of the respective phenothiazine derivatives stressed in the presence of ultraviolet irradiation displayed definite changes in colour. These colour changes were less intense in those samples exposed to the 30 Watt UV Philips Lamp. HPLC analysis of these stressed solutions indicated the presence of several different photoproducts.

Solid

Degradation of the solid material as a result of exposure to the 30 Watt UV Philips Lamp was slow. The initial indication of degradation was observed in terms of a colour change of the surfaces of the phenothiazine derivatives exposed. The colour change of the surfaces of the derivatives stressed under the ultraviolet lamp could not adequately be accounted for in terms of a decrease in the height of the drug peaks, nor in the appearance of additional peaks on the chromatogram.

Fluorescent Light / Diffuse Daylight (350 Lux)

Degradation of the solutions stressed in the presence of the fluorescent lights were slow. All solutions stressed under the fluorescent lights became coloured with some indication of precipitation, the intensity of which varied for the respective derivatives.

Sunlight (Gossen Lunasix 3, Serial Number 2B00971, 44000 Lux)

Degradation of the solutions on exposure to direct sunlight was rapid. The extent of degradation of the stressed solutions after 90 minutes of exposure was approximately equivalent to that observed for samples stressed under the 30 Watt UV Philips Lamp, after 32 days.

Heat

Solution

Of the respective phenothiazine derivatives under investigation, only the two chloro-derivatives: prochlorperazine and perphenazine were affected by heating in solution. The percentage degradation of solutions of prochlorperazine and perphenazine on heating, was 21.18 and 1.46% respectively. Solutions of the trifluoromethyl-derivatives: trifluoperazine and fluphenazine were stable on heating under these conditions.

Pawelczyk et al. report the thermal degradation of aqueous and buffered solutions of the phenothiazine derivatives in the presence of aerial oxygen to proceed due to oxidation of the ring sulphur atom in the phenothiazine nucleus (sulphoxidation) *via* an intermediate step of free radicals for prochlorperazine and trifluoperazine, and that this is dependent on the nature of the R_2 substituent with the trifluoromethyl-derivative more stable than prochlorperazine.[57]

Solid

No degradation was apparent in any of the stressed powder samples, thus the respective phenothiazine derivatives were stable on heating in the solid state.

2.6 Analysis Using Photodiode Array Detection

Photodiode array detectors allow the evaluation of the homogeneity of chromatographic peaks which is important in support of method development as well as in support of the method currently in use.

As selectivity of a method is the ability of the procedure to measure an analyte in the presence of components that may be present in the sample matrix, it is reported that selectivity must be demonstrated by testing mixtures of inactive excipients, drug degradation products, and, as appropriate, synthetic impurities.

The need to ascertain the purity of the drug peaks in the stressed samples in order to justify their use in the development of degradation profiles leads to the use of photodiode array detection, as this technique allows the assessment of peak identity and integrity.

Photodiode Array HPLC Analysis

Reagents

All chemicals were of at least analytical grade. Acetonitrile (HPLC grade) was purchased from Burdick and Jackson. Water for chromatography was obtained using a Milli-Q Plus® water purification system, filtered with a Q Pak, purification pack suitable for water pretreated by reverse osmosis, consisting of an initial 0.5 µm prefilter, activated carbon, nuclear grade ion-exchange resin, and an Organex-Q organic scavenger mixture.

Final Chromatographic Conditions

Mobile Phase:	Acetonitrile: water: glacial acetic acid (60:40:1 v/v), 5×10^{-3} M sodium 1-pentane sulphonate.
Solvent:	Acetonitrile: water (70:30 v/v)
Column:	µBondapak, C18 (3.9 x 300 mm i.d.) 10 µm, 125 Å.
Flow Rate:	1 mL per minute
Injection Volume:	60 µL
Column Temperature:	Ambient

A modular HPLC Beckman System Gold consisting of a Programmable Solvent Modular 126 constant-flow pump, with a System Gold Diode Array Detector Module 168 UV detector set at 254 nm, scanning from 200 to 598 nm, an Olirite Computer System and a rheodyne fixed loop 20 µL injector (Model 7125) was used.

Standard Preparation

In order to provide standards against which the stressed samples could be assessed, a standard preparation of the respective phenothiazine derivatives was prepared by accurately weighing 25 mg of the phenothiazine derivative, and transferring to a 50 mL volumetric flask with addition of solvent to volume.

Sample Preparation

25 mg of the stressed solid material for the respective derivatives was accurately weighed and transferred to a 50 mL volumetric flask, which was made up to volume with solvent.

Method

Stock samples of the derivatives provided standards against which the stressed samples can be evaluated, which involved dissecting the peaks for the stressed and unstressed derivatives at three points: up slope, apex, and down slope with the sections for stressed and unstressed samples overlayed and normalized at 254 nm.

Results and Discussion

It is accepted, with the diode array detection system utilized for these investigations, that the occurrence of a single line on the baseline below the chromatogram signifies the elution of a single compound, while a double line below the peak(s) would indicate the coelution of two compounds. In addition to this, graph analysis of the stressed sample against a standard allows the investigation of the peak for spectroscopic homogeneity by the normalization of sections of the two peaks: stressed *versus* unstressed sample, at the λ_{max}. Two compounds however with identical spectra may coeulte and not be diagnosed. This introduces coefficients of correlation for the normalized regions. Deviation of the correlation coefficients, from 0.95, in conjunction with a double line on the baseline below the peak(s), suggests peak impurity, with the possible coelution of two compounds.

Solid

The colour change of the surfaces exposed to ultraviolet irradiation for the phenothiazine derivatives under investigation suggests the possible degradation of these samples. As no evidence of degradation by the appearance of additional peaks or a decrease in peak height of the drug as compared to a standard in the chromatogram was observed on HPLC analysis, for the solid materials stressed under the 30 Watt Philips UV lamp, photodiode array detection was employed to ascertain the integrity of the drug peaks for the respective derivatives, and to analyze specific areas of the drug peaks. Photodiode array determinations for the phenothiazine derivatives under investigation confirmed the integrity of the stressed samples, in spite of the surface effect observed in these samples, allowing for the only possible explanation to be that of a concentration effect.

Solution

The chromatograms obtained on the use of diode array detection of the stressed samples in solution displayed a single line below the respective parent phenothiazine peak. Photodiode array determinations of solutions of trifluoperazine, however, stressed in the presence of sunlight, as well as solutions stressed in the immersion-well photoreactor, suggested the coelution of two degradation products due to the occurrence of a double line below one of the degradant peaks in the chromatograms.

It has been reported that deviations of correlation coefficient data from 0.95, suggest the coelution of two compounds, this however, should be supported by the occurrence of a double line below that region of the chromatogram which would provide confirmation of impurity. In the absence of a double line, correlation coefficient data is not sufficient to prove the peak to be impure.

The correlation coefficients obtained for solutions under the various stress conditions deviate

in some instances from 0.95, however, the observation of a single line below the chromatogram provides strong evidence of the elution of a single compound. The integrity of the respective drug peaks has thus been proved. This deviation in correlation coefficients for the stressed samples could possibly be due to slight changes in pH of the stressed solutions causing changes in retention times.

Solutions of the phenothiazine derivatives under investigation have been reported to undergo changes in the pH of the solutions after being stressed under the above mentioned conditions.[6]

2.7 Conclusion

Clarke reported that the analysis of drug substances and finished products within the pharmaceutical industry is performed to satisfy both manufacturer and regulatory authority about the quality, integrity and stability of the medicine to be administered to the patient. A survey conducted by the Pharmaceutical Analytical Sciences Group (*PASG*) in 1992 determined the current practice employed in the validation of analytical methods for drug substances and drug products in the United Kingdom (UK) pharmaceutical laboratories. The survey was divided into method validation parameters employed for drug substance and drug products. The investigation indicates that the highest degree of consistency seen in the application of validation parameters to chromatographic techniques, specifically in the case of HPLC, where there was good agreement over which parameters should be applied to method validation. This reflects the universal application and dependence on this technique within the pharmaceutical industry.[49]

The investigation sought to determine the specific details of how each validation parameter was applied to a particular sample type and also the test. Validation parameters in the survey included: accuracy, precision, linearity, limit of detection / quantitation, ruggedness, selectivity,

and system suitability. The survey made evident the validation parameters most frequently applied to the validation of bulk active and finished products by all the pharmaceutical laboratories, to be selectivity, accuracy, precision, LOQ and LOD, solution stability, and linearity. Ruggedness, and sensitivity determinations were only applied in fewer than 60% of the cases.[49] The method for the validation of prochlorperazine, perphenazine, trifluoperazine and fluphenazine considers the validation parameters reported as used by pharmaceutical industries in the UK, which are acceptable to regulatory authorities.

In order to facilitate the evaluation of the validation parameters, acceptance criteria should be universally applicable, numerically and mathematically explicit and complete and achievable. Jenke reports acceptable limits for a number of validation parameters: accuracy, precision, specificity, linearity and sensitivity (LOD and LOQ) in Table 2.13.[58]

The results from the validation study demonstrate excellent precision and accuracy for the four derivatives, with no RSD values above 2% for precision studies and a maximum deviation from theoretical yield of 0.25% for samples of perphenazine only, providing a good indication of the method's performance. The range over which to perform linearity experiments is subject to some discussion. Pharmaceutical industries in the UK report that linearity is determined over a range of 50 - 150% for bulk drug and bulk drug impurity assays, while for finished product assays, the range is 75 - 125%, with a wider range for finished product degradant assays.[49] However, Edwardson et al. report that it is usual practice to perform linearity experiments over a range of 25 - 200% of the nominal concentration of analyte.[52] The range chosen for this investigation was 25 - 125% of the nominal analyte concentration to allow for valid quantitation of the active drug during the degradation studies. Regression analysis of mean peak height *versus* concentration for the four derivatives demonstrated proportional relationships, with correlation coefficients for the regression lines of 0.99 for perphenazine, trifluoperazine and fluphenazine, and 0.98 for prochlorperazine. These values

are within the acceptable limits for linearity.

Table 2.13 - Summary of Acceptable Limits of Validation Parameters.[58]

Validation Parameter	Acceptable Limits
Accuracy	98 -102 % of the theoretical value
Precision	≤ 2.0% RSD
Specificity	Baseline separation between peak of interest and all other analytical responses.
Linearity	Correlation coefficient of best linear least squares regression model between 0.98 - 1.00.
LOD and LOQ	No specific criteria exists.

The lowest amounts of analyte which can be reproducibly quantitated above baseline noise, for which duplicate injections resulted in a RSD ≤ 2% are 4.0×10^{-3}, 3.5×10^{-3}, 4.0×10^{-3}, and 3.0×10^{-3} mg/mL for trifluoperazine, prochlorperazine, fluphenazine and perphenazine respectively, while the lowest amount of analyte which can be detected above baseline noise for the four derivatives is 4.0×10^{-4} mg/mL for trifluoperazine, and 5.0×10^{-4} mg/mL for prochlorperazine, perphenazine, and fluphenazine respectively. The ruggedness of the method can be determined within-laboratory and between laboratories, and is usually assessed from the RSD obtained. In this investigation, ruggedness studies were performed by two analysts between laboratories. No RSD values were in excess of 2%, and the difference in RSD for the derivatives between the two days ranged from 0.2 to 0.97. The assay was also validated for any potential degradants in order that these compounds if present could be quantitatively assayed, which ensured that mass balance was demonstrated throughout the stability study.

The chromatograms of representative stressed samples containing degradation products for the derivatives, Figure 2.7, indicate the separation of the parent / active component from

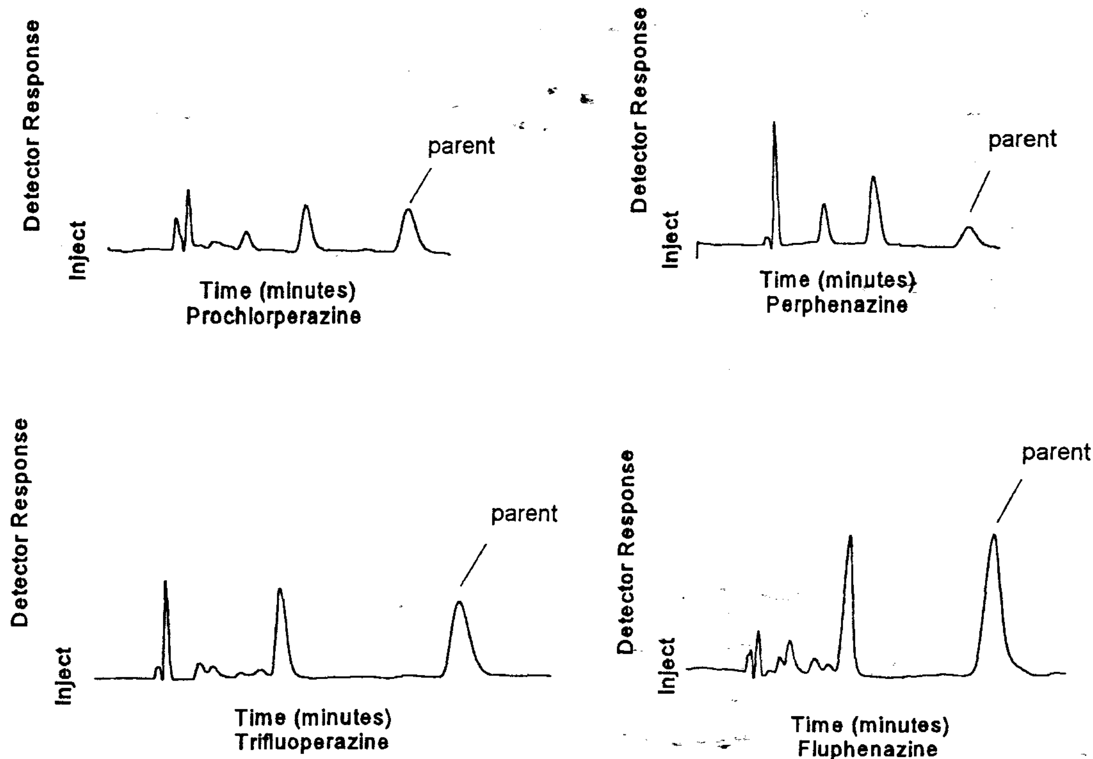


Figure 2.7 - Representative chromatograms of stressed solutions of the derivatives in a 400 Watt High-Pressure Mercury Lamp Immersion-Well Photoreactor.

interferences / degradants. No interfering peaks were observed in the chromatograms. The method is therefore specific / selective for prochlorperazine, perphenazine, trifluoperazine and fluphenazine.

A "typical" system suitability test applied to a chromatographic method includes tests for precision, selectivity, and chromatographic performance, such as tailing factor and column efficiency. The application of these system suitability procedures is extensively used in the UK pharmaceutical industry.[59] The results obtained for the system suitability tests conducted with the four derivatives demonstrate the suitability of the method with different systems for the compounds under investigation, and in addition all experimental results are well within the

limits set for the variables.

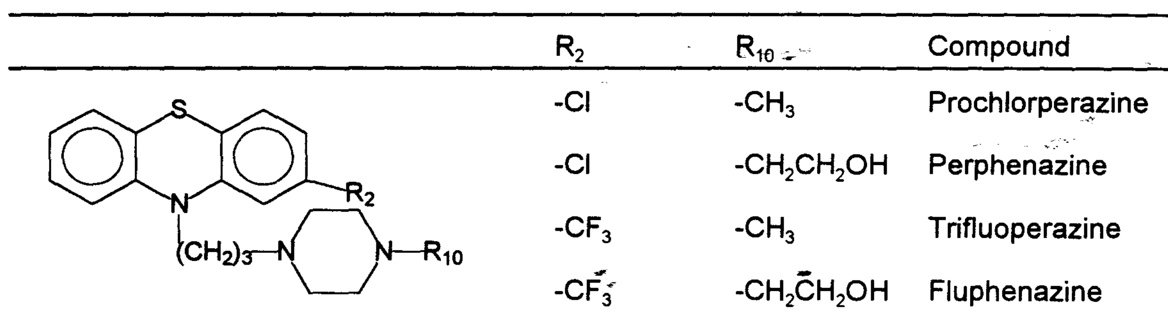
It can therefore be concluded that this initial validation of the analytical method for the four piperazine-substituted phenothiazine derivatives: prochlorperazine, perphenazine, trifluoperazine and fluphenazine provides preliminary assurance of the reliability of the analytical procedures with further changes during development inevitable. This information provides the foundation from which a new validation protocol can be prepared for subsequent investigations. Development and validation of the HPLC method for the assay of the four derivatives has provided some information on the susceptibility of the drugs to degradation with the proposed degradation pathways reported in chapter 5, and ensured the absence of assay interference by degradants and solvents employed in sample preparation. Thus the foundation for the determination of the minor components / degradants has been provided.

CHAPTER 3: IDENTIFICATION AND PRELIMINARY LIGHT STABILITY STUDIES

Chapter 3 deals with: confirmation of the identity of the four piperazine-substituted phenothiazine derivatives used in this study: prochlorperazine dimaleate, perphenazine, fluphenazine dihydrochloride and trifluoperazine dihydrochloride in the form of appearance and solubility characteristics (relevant in chapter 4), comparative literature and experimental melting points, IR, UV, MS and NMR values as well as further information on the preliminary stability studies in the presence of various light sources.

3.1 Identification

Table 3.1 - Four Piperazine-Substituted Phenothiazine Derivatives.



	R_2	R_{10}	Compound
	-Cl	$-CH_3$	Prochlorperazine
	-Cl	$-CH_2CH_2OH$	Perphenazine
	$-CF_3$	$-CH_3$	Trifluoperazine
	$-CF_3$	$-CH_2CH_2OH$	Fluphenazine

Prochlorperazine dimaleate:

2-Chloro-10-[3-(4-methyl-1-piperazinyl)propyl]-10H-phenothiazine dimáleate, is characterized by a white or pale yellow, almost odourless, crystalline powder, with a slightly bitter taste.[59]

Perphenazine:

4-[3-(2-Chlorophenothiazin-10-yl)propyl]-1-piperazineethanol, is characterized by a white or creamy-white odourless or almost odourless powder with a bitter taste.[59]

Trifluoperazine dihydrochloride:

10-[3-(4-Methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine dihydrochloride, is characterized by a white to pale yellow, odourless or almost odourless, hygroscopic, crystalline powder, with a bitter taste.[59]

Fluphenazine dihydrochloride:

4-[3-[2-(Trifluoromethyl)-10H-phenothiazin-10-yl]propyl]-1-piperazineethanol dihydrochloride, is characterized by a white or almost white, odourless, crystalline powder with a bitter taste.[59]

3.1.1 Methodology

Infrared Spectra

The infrared spectra of the four derivatives were recorded on a Perkin Elmer FT-IR Spectrometer, Spectrum 2000, FT01 (KBr-diffuse reflectance). The comparative literature values for trifluoperazine (mineral oil dispersion)[60] and fluphenazine (KBr from methanol)[61] were measured on a Perkin-Elmer Model 457A and 621 respectively.

Ultraviolet Spectrometry

The ultraviolet spectra for all four derivatives were obtained in 95% ethanol on a GBC UV/Vis Model 916, while comparative literature spectra were recorded in 95% ethanol on a Cary Model 15.[60, 61]

Nuclear Magnetic Resonance Spectroscopy

The ^{1}H- and ^{13}C-NMR experiments were performed at 400.14 MHZ using a Bruker NMR spectrometer. The probe temperature was regulated at 303 ± 0.1 K with all spectra being recorded in MeOH-d_4 with the exception of the ^{1}H-NMR spectrum of fluphenazine which was recorded in D_2O. The ^{1}H- and ^{13}C-NMR spectra of trifluoperazine from the literature were recorded in $CDCl_3$ on a Perkin Elmer 32 (^{1}H) / Varian Associates Model FT-80 (^{13}C) with tetramethyl silane as internal standard for trifluoperazine.[60, 61]

Mass Spectrometry

Experimental MS data were obtained on a Hewlett Packard 5890 low resolution mass spectrometer, using the CI mode, while literature reports the mass spectrum of trifluoperazine to be obtained by direct insertion into an Hitachi Perkin-Elmer RMU-6E low resolution mass spectrometer and that of fluphenazine by low resolution on a MS-9.[60, 61]

3.1.2 Results and Discussion

Table 3.2 - Characterization of Four Piperazine-Substituted Phenothiazine Derivatives.

Compound	Molecular Mass[59]	Molecular Formula[59]	Melting Point (°C)		Salt
			Literature[59]	Experimental*	
Prochlorperazine	606.0891	$C_{28}H_{32}ClN_3O_8S$	198.0-203	198.0-200	dimaleate
Perphenazine	404.0000	$C_{21}H_{26}ClN_3OS$	96.0-100	95.0-97	base
Trifluoperazine	480.4177	$C_{21}H_{26}Cl_2F_3N_3S$	242.0-243	242.0-244	dihydrochloride
Fluphenazine	510.4439	$C_{22}H_{28}ClF_3N_3OS$	224.5-226	220.0-223	dihydrochloride

*Gallenkamp Melting Point Apparatus, 50 Hz, 220/240 Volt

Table 3.3 - Solubility Properties of Four Piperazine-Substituted Phenothiazine Derivatives.[59]

Compound	Water	Alcohol	Chloroform	Ether	Acetone	Hydrochloric Acid	Isopropyl Alcohol
Prochlorperazine	Practically Insoluble	Practically Insoluble	Slightly Soluble in Warm	Practically Insoluble	-*	-*	-*
Perphenazine	Practically Insoluble	1 in 20	1 in 1	1 in 80	1 in 13	Soluble in Dilute	-*
Trifluoperazine	1 in 2	1 in 11	1 in 100	Practically Insoluble	-*	-*	Slightly Soluble
Fluphenazine	1 in 10 or less	Slightly Soluble	Slightly Soluble	Practically Insoluble	Slightly Soluble	-*	-*

*no data available

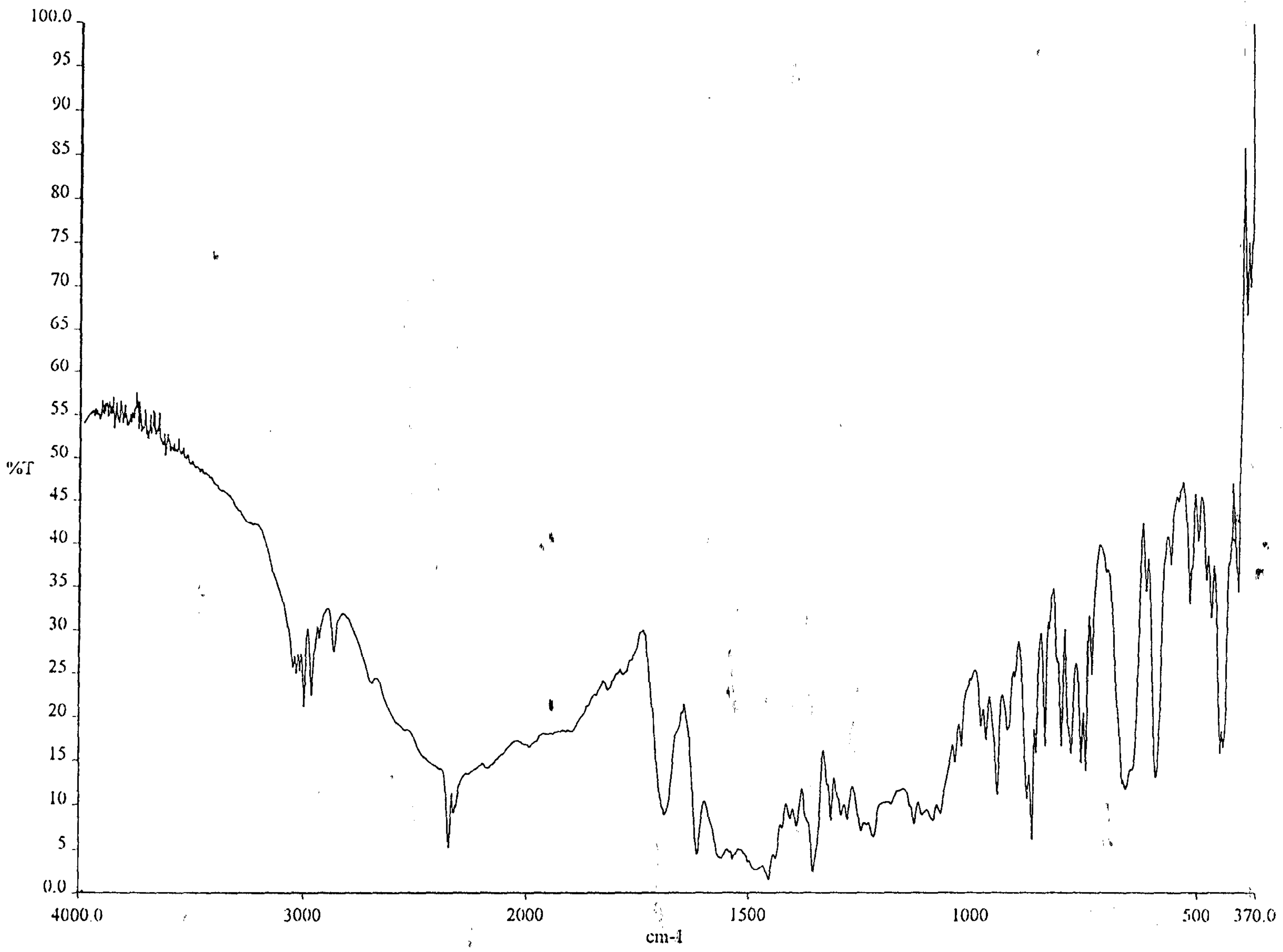


Figure 3.1 - Infrared spectrum for prochlorperazine.

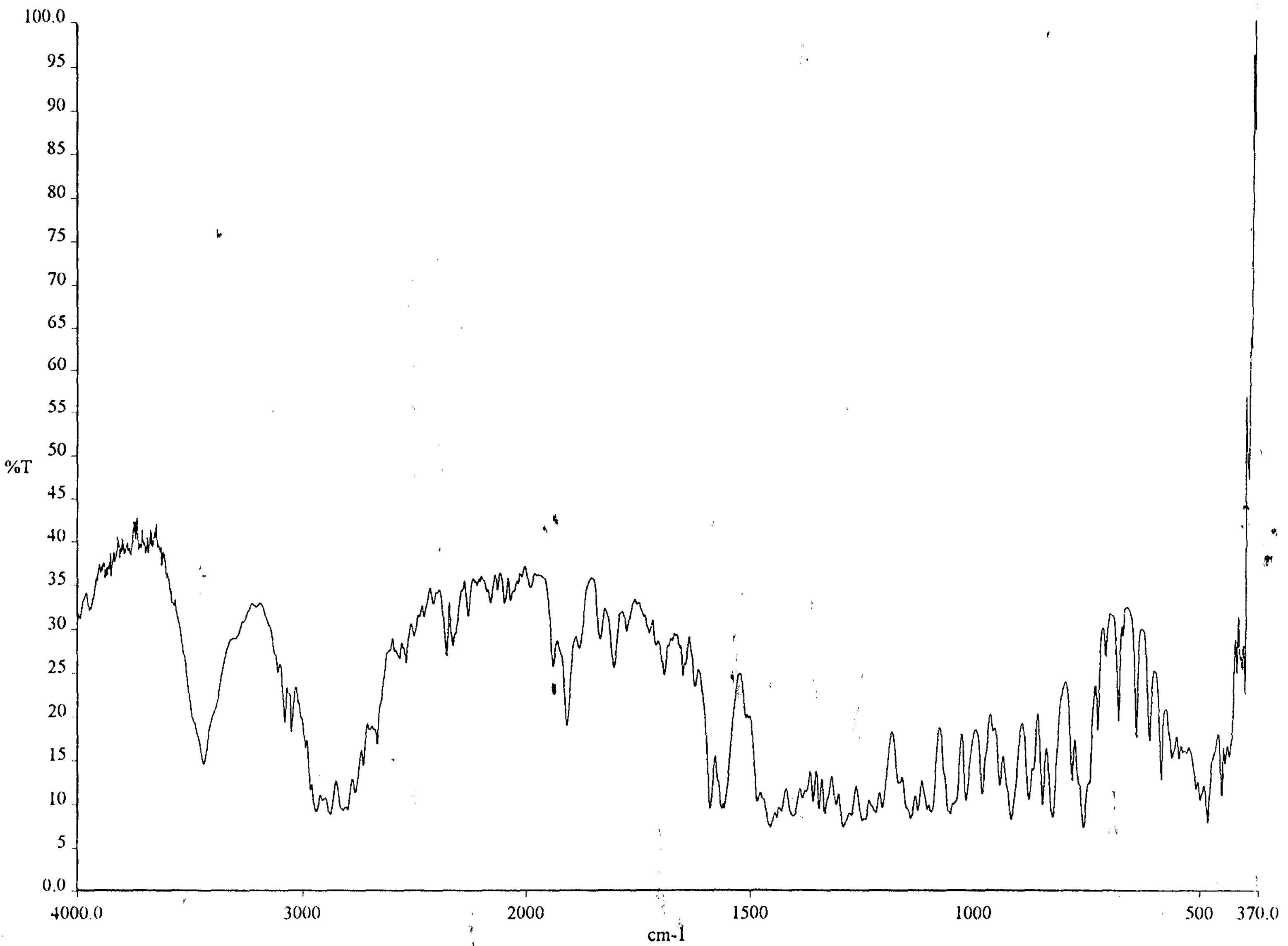


Figure 3.2 - Infrared spectrum for perphenazine.

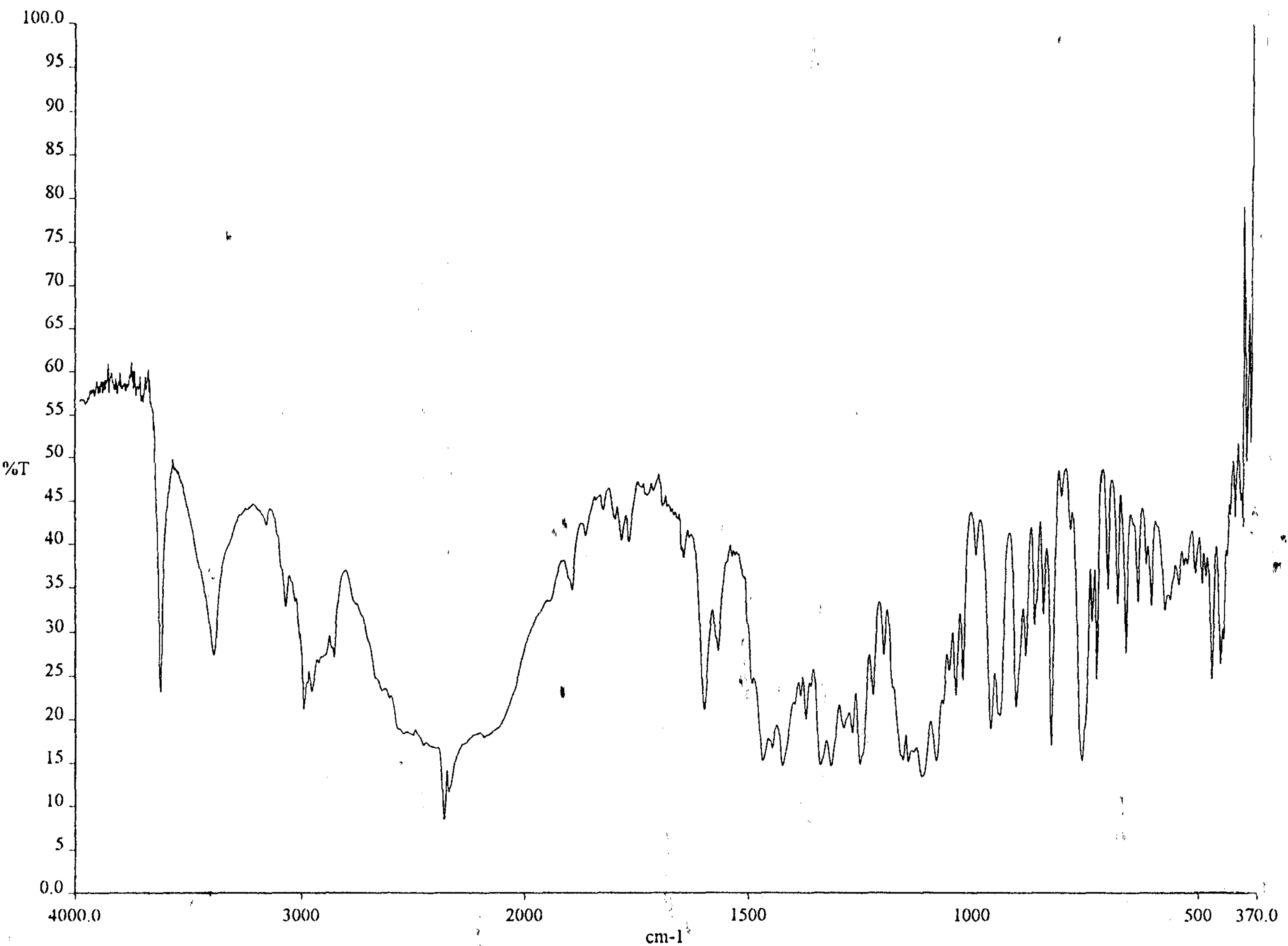


Figure 3.3 - Infrared spectrum for trifluoperazine.

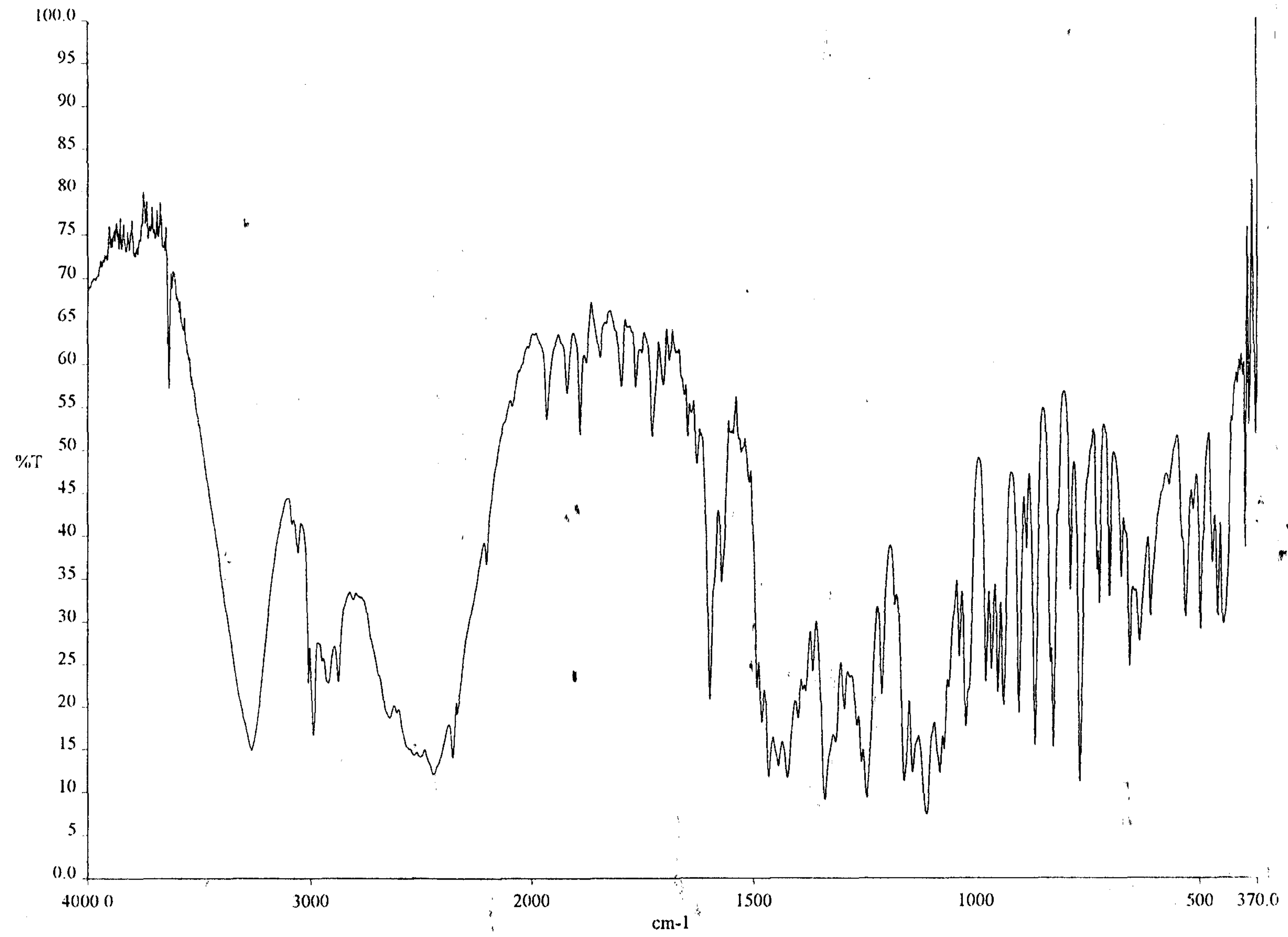


Figure 3.4 - Infrared spectrum for fluphenazine.

Table 3.4 - Infrared Spectral Assignment of Bands for Four Piperazine-Substituted Phenothiazine Derivatives.

Assignment	Prochlorperazine	Perphenazine	Trifluoperazine		Fluphenazine	
	Experimental	Experimental	Literature[60]	Experimental	Literature[61]	Experimental
	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
C=C, aromatic	1630, 1460	1620, 1460, 1410	1600, 1570, 1470	1600, 1430	1600, 1570, 1470	1600, 1470, 1415
CF_3	-*	-*	1320, 1340, 1115	1320, 1370, 1125	1340, 1115	1330, 1125
1,2,4-trisubstituted aromatic	880		829	805	829	815
1,2-substituted aromatic	750	730	760	725	760	730

* not applicable

Table 3.5 - Ultraviolet Spectra of Four Piperazine-Substituted Phenothiazine Derivatives in 95% Ethanol.

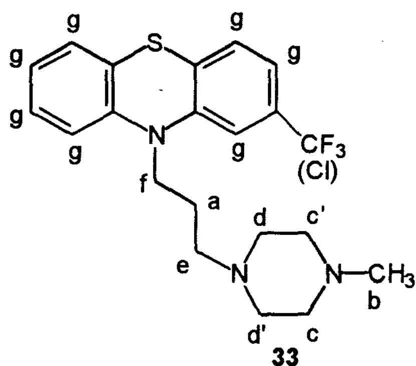
Prochlorperazine		Perphenazine		Trifluoperazine				Fluphenazine			
Experimental		Experimental		Literature[60]		Experimental		Literature[61]		Experimental	
λ (nm)	Log ε	λ (nm)	Log ε	λ (nm)	Log ε	λ (nm)	Log ε	λ (nm)	Log ε	λ (nm)	Log ε
312.0	0.84	310.7	1.21	307.5	3.50	229.8	1.51	239.0	4.28	229.8	1.38
286.4	0.63	287.7	1.01	264.0	4.50	287.4	1.70	264.0	4.59	254.1	1.58
250.6	1.58	251.8	1.84			254.1	0.80	300.0	3.51	287.4	0.66
236.5	1.52	232.6	1.70			313.0	1.02	316.0	3.69	311.7	0.87

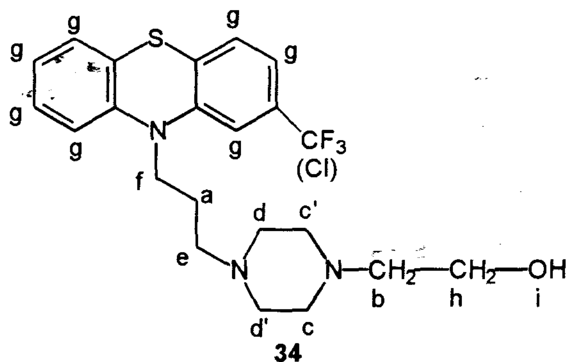
Table 3.6 - Mass Spectrometry Experimental Data for Four Piperazine-Substituted Phenothiazine Derivatives.

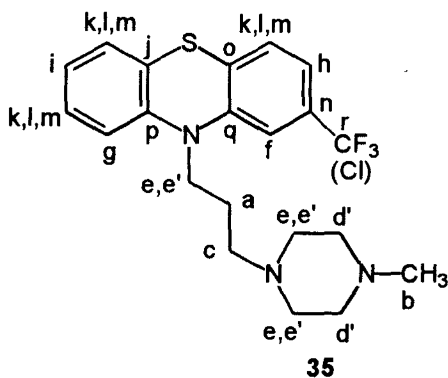
Prochlorperazine			Perphenazine		
Ion	*m/z*	Relative abundance (%)	Ion	*m/z*	Relative abundance (%)
M^+	373	18.33	M^+	403	22.40
$(M-C_5H_{13}N_2)^+$	272	10.07	$(M-C_6H_{15}N_2)^+$	272	8.37
$(M-C_7H_{15}N_2S)^+$	214	10.66	$(M-C_7H_{17}N_2O)^+$	246	100.00 (base peak)
$(C_6H_{13}N_2)^+$	113	100.00 (base peak)	$(C_7H_{15}N_2O)^+$	143	0.73
$(C_5H_{10})^+$	70	77.74			

Table 3.6 continued

Trifluoperazine			Fluphenazine		
Ion	*m/z*	Relative abundance (%)	Ion	*m/z*	Relative abundance (%)
M^+	407	23.82	M^+	437	22.06
$(M-C_5H_{13}N_2)^+$	306	11.49	$(M-C_6H_{15}N_2)^+$	306	6.50
$(M-C_7H_{15}N_2S)^+$	248	17.83	$(M-C_7H_{17}N_2O)^+$	280	100.00 (base peak)
$(C_6H_{13}N_2)^+$	113	0.69	$(C_7H_{15}N_2O)^+$	143	73.32
$(C_5H_{10})^+$	70	100.00 (base peak)	$(C_5H_{10})^+$	70	64.91







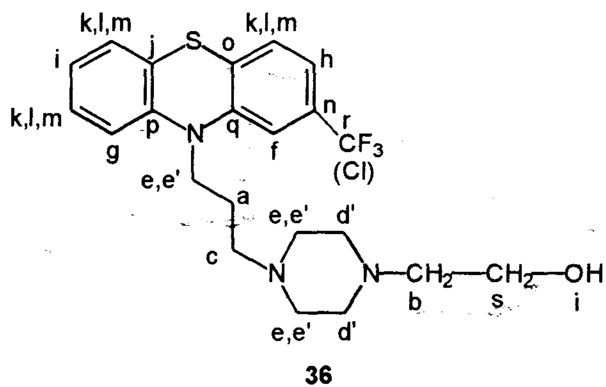


Figure 3.5 - Assignment of protons for a typical piperazine-substituted phenothiazine (33) methyl derivative and (34) β-hydroxyethyl derivative (^{1}H-NMR), (35) methyl derivative and (36) β-hydroxyethyl derivative (^{13}C-NMR).[60]

Table 3.7 - ^{1}H-NMR Data for Four Piperazine-Substituted Phenothiazine Derivatives.

Protons	Assignment	Prochlorperazine	Perphenazine	Trifluoperazine		Fluphenazine
		Experimental	Experimental	Literature[60]	Experimental	Experimental
		ppm	ppm	ppm	ppm	ppm
a	Multiplet	1.3509	1.9640	1.94	1.3107	2.1633
b	Singlet / Triplet*	2.1957	2.2587	2.25	2.2819	3.2912
c, c'						
d, d'	Broad Multiplet	2.0009 - 2.3526	2.5379 - 2.5906	2.3 - 2.6	2.1603 - 2.3378	3.4993 - 3.7407
e						
f	Triplet	4.0752	3.9996	3.96	4.1923	4.0214
g	Broad Multiplet	6.9760 - 7.4148	6.9655 - 7.1742	6.75 - 7.30	7.0525 - 7.3560	6.8586 - 7.2723
h	Triplet		3.7389			3.4993
i	Broad Singlet		3.4153			3.8849

Table 3.8 - ^{13}C-NMR Data for Four Piperazine-Substituted Phenothiazine Derivatives.

Carbon	Prochlorperazine	Trifluoperazine	
	Experimental	Literature[60]	Experimental
a	24.89	24.15	22.89
b	46.27	45.23	45.29
c	46.79	45.95	45.75
d	52.80	53.26	51.30
e	54.60	55.16	55.77
f	113.40	110.80	113.45
g	116.80	115.88	117.85
h	119.60	118.83	120.74
i	123.70	122.96	124.61
j	125.60	123.84	126.63
k	128.35	127.54	128.79
l	128.76	127.39	129.01
m	128.99	127.30	129.29
n	126.26	129.56	131.50
o	133.67	129.79	132.50
p	145.60	144.23	145.18
q	146.80	145.68	147.28
r	-*	124.29	130.57

* not applicable

Table 3.8 continued

Carbon	Perphenazine Experimental	Fluphenazine Experimental
a	24.91	22.90
b	46.13	45.28
c	46.75	45.78
d, d'	54.01	55.81
e, e'	56.33	56.54
f	117.09	113.44
g	117.38	117.86
h	123.31	120.74
i	124.13	124.96
j	125.10	126.32
k	128.38	128.80
l	128.73	129.02
m	128.97	129.30
n	126.04	132.58
o	134.37	134.37
p	145.79	145.20
q	148.03	147.30
r	-*	131.05
s	59.80	59.52

* not applicable

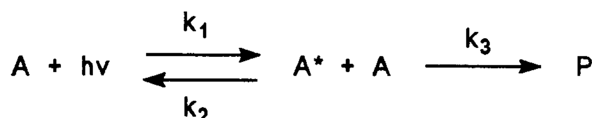
3.2 Light Stability Studies

3.2.1 Introduction

A study of instability problems in pharmaceutical products is important since there are at least six possible results of drug product instability i.e. loss of the active drug, vehicle and content uniformity, reduction of bioavailability, impairment of pharmaceutical elegance and production of potentially toxic materials. Light stability studies may have implications in terms of loss of

the active drug, its effect on the elegance of the preparation and the production of potentially toxic photoproducts and this study focuses on the loss of the active drug and the resulting production of degradants.[62] Since normal stress long-term stability studies are sometimes unacceptable to the industry in terms of time constraints there is a need to accelerate physicochemical changes through high stress short-term situations, however, these predictive measures are more risk-orientated the higher the stress employed, since it is possible that such conditions will give rise to reaction rates, mechanisms and by-products which are different from those under normal stress conditions. There are various factors affecting the stability of a solid dosage form, including moisture and pH, oxygen, light, and the crystalline state. Many drugs are subject to oxidative degradation on exposure to dry heat, while others undergo air oxidation or other chemical reactions in the presence of oxygen. Pharmaceutical compounds such as vitamins, sulphonamides, antibiotics, local anaesthetics, steroids and phenothiazine derivatives are susceptible to photolytic deterioration.[63]

A simple method for photolytic degradation is illustrated in Scheme 3.1 where A is excited to



Scheme 3.1[63]

A* which may return to the ground state or react with a second molecule and decompose into products (P). On irradiation of coloured solid dosage forms, the fading rate is greatest during the initial stage confirming that photolysis is a surface phenomenon. Drug stability is affected by both the intensity and the spectral character of radiation with ultraviolet light, not withstanding its lower energy than X- and γ-rays causing as much photolytic damage. Protection for a UV-sensitive formulation can be effected by packaging or the inclusion of an excipient which absorbs in the desired UV region.[63] A stability liability with respect to certain liquid dosage forms, particularly small volume parenterals is photochemical and oxidative decomposition reactions. Although both of these reactions are free radical mediated, it must

be noted that a photochemical reaction can occur in the absence of oxygen and oxidative reactions can occur in the absence of the catalytic effect of light. In the case of ampoules, the large surface area to formulation volume ratio allows maximum light impingement on a relatively dilute drug solution and the short path length. In addition, the head space gas to formulation volume offers a presence of molecular oxygen. As previously mentioned in the solid state studies, stress testing may not be meaningful for stability projections, especially for a true chain reaction with an efficient propagation step. Irradiation stress testing is widely used from a qualitative point of view, although the quantitative estimate of photolytic decomposition based on stress testing has proved to be difficult. From a practical point of view for the pharmaceutical scientist the first 10-20% degradation on irradiation of a commercial dosage form is most significant. Approximate first-order behaviour has been predicted for dilute solutions, but approaches pseudo-zero-order kinetics in more concentrated solutions. In the work by Mendenhall further research into the use of accelerated light stability to accurately project decomposition rates under normal room lighting is continuing. Purging the solution and head space with nitrogen, inclusion of anti-oxidants and chelating agents and the use of light protective packaging in the form of amber glass ampoules are available to the formulator to enhance stability. However, in addition to inspection difficulties and cost considerations associated with amber glass ampoules, leaching of iron and / or manganese present in amber glass may cause decreased stability associated with some drug products.[64]

The degradation of pharmaceutical products may be classified according to chemical, physical, biological or a combination of more than one of these mechanisms. Although kinetics has received recognition in assisting to deal with chemical problems, there are certain limitations to be considered. Rhodes reports the limitations that the accuracy, precision, sensitivity and specificity of the assay places on the kinetic interpretation of data and the fact that often only the first 10% of the degradation reaction is of interest, such that the range of experimental concentration values are small and inhibit the ability to differentiate a zero- from a first-order reaction. These limitations expressed by Rhodes are partially offset by the fact

that chromatographic methods, especially HPLC allow the development of stability- indicating assays of acceptable precision.[62] Moore further expresses reservations about the kinetic treatment of, in this case photochemical reactions. Although the rate of photodegradation has been quantified in terms of a rate constant, the difficulty associated with reporting kinetics photochemically as opposed to the thermal reaction is due to the dependence of such reactions on wavelength and intensity of the irradiating source and shape and distance of the reaction vessel from the source. Thus although the rate of photodegradation of a dilute solution of a drug may approximate first-order kinetics, these mixtures may only be compared if exactly the same irradiation conditions are applied. For comparison of photoreaction rates under different irradiation conditions, the experimental arrangement should be calibrated using the ferrioxalate chemical actinometer.[65]

Various light stability studies have been reported under aerated conditions and are discussed in terms of the methodology used, its influence on and the kinetics of photodecomposition. Midazolam was irradiated in ethanol due to its faster decomposition than in aqueous solution with the formation of the same photoproducts other than the solvent addition product[66]. However, while irradiation of furosemide in aqueous solution gave rise to the dechlorinated product, in methanol photoreduction and hydrolysis were reported.[67] In a study on the light degradation of ketorolac tromethamine, photolysis in ethanol resulted in 94-101% of reacted drug being accounted for, while in water the balance of the products was pH-dependent.[68] The degradation of midazolam, furosemide and ketorolac tromethamine all followed good first-order kinetics and in the case of the tromethamine salt at a concentration of $\leq$ 2 µg/mL, however at a concentration $\geq$ 10 µg/mL non first-order kinetics were displayed. The fact that at this concentration the solution is no longer optically thin is likened to the increase in colour intensity of solutions of midazolam (15 mM and 20 mM), slowing down the rate of reaction and resulting in a departure from linearity.[66, 67, 68]

Of direct relevance to this study are the reports by Pawelczyk et al. who discuss the thermal and photochemical degradation of aqueous and buffered solutions of perazine derivatives and the fact that the quantum yield of photooxidation was used for correlation with substituent volumes highlights the importance of the R_2 substituent in these reactions.[41, 42, 57]

Due to information available on the photochemical degradation it is assumed that thermal degradation results in sulphoxidation of the majority of these compounds. In these three reports findings in respect of prochlorperazine and trifluoperazine are relevant to this investigation.[41, 42, 57] Hammett plots and calculated thermodynamic parameters indicate that a halogen substituent of type X or CX_3 at the 2-position on the phenothiazine nucleus results in an increase in electron density on the sulphur atom by induction and resonance causing inhibition of oxidation and thus a decrease in reactivity in relation to the unsubstituted compound. Thus the R_2 substituent influences the thermal degradation of these compounds with trifluoperazine exhibiting greater stability than prochlorperazine.[57] When considering the photochemical degradation in acidic aqueous solutions of these perazine derivatives, it is reported that only perazine gives rise to two parallel reactions of photolysis and photooxidation with R_2 substituents such as Cl and CF_3 preventing photolysis. In a further paper Pawelczyk describes the effect of substituents on the photochemical stability of perazine derivatives by determining physicochemical parameters such as the quantum yield of photooxidation, values of pK_{a1} and pK_{a2} and volumes of the substituents at the 2-position on the phenothiazine nucleus in order to allow for a broader interpretation of the kinetic results of their previous papers.[41, 42]

Results showed that the basicity of these compounds increases with an increase in the substituent volume at the 2-position on the phenothiazine nucleus and the Hammett plot confirms a relationship between the basicity and the rate of degradation i.e. the greater the substituent volume at the 2-position on the phenothiazine nucleus, the lower the degradation under the influence of light. The double lined Hammett plot suggesting two different mechanisms of the reactions confirms the findings of the previous paper. To conclude the

findings based on substituent volumes it is observed that trifluoperazine is more stable to photochemical degradation under these conditions (a low-pressure TUV 30 Philips UV lamp with 254.7 nm of wavelength) than prochlorperazine. Thus these results indicate similar thermal and photochemical stability for these two derivatives of perazine.[41, 42, 57]

3.2.2 Methodology

A 1 mg/mL solution (in triplicate) of all four piperazine-substituted phenothiazine derivatives in buffer (KH_2PO_4 / NaOH, pH 6.4) was exposed to the following light conditions, Table 3.9 with subsequent analysis by HPLC (chapter 2).

3.2.3 Results and Discussion

Tables 3.10 to 3.13 summarise the percentage drug remaining for each of the four derivatives stressed under the four light conditions studied.

Exposure of the propyl piperazine-substituted derivatives to the 30 W Philips UV source, sunlight and fluorescent 55 W Philips source / diffuse daylight does allow for some comparison to be made in respect of their degradation rates, the systems were not de-aerated. Since the study on the four derivatives in the presence of each light condition was conducted simultaneously the rates of degradation are thus dependent only on the nature of the derivative and if any comparisons are made between the various light conditions, the light source and the R_2 and R_{10} substituents must be considered. In order to quantify the percentage of the derivative remaining the HPLC method validation linearity parameter needs to be confirmed over the required concentration range especially at the lower concentrations. The lowest concentration at 4.51×10^{-2} mg/mL falls within the range of 6.0×10^{-2} mg/mL and 1×10^{-3} mg/mL reported in chapter 2 and the integrity of the drug peak in the HPLC chromatogram is ensured due to selectivity / specificity results of the method validation (chapter 2).

Table 3.9 - Conditions of Light Stability Studies.

Light Source	Container (Volume)	Temperature	Relative Humidity	Irradiation Period	Sampling Interval
400 Watt High-Pressure Mercury Lamp (UV-Vis range)	Quartz Immersion-Well Photoreactor (400 mL)	40 ± 2 °C	40 - 60% (ambient)	90 minutes	10 minutes for 30 minutes, 15 minutes to 60 minutes then 30 minutes
30 Watt Philips UV Lamp (G30TH)	Clear Glass Ampoules (10 mL)	25 ± 2 °C	40 - 60% (ambient)	32 days	4 days
Sunlight (Windowsill in direct sunlight)	Clear Glass Ampoules (10 mL)	35 ± 5 °C (morning)	40 - 60% (ambient)	90 minutes	15 minutes
Fluorescent / Diffuse Daylight Phillips 55 Watt/33 Lamp (S72T12)	Clear Glass Ampoules (10 mL)	25 ± 2 °C	40 - 60% (ambient)	32 days	4 days

Table 3.10 - 400 Watt-High Pressure Mercury Lamp (UV-Vis Range).

Time (Minutes)	Phenothiazine Derivative % Remaining			
	Prochlorperazine	Perphenazine	Trifluoperazine	Fluphenazine
0	100.00	100.00	100.00	100.00
10	44.24	84.76	66.92	86.17
20	34.48	79.29	63.39	50.65
30	25.89	65.10	40.60	30.96
45	15.87	43.05	22.56	21.40
60	10.11	24.64	11.28	12.84
90	-*	11.79	4.51	7.70

* % remaining is outside the range

Table 3.11 - 30 Watt Philips-UV Lamp.

Time (Days)	Phenothiazine Derivative % Remaining			
	Prochlorperazine	Perphenazine	Trifluoperazine	Fluphenazine
0	100.00	100.00	100.00	100.00
4	44.10	44.10	73.45	56.56
8	46.76	33.06	47.19	47.37
12	38.46	19.27	55.08	40.42
16	30.04	19.70	46.88	40.55
20	29.67	18.04	46.67	36.95
24	25.30	16.71	46.50	30.89
28	20.83	15.98	41.96	25.99
32	24.29	13.23	43.01	22.42

Table 3.12 - Sunlight (Windowsill in direct sunlight).

Time (Minutes)	Phenothiazine Derivative % Remaining			
	Prochlorperazine	Perphenazine	Trifluoperazine	Fluphenazine
0	100.00	100.00	100.00	100.00
15	57.38	35.54	70.35	67.07
30	31.69	15.68	52.90	47.39
45	26.23	14.11	39.54	41.77
60	18.57	13.92	34.31	35.74
75	17.49	11.50	24.42	30.72
90	12.57	7.19	-*	22.49

* % remaining is outside the range

Table 3.13 - Fluorescent / Diffuse Daylight - Philips 55 Watt/33 Lamp.

Time (Days)	Phenothiazine Derivative % Remaining			
	Prochlorperazine	Perphenazine	Trifluoperazine	Perphenazine
0	100.00	100.00	100.00	100.00
4	79.76	58.54	55.52	81.48
8	71.43	54.77	75.10	62.09
12	69.64	38.64	72.24	72.71
16	61.33	41.19	61.20	69.31
20	62.20	33.86	57.23	53.95
24	48.77	35.98	61.02	62.58
28	60.25	40.07	62.07	65.09
32	57.89	31.81	58.70	61.64

The order of degradation on exposure to the 30 W UV Philips lamp and sunlight half-way through the studies is from the most to the least stable, trifluoperazine, fluphenazine, prochlorperazine and perphenazine. In the case of the fluorescent lighting, at 16 days the degradation of perphenazine is 59% with trifluoperazine, fluphenazine and prochlorperazine all at ± 40 - 50% with an overall slower degradation rate compared to the other sources. Irradiation of the derivatives in the photoreactor / 400 W UV source does not allow for

comparison between the derivatives since the experiments were not performed simultaneously and no comparison can be made with the 30 W UV Philips UV source as irradiation is through quartz as opposed to clear glass. However, the order of degradation does appear to be reversed with perphenazine, the most stable. This requires further investigation and the use of actinometry in order to allow for comparisons. It must however be considered in the systems stressed over a long period of time, that the presence of oxygen may be responsible for a changed reaction mechanism due to its complete consumption in those systems, 30 W UV Philips UV and Philips 55 W Lamp Fluorescent / Diffuse Daylight.

As seen in Figures 3.6 - 3.9 the logarithm of the residual phenothiazine derivative concentration (D) expressed as a percentage of initial concentration (D_o) is directly proportional to the irradiation time.

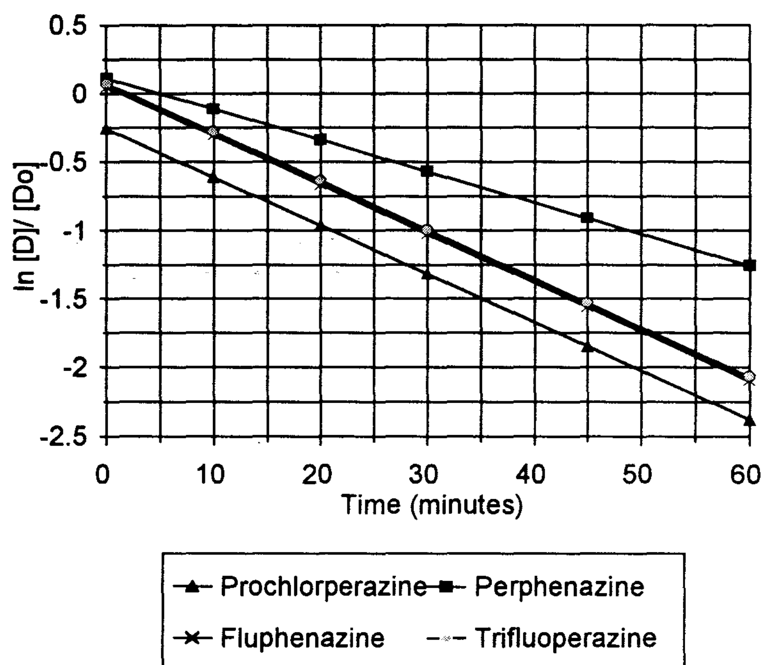


Figure 3.6 - 400 Watt UV - Photoreactor.

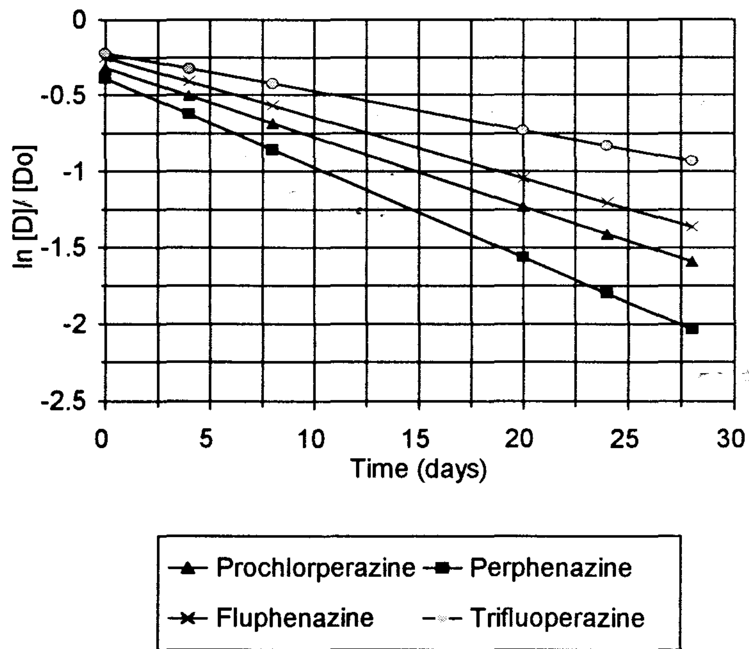


Figure 3.7 - 30 Watt Philips UV- Glass Ampoule.

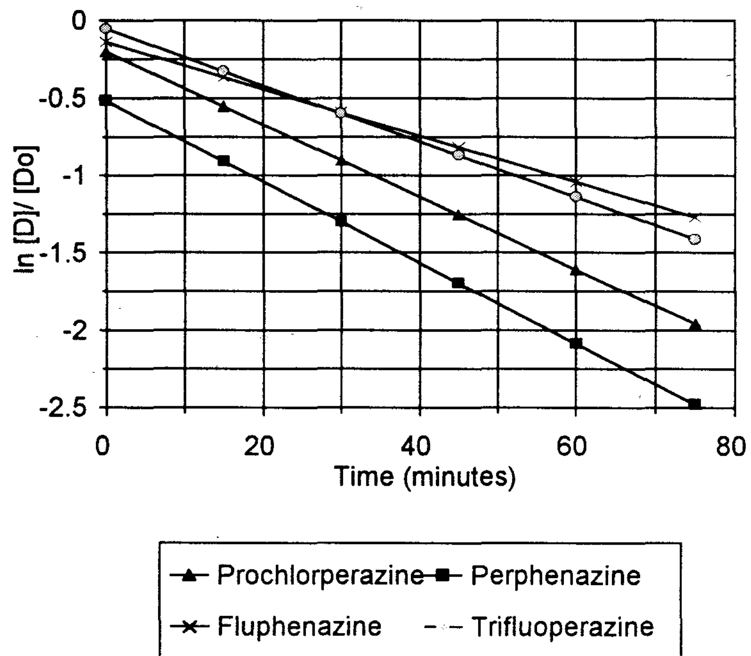


Figure 3.8 - Sunlight - Glass Ampoule

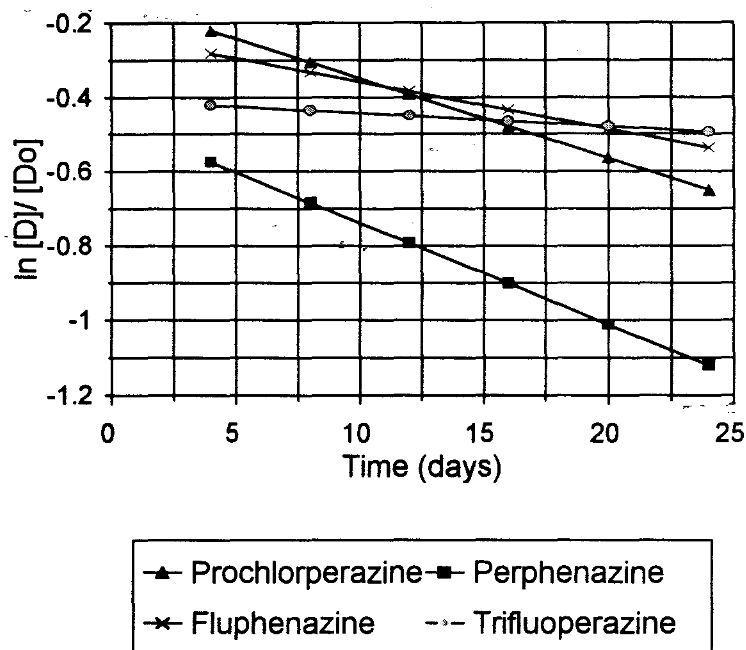


Figure 3.9 - Fluorescent light - Glass Ampoule

The degradation of these compounds displayed first-order kinetics with the pseudo first-order rate constants (k) determined from the slopes of the linear plots and correlation coefficients for these apparent first-order kinetic reactions presented in Table 3.14.

Table 3.14 - Apparent First-Order Rate Constants (Correlation Coefficients).

Phenothiazine Derivative	400 W UV (min^{-1})	30 W UV (day^{-1})	Fluorescent (day^{-1})	Sunlight (min^{-1})
Prochlorperazine	0.0353	0.0334	0.0214	0.0217
	(0.9607)	(0.9437)	(0.8983)	(0.9404)
Perphenazine	0.0246	0.1314	0.0732	0.0618
	(0.9756)	(0.9647)	(0.9203)	(0.9955)
Trifluoperazine	0.0354	-	0.0279	0.0181
	(0.9894)		(0.9237)	(0.9895)
Fluphenazine	0.0302	0.0309	0.0263	0.0154
	(0.9664)	(0.9694)	(0.9155)	(0.9622)

Although Rhodes comments on the limitations placed on the kinetic interpretation of data due to the lack of accuracy, precision, sensitivity and specificity of the assay[62] this is not the case

in this investigation with precision confirmed with a % RSD at a concentration of 5 x 10^{-3} mg/mL of not greater than 1.86% and 4 x 10^{-2} mg/mL of not greater than 0.91%. All other validation parameters are accounted for in chapter 2. These rate constants confirm the instability of the chlorine-containing derivatives, especially perphenazine.

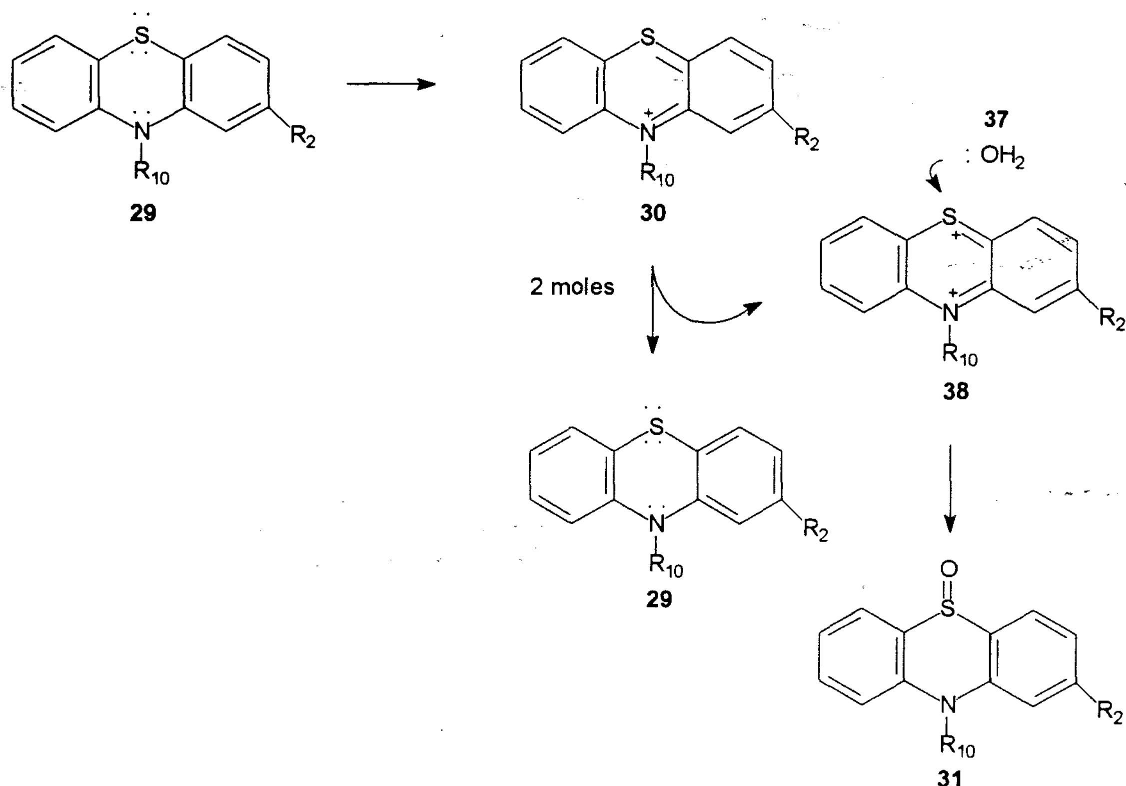
In these stability studies the rate of degradation presented as a semi-logarithmic plot of the percentage remaining phenothiazine derivative expressed as a percentage of the original *versus* time produces a series of straight lines. As mentioned by Moore these rate constants can only be compared if these reaction mixtures of different phenothiazines are studied under the same irradiation conditions.[65] In the study this has allowed comparisons to be made and the order of stability of the various derivatives predicted under each of the light conditions. Gu reports that drugs with absorption of greater than 280 nm have the potential for decomposition in sunlight.[68] This may account for the rates of degradation of these phenothiazine derivatives in the presence of sunlight conditions with a further observation by Bundgaard that rate acceleration can be due to the higher temperatures of the solutions exposed to sunlight contributing to these results.[67] A comment by Andersin that an increase in colour of the solutions hinders UV absorption of the reacting molecules thus slowing down the rate of reaction[66] is relevant here, firstly since irradiation does produce colouration of these solutions and secondly, this may explain the increased degradation from zero time to the first sampling as compared to subsequent readings.

In conclusion these results in part concur with those of Pawelczyk et al. in that trifluoperazine is found to be more stable than prochlorperazine. However, this study has extended the investigation to include not only the effect of the R_2 substituent but also the R_{10} group. Since perphenazine is more susceptible to degradation than fluphenazine it is obvious that the R_2 substituent is a primary influence, however, the fact that perphenazine is less stable than prochlorperazine highlights the contribution of the R_{10} substituent to stability making this worthy of further investigation.[41, 42, 57]

CHAPTER 4: SULPHOXIDATION

4.1 Introduction

The photochemical breakdown of chlorpromazine and subsequent formation of the sulphoxide and hydroxy derivatives in solutions of chlorpromazine exposed to ultraviolet irradiation has been reported.[6, 8, 33, 36] Early investigators reported the oxidation of the phenothiazine derivatives in the presence of ultraviolet light to their sulphoxides to occur as a result of aerial oxidation, while sulphoxidation is also reported as a common biotransformation pathway for all phenothiazines.[8] The following scheme was proposed by Felmeister and Discher as a probable mechanism for the formation of chlorpromazine sulphoxide:[6]



Scheme 4.1[6]

It is believed that the main oxidative decomposition products of the phenothiazines are the monosulphoxides, some of which have been found to be significantly less potent than the parent compounds although chlorpromazine sulphoxide, has been reported to be

pharmacologically active and in sufficiently high concentrations has also been implicated in the phototoxic side-effects associated with chronic administration of the drug.[6, 8, 33]

Phenothiazine ring sulphoxides attain higher steady-state plasma concentrations than the parent drugs during maintenance therapy. With the exception of chlorpromazine sulphoxide, it is unlikely that the sulphoxides contribute to the antipsychotic activity of this series of drugs, as they have demonstrated negligible dopamine receptor binding.[7]

The poor stability of the phenothiazine series resulting in the prominent formation of the 5-sulphoxide in both *in vitro* degradation studies and *in vivo* metabolism, the accumulation of higher concentrations of the sulphoxide than the parent compounds during maintenance therapy with the phenothiazines, their lack of neuroleptic activity, and the implication of the sulphoxides in the adverse effects commonly associated with this group of drugs, emphasises the importance of the synthesis of standards for comparison to confirm the presence of sulphoxides as degradants in the photostudies undertaken in chapter 5.[7, 18, 25,]

Several methods for the oxidation of the phenothiazine derivatives have been reported, [21, 24, 45, 47, 69, 70] with hydrogen peroxide most frequently used as the oxidising agent. Variations within this application are evident from literature reports: Huang et al. determined a method for the oxidation of trifluoperazine hydrochloride by means of the oxidation of an aqueous solution of the drug with 0.3 mL, 30% hydrogen peroxide. The mixture was left overnight at room temperature. After evaporation to dryness, the sulphoxide was recrystallized from methanol.[24] This method was utilised by West et al. in the synthesis of the sulphoxides for the establishment of assay procedures for thioridazine, trifluoperazine, and their sulphoxides and in the determination of the urinary excretion of these compounds in mental patients.[70] An alternative oxidation method developed by Davidson was used in a study conducted by Al-Obaid et al. where methanolic solutions of perphenazine, trifluoperazine and triflupromazine

(0.25 mg/mL) were oxidised to the corresponding sulphoxides with a solution of peroxyacetic acid, which was prepared by diluting 5 mL, 30% hydrogen peroxide solution to 500 mL of glacial acetic acid. After the addition of the peroxyacetic acid to the phenothiazine solution, the reaction mixture was left to stand at room temperature for 15 minutes, or until the reaction had gone to completion, confirmed by the observation of a single compound on thin layer chromatography.[47] A recent report on the development of a photostability-indicating HPLC method for the determination of trifluoperazine in bulk form and pharmaceutical formulations by Abdel-Moety et al. included the preparation of the sulphoxide by heating 100 mg of the drug (base) with 10 mL of 15% hydrogen peroxide and 0.2 mL glacial acetic acid on a water-bath at 60°C for 30 minutes. The solvent was evaporated under vacuum and the sulphoxide recrystallized from ethanol.[45]

Owens et al. report the oxidation of several phenothiazine salts to their sulphoxides using a hydrogen peroxide, as well as an aqueous nitrous acid method of oxidation. In the former method 10 mL of 15% hydrogen peroxide was added to the phenothiazine derivative, made up to 100 mL with water and stirred for 3 days. The reaction mixture was made basic with an ammonium hydroxide solution, the sulphoxide extracted into chloroform and the resulting extracts evaporated to dryness with subsequent recrystallization of the sulphoxide from ethyl acetate. The aqueous nitrous acid method of oxidation is considered at length in the methodology,[7] as it is the preferred method for the oxidation of prochlorperazine, perphenazine, trifluoperazine and fluphenazine to their sulphoxides. The solubility of trifluoperazine and fluphenazine parent compounds, however created the need to adapt the reported method of oxidation if it is to be used for all four derivatives.

4.2 Methodology

Reagents

All chemicals were of at least analytical grade. Benzene, ammonium hydroxide solution

(25%), ammonium acetate and sodium hydroxide were obtained from Unilab, Saarchem (Pty) Ltd, heptane from E. Merck, Darmstadt, and methanol (HPLC grade) from Romil Ltd, Cambridge. Water for chromatography was obtained using a Milli-Q Plus ® water purification system, filtered with a Q Pak, purification pack suitable for water pretreated by reverse osmosis, consisting of an initial 0.5 µm prefilter, activated carbon, nuclear grade ion-exchange resin, and an Organex-Q organic scavanger mixture.

Prochlorperazine, perphenazine, trifluoperazine and fluphenazine were kindly donated by Lennon Ltd (Port Elizabeth, South Africa) and Fine Chemicals Ltd (Cape Town, South Africa).

HPLC Standard Preparation

20 mg of each phenothiazine sulphoxide was accurately weighed and transferred to a 50 mL volumetric flask and made up to volume with solvent, to prepare a 2×10^{-1} mg/mL standard solution.

Equipment

Φ 32 pH Meter, Beckman 015-247665-A

Precisa 120 A, Mass Balance

Final Chromatographic Parameters

Column:	µBondapak, C18 (3.9 x 300 mm i.d.) 10 µm, 125 Å
Mobile phase:	Methanol: 30 mM ammonium acetate, (80:20 v/v), pH 6.5
Flow rate:	1.0 mL per minute
DetectorWavelength:	254 nm
Injection Volume:	60 µL
Column Temperature:	Ambient

A modular HPLC system consisting of an Iso Chrom LC (SP 8700) constant-flow pump (Spectra-Physics), with a Lambda-Max LC variable wavelength UV detector (Pye-Unicam), a Spectra-Physics integrator (SP 4290), and a rheodyne fixed loop 20 µL injector (Model 7125) was used.

Aqueous Nitrous Acid Oxidation[7]

Prochlorperazine and Perphenazine Sulphoxide

Prochlorperazine / perphenazine was accurately weighed into a 500 mL Erlenmeyer flask with the addition of 250 mL of deionized water and 9 M HCl with stirring until a solution was obtained. Excess aqueous sodium nitrite (1 g/ 10 mL) was added and the mixture stirred until the reaction had gone to completion (HPLC). Nitrogen gas was bubbled through the solution for approximately 2 hours to remove nitrogen oxide gases prior to work up. The solution was extracted twice with 40 mL portions of chloroform to remove impurities, and the chloroform discarded. Ammonium hydroxide solution (25%) was added (pH 10) to release the sulphoxide free base, which was extracted with two 100 mL portions of chloroform. The combined chloroform solution was washed twice with 100 mL portions of water, allowed to stand for 10 -15 minutes and then filtered. After removal of the chloroform by evaporation, the resulting sticky residue was crystallized by repeated trituration with of hot heptane. The final product was then recrystallized from ethyl acetate.

Trifluoperazine and Fluphenazine Sulphoxide

The method of synthesis is essentially as above. While the chloro-derivatives require the addition of acid in order to facilitate dissolution in aqueous media, the trifluoromethyl-derivatives are readily soluble in water, and the addition of acid prior to the dissolution causes precipitation. It is thus essential to first obtain a solution of the trifluoromethyl-derivatives in water before the addition of the required amount of sodium nitrite and acid.

The free sulphoxide base is extracted with ether and after evaporation the oily residue

dissolved in benzene. This extraction and crystallization procedure was adapted from the method for the synthesis of fluphenazine sulphoxide proposed by Kreyenbuhl et al.[71] After the addition of a small volume of hexane, refrigeration overnight induced crystallization. The resultant product was recrystallized from ethyl acetate.

Table 4.1 - Preparation of Four Piperazine-Substituted Phenothiazine Sulphoxide Derivatives.

Phenothiazine Derivative	Amount Weighed	9 M HCl (mL)	Sodium Nitrite (mL)	% Yield
Prochlorperazine	3.0325 g	30.0	15	56.24
Perphenazine	4.7704 g	16.5	25	87.93
Trifluoperazine	0.9613 g	15.0	50	82.06
Fluphenazine	2.5125 g	5.0	25	29.65

4.3 Results and Discussion

To confirm the identity and purity of the sulphoxides the following methods were employed: HPLC, TLC, mp, accurate mass and elemental analyses, MS, UV, IR, and NMR.

HPLC

The presence of a single peak in each of the HPLC chromatograms confirmed the oxidation of the parent drugs to a single compound, the retention times of which are shown in Table 4.2. Due to the polar nature of the sulphoxides, as a result of the addition of the oxygen atom into the molecule, the retention times were significantly shorter than those of the parent phenothiazines.

Table 4.2 - HPLC Retention Times of the Sulphoxides and Parent Phenothiazines.

Phenothiazine Derivative	Parent Drug R_t (min)	Sulphoxide R_t (min)
Prochlorperazine	~ 12.5	~ 7.8
Perphenazine	~ 7.3	~ 5.3
Trifluoperazine	~ 12.0	~ 7.4
Fluphenazine	~ 7.3	~ 5.2

TLC

Thin layer chromatography of the respective sulphoxides dissolved in methanol, was undertaken using precoated, chromatographic plates, Silica gel $60F_{254}$ on aluminium support, E. Merck, D-6100 Darmstadt, Federal Republic Germany. The plates were developed using two different solvent systems and then air-dried and visualised under short-wave ultraviolet light. The R_f values for the synthesised sulphoxides are compared with those documented in the literature, and the results are given in Table 4.3.

Table 4.3 - Comparative R_f Values for the Sulphoxides.

Phenothiazine Derivative	R_f Values (Experimental)		R_f Values (Literature)[71]	
	A*	B*	A	B
Prochlorperazine sulphoxide	0.40	0.20	0.31	0.23
Perphenazine sulphoxide	0.49	0.34	0.44	0.28
Trifluoperazine sulphoxide	0.47	0.41	-	-
Fluphenazine sulphoxide	0.43	0.32	0.51	0.29

* Solvents A: ammonium acetate 3 g, water 20 mL to 100 mL with methanol.

B: methanol-benzene, 50:50 v/v.[71]

In addition to the similarities in R_f values between the synthesised sulphoxides and literature values, a single compound on the TLC plates for the four synthesised sulphoxides verified their purity.

Melting points determined in open capillary tubes with a Gallenkamp Melting Point Apparatus, 50 Hz, 220/240 Volts, are reported uncorrected. Accurate mass determinations were performed by the Mass Spectrometry Unit, Cape Town and elemental analyses of the sulphoxides obtained from the CSIR, Pretoria are listed in Table 4.4.

Table 4.4 - Comparative Melting Points, Accurate Masses and Elemental Analyses of the Sulphoxides.

Phenothiazine Derivative	Melting Point (°C)		Accurate Mass		Elemental Analysis	
	Literature[71, 24]	Experimental	Theoretical	Experimental	Theoretical	Experimental
Prochlorperazine	164	160 - 163	$C_{20}H_{24}ClSN_3O$, 389.1340	$C_{20}H_{24}ClSN_3O$, 389.1329	C: 61.60%, N: 10.78%, H: 6.20%	C: 61.73%, N: 10.80%, H: 6.26%
Perphenazine	142 - 145	143 - 145	$C_{21}H_{26}ClSN_3O_2$, 419.1127	$C_{21}H_{26}ClSN_3O_2$, 419.1121	C: 60.06%, N: 10.01%, H: 6.24%	C: 60.05%, N: 9.96%, H: 6.19%
Trifluoperazine	134 - 137	141 - 142	$C_{21}H_{24}F_3SN_3O$, 423.1595	$C_{21}H_{24}F_3SN_3O$, 423.1592	C: 59.56%, N: 9.92%, H: 5.71%	C: 60.11%, N: 10.04%, H: 5.77%
Fluphenazine	140	139 - 140	$C_{21}H_{26}F_3SN_3O_2$, 453.1710	$C_{21}H_{26}F_3SN_3O_2$, 453.1698	C: 58.26%, N: 9.27% H: 5.78%	C: 58.26%, N: 9.24%, H: 5.83%

MS

Table 4.5 - Experimental Mass Spectral Data for the Sulphoxides.

Prochlorperazine			Perphenazine		
Ion	*m/z*	Relative Abundance (%)	Ion	*m/z*	Relative Abundance (%)
M^+	389	0.76	$(M + H)^+$	420	0.25
$(M\text{-}C_3H_4NO)^+$	319	50.84	$(M\text{-}C_4H_6NO_2)^+$	319	0.52
$(M\text{-}C_7H_{15}N_2O)^+$	246	100.00 (base peak)	$(M\text{-}C_8H_{17}N_2O_2)^+$	246	100.00 (base peak)
$(M\text{-}C_8H_{17}N_2O)^+$	232	43.16	$(C_9H_{17}N_2O)^+$	169	17.80
$(C_8H_{15}N_2)^+$	139	26.70	$(C_8H_{15}N_2)^+$	139	12.77
$(C_6H_{13}N_2)^+$	113	48.44			

Table 4.5 continued

Trifluoperazine			Fluphenazine		
Ion	*m/z*	Relative Abundance (%)	Ion	*m/z*	Relative Abundance (%)
M^+	423	0.73	M^+	453	0.59
$(M\text{-}C_3H_4NO)^+$	353	39.59	$(M\text{-}C_4H_6NO_2)^+$	353	32.82
$(M\text{-}C_7H_{15}N_2O)^+$	280	100.00 (base peak)	$(M\text{-}C_8H_{17}N_2O_2)^+$	280	100.00 (base peak)
$(C_6H_{13}N_2)^+$	113	64.42	$(C_9H_{17}N_2O)^+$	139	23.19

The MS data, Table 4.5, obtained for the synthesised sulphoxides show similar fragmentation patterns for both the chloro- and trifluoromethyl-derivatives. The MS data for the chloro-derivatives illustrate common product ions at *m/z* 319, 246, and 139. Partial cleavage of the piperazine ring in the side chain results in the formation of the product ion at *m/z* 319 for prochlorperazine and perphenazine sulphoxides. Subsequent loss of 73 daltons (C_3H_7NO) gives rise to a common product ion at *m/z* 246. The common product ion at *m/z* 139 for the chloro-derivatives is a derivative of the side chain $(C_8H_{15}N_2)^+$. Differences between the two chloro-derivatives are indicated by product ions at *m/z* 232, and 169 for prochlorperazine and perphenazine respectively. The loss of 14 daltons to form the product ion at *m/z* 232 for prochlorperazine, is due to the cleavage of the methyl group from the ring nitrogen atom, while in perphenazine the product ion at *m/z* 169 is a derivative of the side chain.

The trifluoromethyl-derivatives, undergo fragmentation to form the product ions at *m/z* 353, and 280, which are identical to those observed for the chloro-derivatives, with the difference attributed to the substituent at the 2-position on the phenothiazine nucleus. Differences between these two derivatives give rise to the formation of product ions at *m/z* 139 and 113 for samples of fluphenazine and trifluoperazine sulphoxide respectively. However for all four derivatives the base peak is due to α-cleavage of the side chain.

UV

The method of oxidation used is specific for the synthesis of the respective phenothiazine sulphoxides. However, further oxidation of the sulphoxide can result in the formation of the sulphone, which has been reported by Wallace.[72] Although these compounds have structural similarities, their ultraviolet absorption spectra are different and characteristic for the specific functional groups. Table 4.6 shows the differences in the ultraviolet absorption data for a phenothiazine derivative, its sulphoxide and sulphone.[60] Davidson confirmed the presence of the sulphoxide by a shoulder around 345 nm in the absorption spectra of aqueous solutions

of the respective phenothiazines.[39]

Table 4.6 - UV Absorption Data for Trifluoperazine, its Sulphoxide and Sulphone, in 95% Ethanol.[60]

Phenothiazine Derivative	UV Absorption Data (nm)			
Trifluoperazine	-	310	-	254
Trifluoperazine Sulphoxide	342	300	276	240
Trifluoperazine Sulphone	335	295	272	237

A shoulder in the absorption spectra of fluphenazine and trifluoperazine sulphoxides was observed around 349.1 nm, while in the case of the two chloro-derivatives, the perphenazine and prochlorperazine sulphoxides, the presence of a shoulder at 342.7 nm, for ethanolic solutions of the respective samples was evident. The ultraviolet absorption spectra for trifluoperazine, prochlorperazine, perphenazine and fluphenazine sulphoxides in 95% ethanol are shown in Figures 4.1 - 4.2.

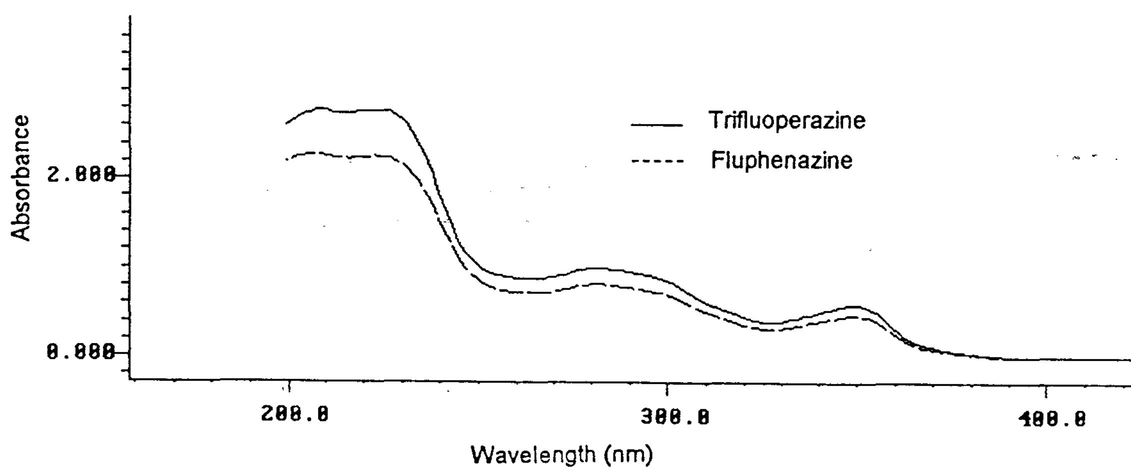


Figure 4.1 - Ultraviolet spectra for trifluoperazine and fluphenazine sulphoxide.

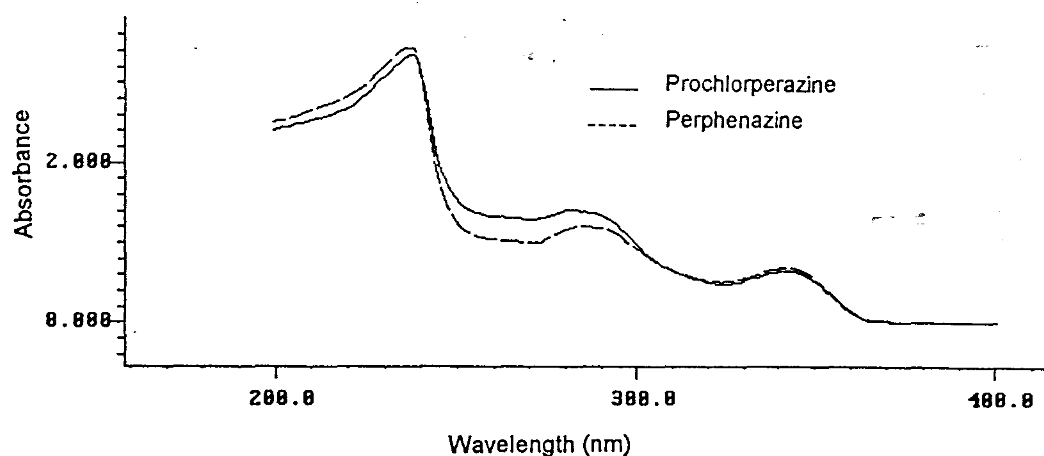


Figure 4.2 - Ultraviolet spectra for prochlorperazine and perphenazine sulphoxide.

IR

It has been reported that the infrared, S=O, frequency for alkyl and aryl sulphoxides falls in the 1070 - 1035 cm^{-1} range. The sulphoxide is thus readily identifiable by the presence of a strong absorption band in the 1050 cm^{-1} region.[73] The infrared spectra of trifluoperazine, prochlorperazine, perphenazine and fluphenazine sulphoxides were recorded on a Perkin Elmer FT-IR Spectrometer, Spectrum 2000, FT01 (KBr - diffuse reflectance). Table 4.7 contains the infrared data for the respective sulphoxides, confirming the presence of the S=O in each case.

Table 4.7 - Infrared Data for the Sulphoxides.

Phenothiazine Derivative	IR Literature Data[7, 71] (S=O stretching)	IR Experimental Data (S=O stretching)
Prochlorperazine	1030	1049.8 cm^{-1}
Perphenazine	1000	1060.0 cm^{-1}
Trifluoperazine	1000	1048.0 cm^{-1}
Fluphenazine	1000	1054.7 cm^{-1}

NMR

NMR spectra of the parent phenothiazines and their oxidized products were recorded using the same conditions as specified in chapter 3 on a Bruker AMX 400, and chemical shifts are expressed in δ (ppm). The assignment of the protons (^{1}H-NMR) and carbons (^{13}C-NMR) refers to Figure 4.3.

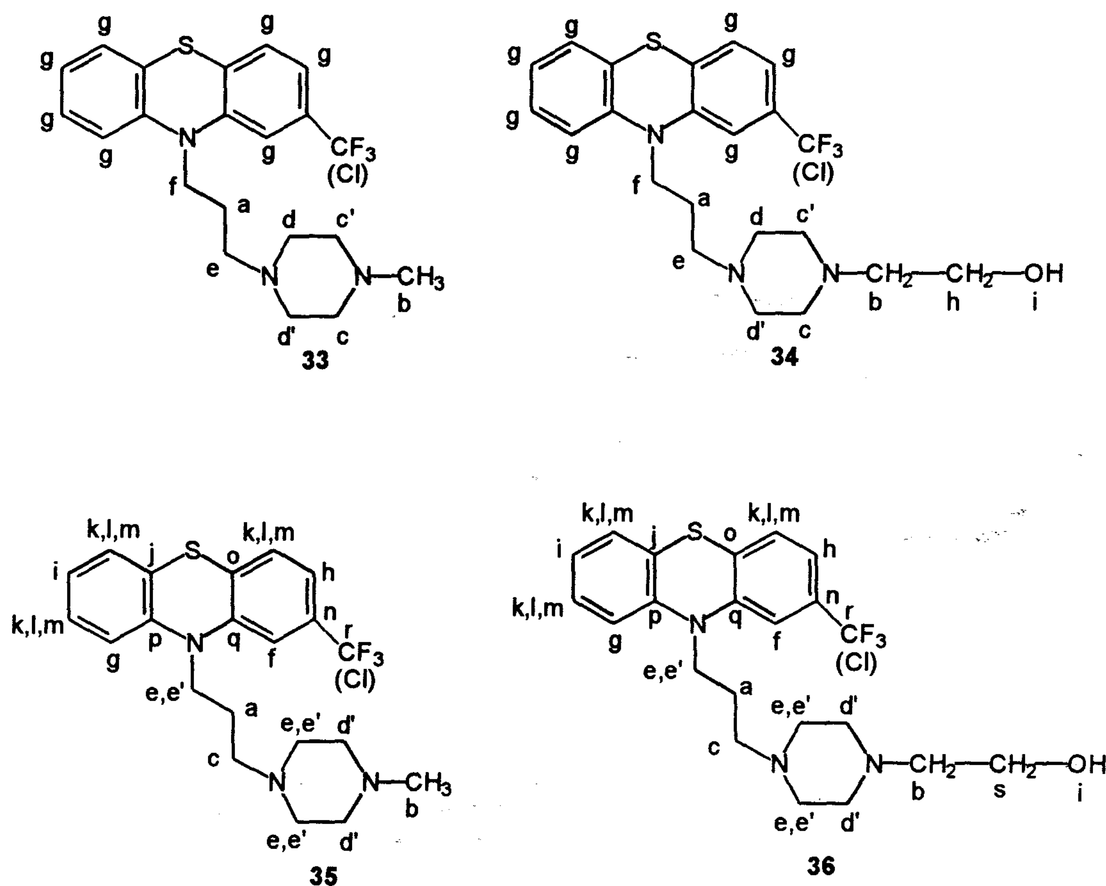


Figure 4.3 - Assignment of protons 33, 34 (^{1}H-NMR) and carbons 35, 36 (^{13}C-NMR) for a typical piperazine-substituted phenothiazine.

Since the protons (^{1}H-NMR spectra) and carbons (^{13}C-NMR spectra) are assigned in chapter 3 for all the derivatives the purpose of this chapter is to highlight the differences which confirm the identity of the sulphoxides. Figure 4.3 assigns the protons and carbons for both a methyl- and β-hydroxyethyl-derivative while Table 4.8 and 9 provide comparative ppm values for selected protons and carbons. The introduction of the oxygen is expected to result in a

Table 4.8 - Comparative ^{1}H-NMR Data (ppm) for Four Piperazine-Substituted Phenothiazine Sulphoxides.

Protons	Assignment	Prochlorperazine	Prochlorperazine SO	Perphenazine	Perphenazine SO
f	Triplet	4.0752	4.4892	3.9996	4.4410
g	Broad Multiplet	6.9760 - 7.4148	7.3243 - 8.0131	6.9655 - 7.1742	7.300 - 8.050

Table 4.8 continued

Protons	Assignment	Trifluoperazine	Trifluoperazine SO	Fluphenazine	Fluphenazine SO
f	Triplet	4.1923	4.6038	4.0214	4.4470
g	Broad Multiplet	7.0525 - 7.3560	7.4294 - 8.2251	6.8586 - 7.2723	7.4744 - 8.1088

Table 4.9 - Comparative ^{13}C-NMR Data for Parent Phenothiazines and their Sulphoxides (-SO).

Carbon	Prochlorperazine	-SO	Perphenazine	-SO	Trifluoperazine	-SO	Fluphenazine	-SO
k	128.35	132.51	128.38	132.51	128.79	132.42	128.80	132.27
l	128.76	134.00	128.73	134.00	129.01	133.59	129.02	133.48
m	128.99	134.92	128.97	134.51	129.29	135.07	129.30	135.11

downfield shift of certain aromatic protons to a greater extent than others. Since these aromatic protons have not been individually assigned, a downfield shift for the centre of the broad multiplet of between 0.47 and 0.72 ppm is noted. However the carbons expected to be most affected by the introduction of the oxygen (k, l, m) have been assigned and downfield shifts are reported (Table 4.9) as compared to those values obtained for the parent drugs with the two chloro-derivatives, perphenazine and prochlorperazine and trifluoromethyl-derivatives, fluphenazine and trifluoperazine, showing chemical shifts of similar magnitude. Based on a knowledge of the assignment of the carbons in the ^{13}C-NMR, HMQC experiments to assign the aromatic protons individually within the broad multiplet have proved unsuccessful at present. Kreyenbuhl et al report a characteristic shift in the signal (triplet) due to the N_{10}-CH_2-protons from 3.9 ppm to 4.4 ppm.[71] This is explained in terms of the increased electronegativity of the sulphoxide group causing the shielding of these protons to be decreased. A downfield shift of a similar magnitude is observed for all four sulphoxides (Table 4.8) and is shown in Figure 4.4 for prochlorperazine as a representative example.

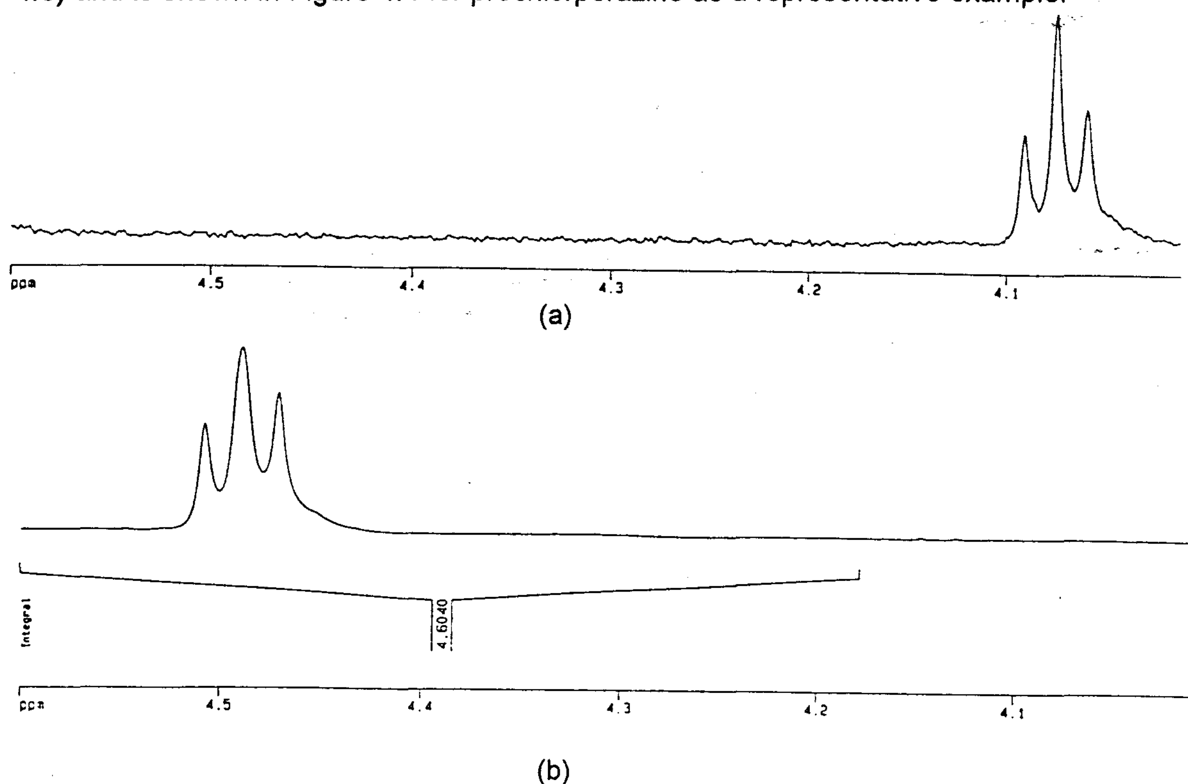


Figure 4.4 - ^{1}H-NMR spectra of (a) prochlorperazine and (b) prochlorperazine sulphoxide.

Because of the detection of the sulphoxides *in vivo* they have been studied in terms of the conditions under which they are formed and this has led to extensive stability studies investigating the influence of both light and oxygen. This chapter also includes numerous references to the synthesis of various phenothiazine sulphoxides and proposes a method which has application to all four derivatives included in this study.[21, 24, 45, 47, 69, 70] The four sulphoxides were synthesised in percentage yields ranging between 30 - 88% similar to those achieved by Kreyenbuhl et al.[71] and their identity and purity confirmed by HPLC, TLC, mp, accurate mass, elemental analyses, MS, UV, IR and NMR, including comparative literature values where available.

CHAPTER 5: PHOTOPRODUCT CHARACTERIZATION

5.1 Introduction

Current international regulations of drug purity control within the International Conference on Harmonisation (ICH) forum, require that impurities exceeding the 0.1% level are examined and preferably identified. These impurities may result from degradation, impure starting materials, or the synthetic procedure.[74] The selection of four structurally related phenothiazine derivatives available in a variety of forms: liquid, tablet, suppository and as fine chemicals, together with their associated poor stability prompted a detailed study to identify degradants in solid state and solution samples stressed under various light conditions.

During the course of the HPLC method development and validation, the four phenothiazine derivatives were stressed under various conditions such as temperature, acid, base and oxidizing conditions, and light: ultraviolet irradiation, sunlight, and fluorescent light / diffuse daylight. These stress conditions were used to validate the analytical monitoring method developed in chapter 2 and serve as some indication of the nature of future stability studies required.

Table 5.1 - Light Conditions for Stability Studies.

	400 W - UV	30 W - UV	Sunlight	55 W Fluorescent /Diffuse Daylight
Solution	✓	✓	✓	✓
Solid		✓		

Stability studies on solutions of the derivatives stressed under the various light conditions, were performed under controlled pH conditions. During the course of the stability studies, several low level degradants were observed in the HPLC chromatograms and UV spectra. The presence of the buffer however restricted the isolation of pure degradants from the stressed samples using semi-preparative HPLC. While, solid state studies on samples of the

four derivatives allowed an initial investigation into the use of semi-preparative HPLC to isolate the degradant(s) and parent compound(s), with a view to further characterization by MS, NMR, UV, IR and elemental analysis.

As no detailed structural information could be obtained from the analytical LC studies and the inability to adapt the mobile phase to achieve separation in the absence of buffers precluded the use of a semi-preparative HPLC system to generate pure degradants in the solid degradation studies, liquid chromatography-mass spectrometry (LC-MS) was used to identify the degradants from the solid state and solution studies.

Mass spectrometry (MS) provides information based on molecular mass and thus the combination of HPLC and MS (LC-MS) has been reported as a powerful and rapid method for the analysis of drugs and biological substances. Analyses combined optimized HPLC separation conditions on-line with an electrospray-MS interface to obtain molecular mass information from LC-MS chromatograms and structural information from LC-MS-MS spectra. This technique is extensively used in the pharmaceutical industry and related laboratories for drug identification, as well as screening and quantification.[75] Examples of such a use are illustrated in studies by Erickson et al., Qin et al. and Chen et al.[74, 75, 76] Erickson et al. employed LC-MS to elucidate the structure of an impurity found at levels between 0.2 and 0.3% in some batches of metoprolol tartrate of non-Astra origin.[74] While Beaulieu et al. reported famatidine to degrade *via* hydrolysis and oxidation reactions to yield the respective degradants, Qin et al. detected an unknown package-related famotidine degradate in an over-the-counter formulation. LC-MS with an atmospheric pressure chemical ionization (APCI) source was successfully applied to characterize the degradate.[75] Terfenadine, a non-sedating antihistamine which undergoes first pass metabolism to result in the formation of two metabolites, an "acid" and "alcohol" metabolite, which had been identified and previously documented, however, on LC-MS analysis of a human urine sample after oral administration

of terfenadine tablets, three additional terfenadine-related metabolites were detected by Chen et al. Thermospray LC-MS was applied to elucidate the structures of these metabolites.[76]

5.2 Methodology

Reagents

All chemicals were of at least analytical grade. Ammonium acetate was obtained from Univar, SaarChem (Pty) Ltd, and methanol (HPLC grade) from Romil Ltd, Cambridge. Water for chromatography was obtained using a Milli-Q Plus ® water purification system, filtered with a Q Pak, purification pack suitable for water pretreated by reverse osmosis, consisting of an initial 0.5 µm prefilter, activated carbon, nuclear grade ion-exchange resin, and an Organex-Q organic scavenger mixture.

Prochlorperazine, perphenazine, trifluoperazine and fluphenazine were kindly donated by Lennon Ltd (Port Elizabeth, South Africa) and Fine Chemicals Ltd (Cape Town, South Africa).

Standard Preparation for Analytical HPLC Analysis

Solution Studies

A 1.0 mg/mL stock solution of each derivative was prepared in a buffer solution, pH 6.4 (chapter 2), and stressed under the conditions in Table 5.2. The transfer of 10.0 mL of the stressed stock solutions to a 50 mL volumetric flask, made up to volume with solvent (methanol: water, 70:30 v/v), was necessary for the preparation of 2.0×10^{-1} mg/mL standard solutions used in LC and LC-MS analyses. All preparations were protected from light.

Solid Studies

A 2.0×10^{-1} mg/mL standard solution for each derivative was prepared by the transfer of 10 mg of stressed powder sample to a 50 mL volumetric flask and made up to volume with solvent (methanol: water, 70:30 v/v).

Table 5.2 - Conditions of Solution Stability Studies for Prochlorperazine, Perphenazine, Trifluoperazine and Fluphenazine.

Light Source	Container (Volume)	Temperature	Relative Humidity	Irradiation Period	Sampling Interval
400 Watt High-Pressure Mercury Lamp (UV-Vis range)	Quartz Immersion-Well Photoreactor (400 mL)	40 ± 2 °C	40 - 60% (ambient)	90 minutes	10 minutes for 30 minutes, 15 minutes to 60 minutes then 30 minutes
30 Watt Philips UV Lamp (G30TH)	Clear Glass Ampoules (10 mL)	25 ± 2 °C	40 - 60% (ambient)	32 days	4 days
Sunlight (Windowsill in direct sunlight)	Clear Glass Ampoules (10 mL)	35 ± 5 °C (morning)	40 - 60% (ambient)	90 minutes	15 minutes
Fluorescent / Diffuse Daylight Philips 55 Watt/33 Lamp (S72T12)	Clear Glass Ampoules (10 mL)	25 ± 2 °C	40 - 60% (ambient)	32 days	4 days

Standard Preparation for Semi-Preparative LC Analysis

Solid Studies

A 5.0 mg/mL standard solution for each derivative was prepared by the transfer of 50 mg of a stressed powder sample to a 10 mL volumetric flask and made up to volume with solvent. The final solution was filtered through a 0.45 µm hydrophilic filter and protected from light.

Final LC-MS Conditions

Liquid chromatographic separations were performed on a VG Quattro Triple Quadropole Mass Spectrometer fitted with an electrospray ionisation source by the University of Stellenbosch, Department of Biochemistry, using a µBondapak, C18 column (3.9 x 300 mm i.d.), 10 µm, 125 Å. The mobile phase was methanol: 30 mM ammonium acetate, (80:20 v/v), pH 6.5, at a flow rate of 1.0 mL/min, with a post column split to give a flow of 90 µL/min into the mass spectrometer. The source temperature was 80°C, the capillary voltage was 3.5 kV and the cone voltage 30 V, except during the fragmentation runs, where it was 90 V.

Final Analytical LC Conditions

In the analytical LC studies, reverse phase liquid chromatography using the above chromatographic conditions was employed. The apparatus used in the investigation consisted of a modular HPLC system with an Iso Chrom LC (SP 8700) constant-flow pump (Spectra-Physics), a Lambda-Max LC variable wavelength UV detector (Pye-Unicam), an integrator (Spectra-Physics, SP 4290) and a rheodyne fixed loop 20 µL injector (Model 7125).

Detector Wavelength:	254 nm
Injection Volume:	60 µL
Column Temperature:	Ambient
Solvent:	Methanol: water (70:30 v/v)

Final Semi-Preparative LC Conditions

The apparatus used consisted of a reverse phase LiChrospher, C18 column (10 x 250 mm

i.d.), 10 µm, a guard column and an in-line filter. The mobile phase was methanol: 30 mM ammonium acetate (50:50 v/v) at a flow rate of 1.0 mL per minute. A modular HPLC Beckman System Gold consisting of a Programmable Solvent Modular 126 constant-flow pump, with a System Gold Diode Array Detector Module 168 UV detector, an Olirite Computor System and a rheodyne fixed loop 500 µL injector (Model 7125) was used in the investigation.

Detector Wavelength:	254 nm
Scanning Range:	200 - 598 nm
Injection Volume:	250µL
Column Temperature:	Ambient
Solvent:	Methanol: Water (70:30 v/v)

The interfaces of LC-MS thus far developed do not allow for the use of non-volatile buffers, due to the ionization in MS which is subject to interference by these non-volatile buffer components.[68] Although recent studies report that this can be overcome with the use of frit-fast atom bombardment MS (frit-FAB LC-MS),[77] the traditional LC-MS system utilizes ammonium acetate, a relatively volatile buffer. This led to the need to develop a mobile phase which provided similar separation of the degradants and parent compounds as compared to that used in the analytical LC studies, in the absence of non-volatile buffer components or ion-pairing reagents.

5.3 Results and Discussion

Solution Studies

In the study on the stability of solutions of the four derivatives, between 5 and 8 unknown peaks eluting at retention times shorter than the parent compounds were seen in the chromatograms, implying degradants to be more polar in nature than the parent derivatives. As no further information could be obtained on the nature of the degradants using LC, a detailed LC-MS study to determine the molecular mass and thus assist in structural

elucidation (LC-MS-MS) of the degradants and subsequent proposal of a degradation pathway was undertaken.

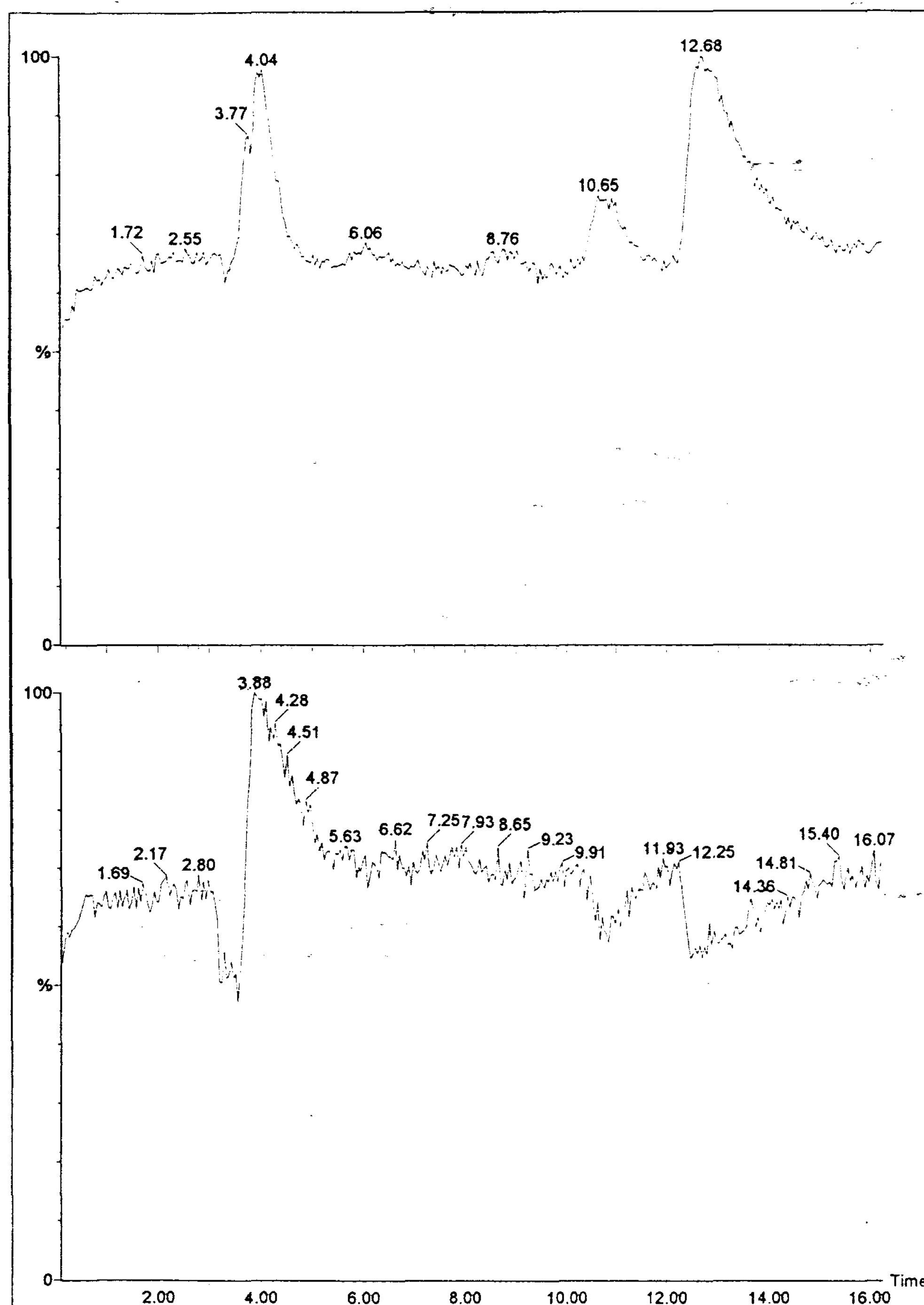


Figure 5.1 - Total Ion Current chromatograms of prochlorperazine stressed in fluorescent / diffuse daylight.

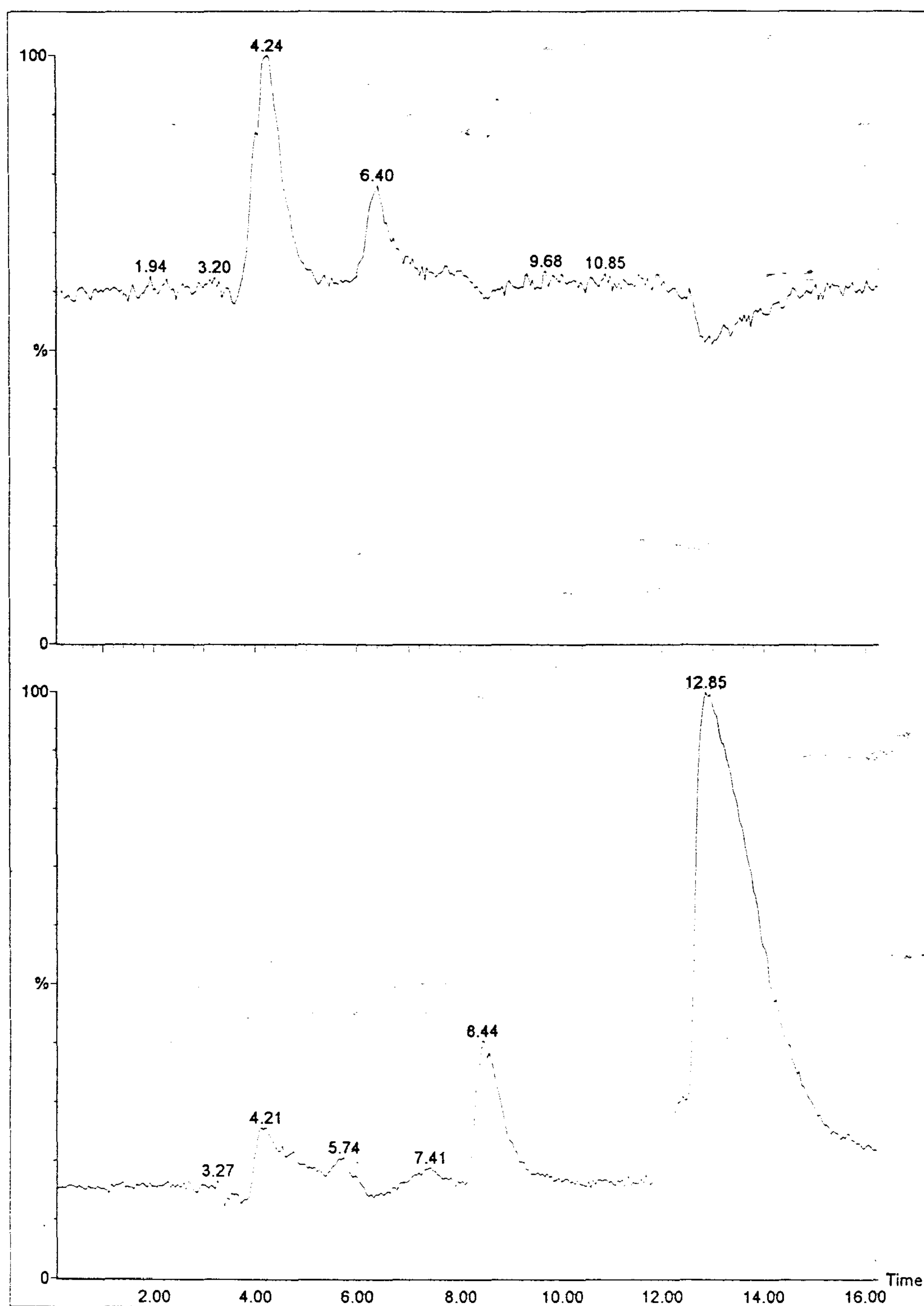


Figure 5.2 - Total Ion Current chromatograms of perphenazine stressed in fluorescent / diffuse daylight.

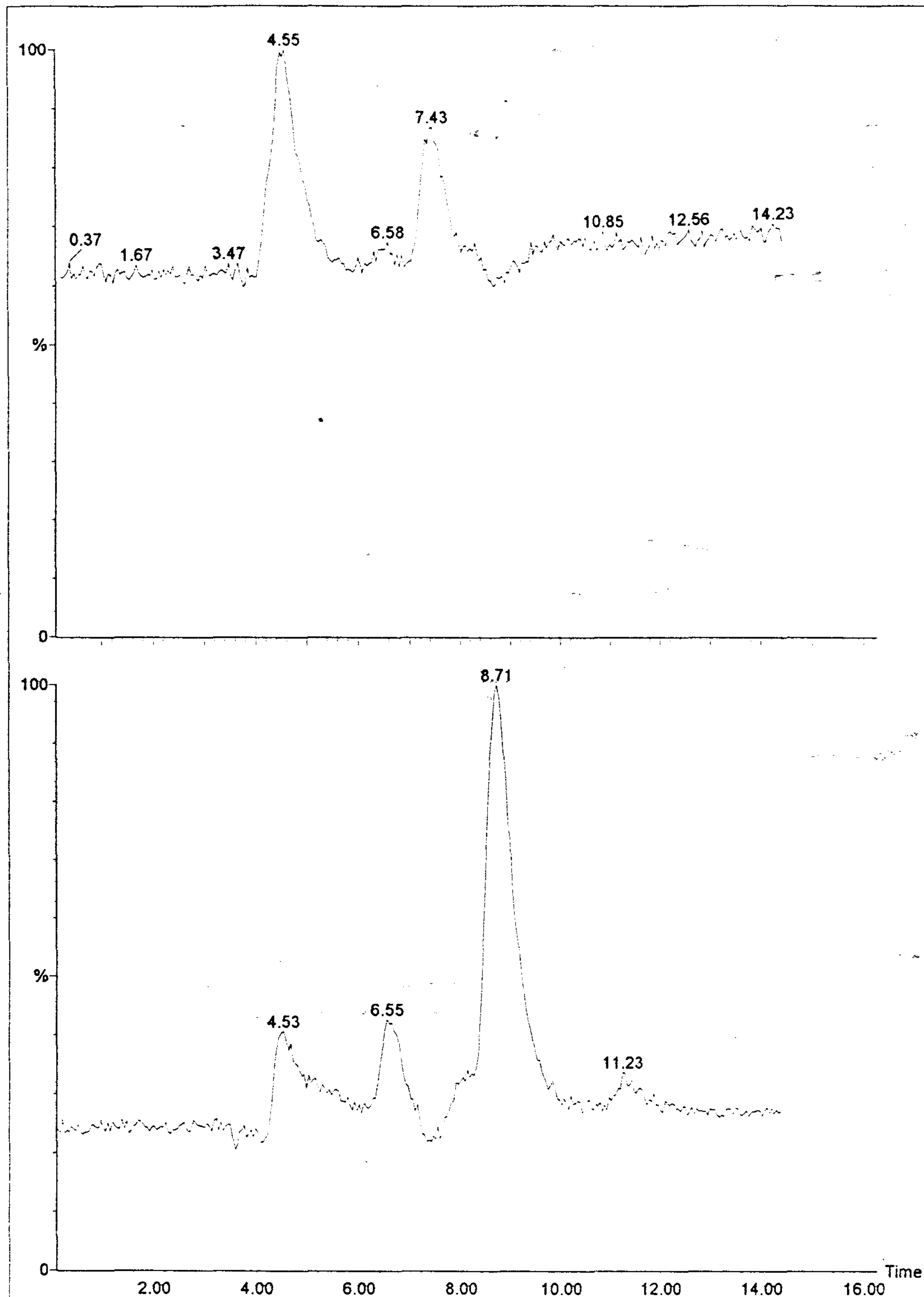


Figure 5.3 - Total Ion Current chromatograms of trifluoperazine stressed in fluorescent / diffuse daylight.

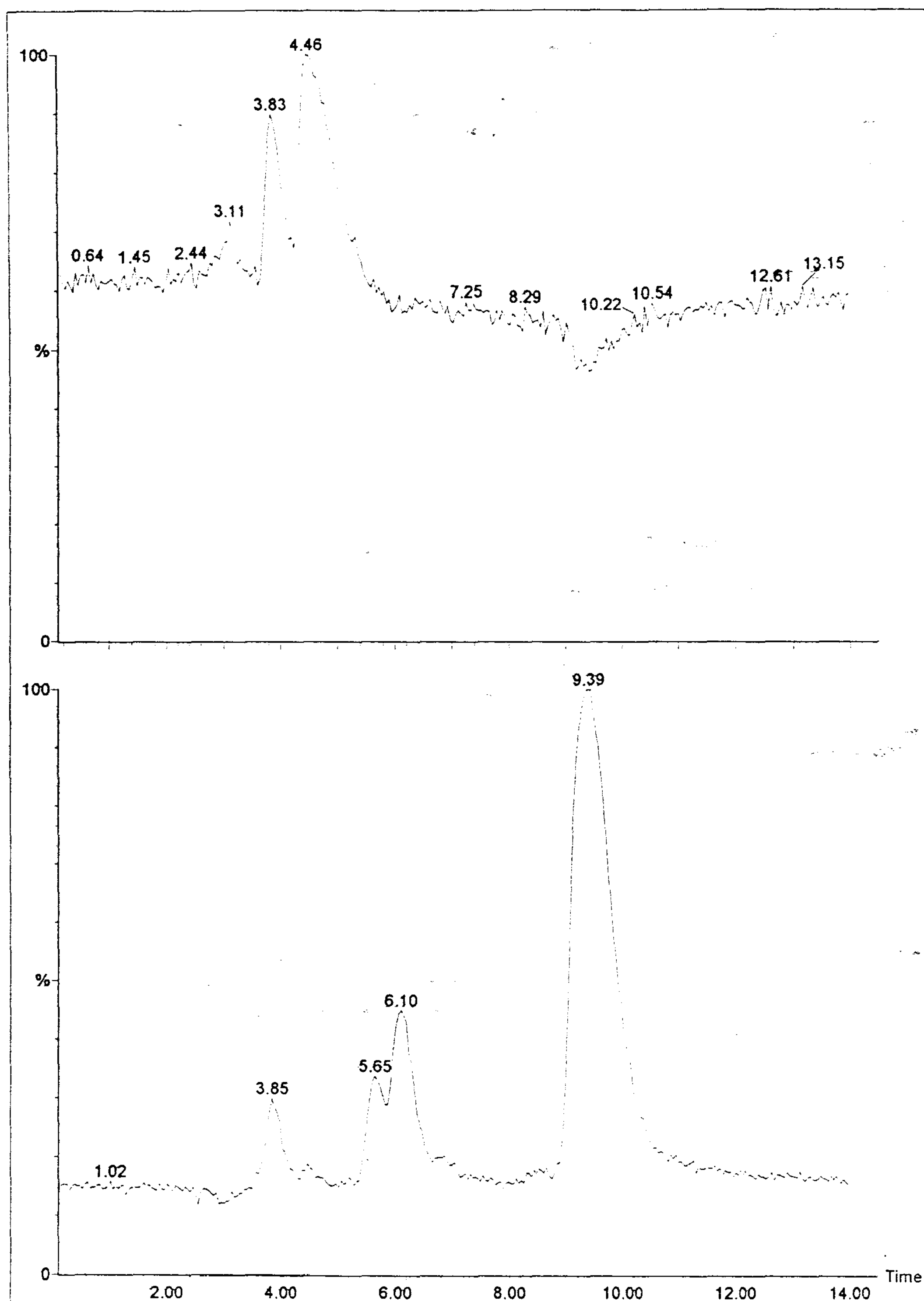


Figure 5.4 - Total Ion Current chromatograms of fluphenazine stressed in fluorescent / diffuse daylight.

Figures 5.1 - 5.4 show the Total Ion Current (TIC) chromatograms of representative stressed samples for each derivative, to illustrate the resolution of the chromatographic peak of the parent compounds at 12.68, 12.85, 11.23 and 9.39 minutes for prochlorperazine, perphenazine, trifluoperazine and fluphenazine respectively and their degradants. The presence of the ammonium acetate in the solvent accounts for a very strong signal, *m/z* 391, centred in the scanning range thus inclusion of this peak in the mass spectrum would cause the disappearance of some of the weaker ions. Two scanning functions were established from *m/z* 100 to 390, and from *m/z* 391 to 550, generating a set of chromatograms for each of these ranges.

Prochlorperazine - In the solution studies on prochlorperazine, 7 unknown compounds with retention times of 4.0, 4.2, 4.5, 6.0, 8.7, 9.2 and 10.7 minutes were observed on HPLC analysis, with the parent compound eluting at approximately 12.68 minutes. These peaks have essentially the same retention times in the HPLC chromatograms as were detected in the TIC chromatograms for the sample of prochlorperazine, stressed in diffuse daylight, Figure 5.1. Figure 5.5 shows the LC-MS chromatograms of each unknown degradant. These chromatograms each consist of an intense peak representative of the protonated species (unknown + H^+). LC-MS confirmed the molecular mass of prochlorperazine to be 374, and degradants **40, 41, 42, 43** and **44**, are shown in Figure 5.6. There appears to be a common pathway representative of all light stress conditions to result in the formation of these degradants. Degradant **40**, formed by the elimination of the chlorine atom from the 2-position on the phenothiazine ring is proposed to be the photoreduced product. The addition of an hydroxyl group at the 2-position results in the formation of the 2-hydroxy derivative, degradant **42**. The elimination of the chlorine atom from the 2-position on the phenothiazine nucleus makes it available to interact with other species present in the solution, accounting for the formation of **43**, which results from cleavage of the side chain, with the addition of the chlorine group. Loss of the chlorine group from **43**, occurs to form degradant **44**. A common *in vitro*

degradation product and *in vivo* metabolite, which results due to oxidation of **39** is the 5-sulphoxide **41**.

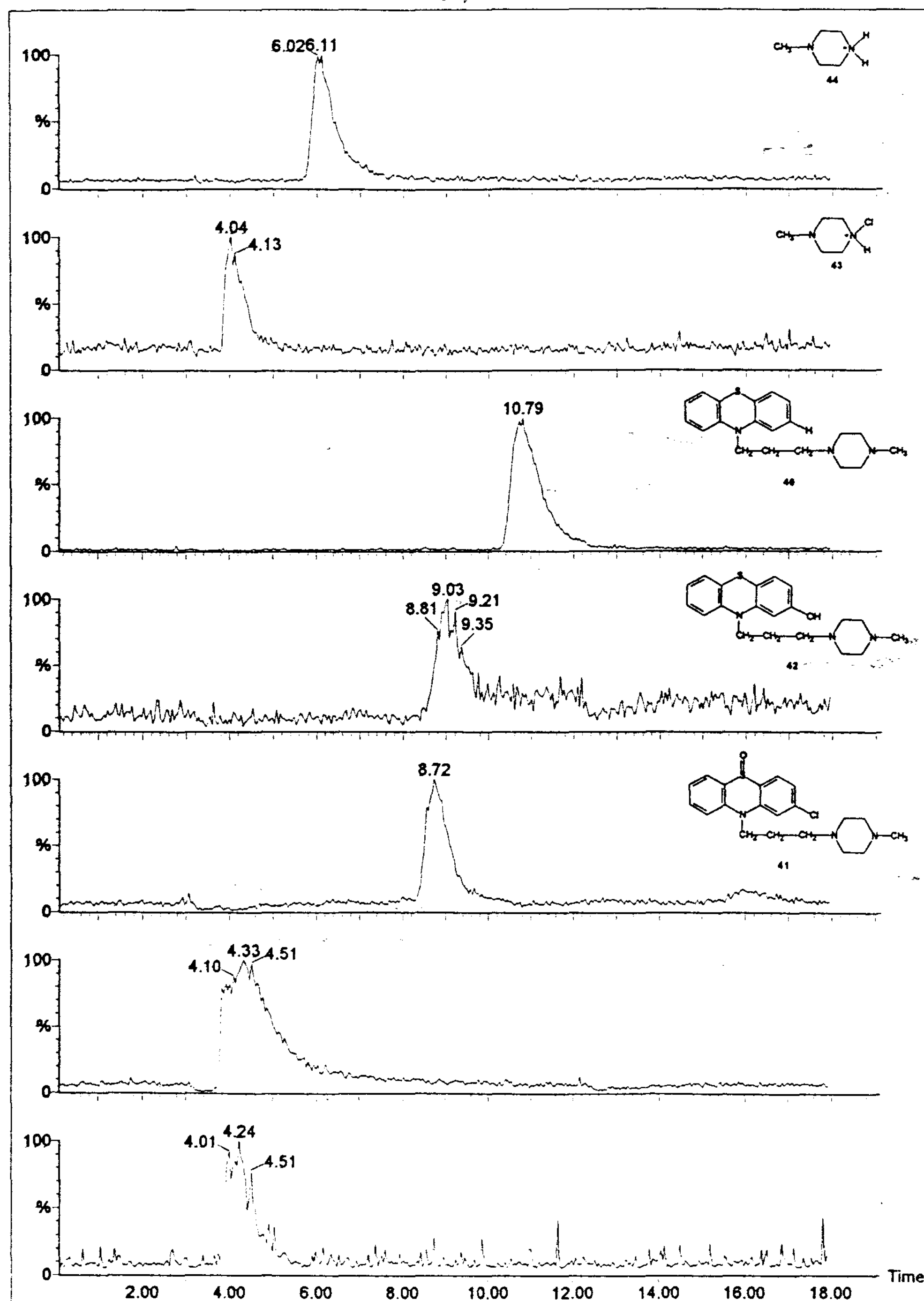


Figure 5.5 - LC-MS chromatograms of unknown compounds detected in stressed samples of prochlorperazine.

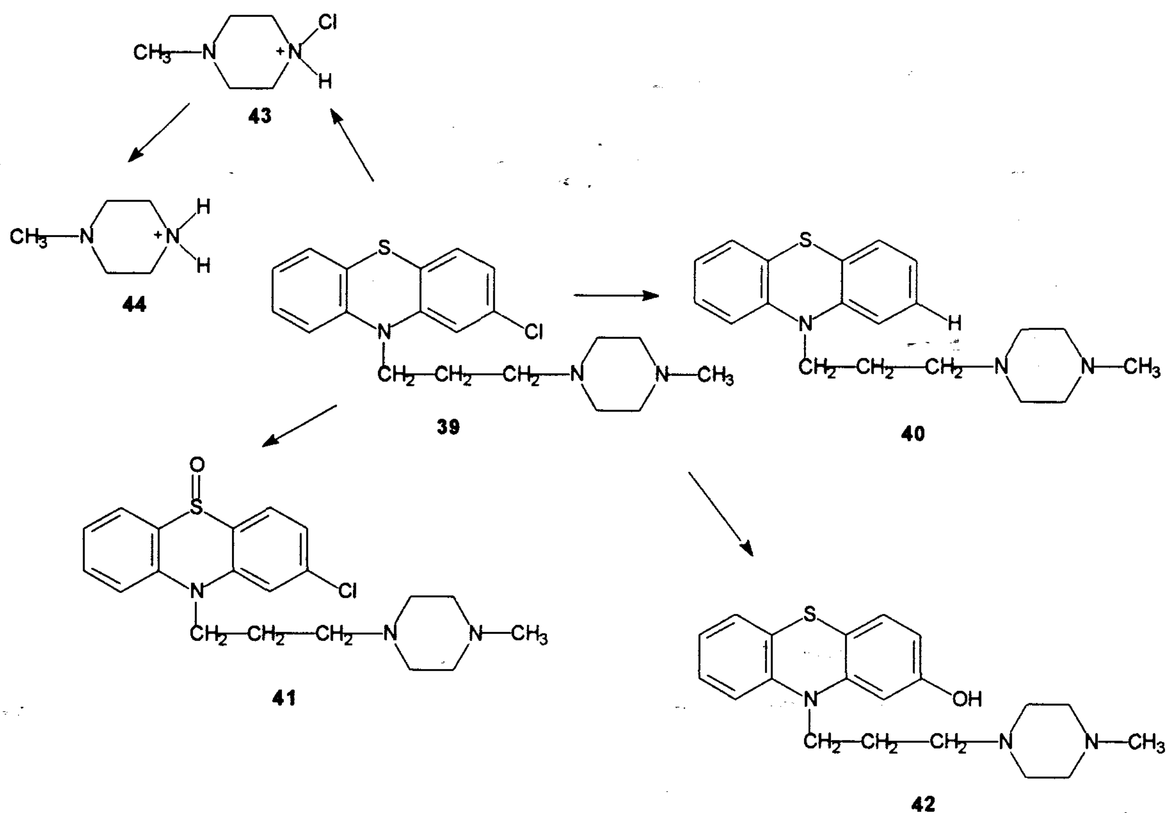


Figure 5.6 - Proposed degradation pathways for prochlorperazine.

The difference between the molecular mass of prochlorperazine and its degradants is indicative of the substructural differences between these compounds. The structures are based on the premise that the degradants retain much of the original prochlorperazine structure, **39**. Common product ions of prochlorperazine and those of the degradants were evidence for common substructures and differences were indicative of variance in those substructures. Figure 5.7 illustrates the MS-MS product ion spectrum of prochlorperazine at *m/z* 374.38 (M + H^+). Partial loss of the propyl piperazine side chain yields an ion at *m/z* 246.07, one of the primary product ions, followed by the loss of 32 daltons which corresponds to the loss of the sulphur atom from the phenothiazine nucleus, to yield the product ion at *m/z* 214.16. Other diagnostic product ions common to all the degradants of prochlorperazine are *m/z* 141.15 $(C_8H_{17}N_2)^+$ and 113.17 $(C_6H_{13}N_2)^+$, due to cleavage of the side chain.

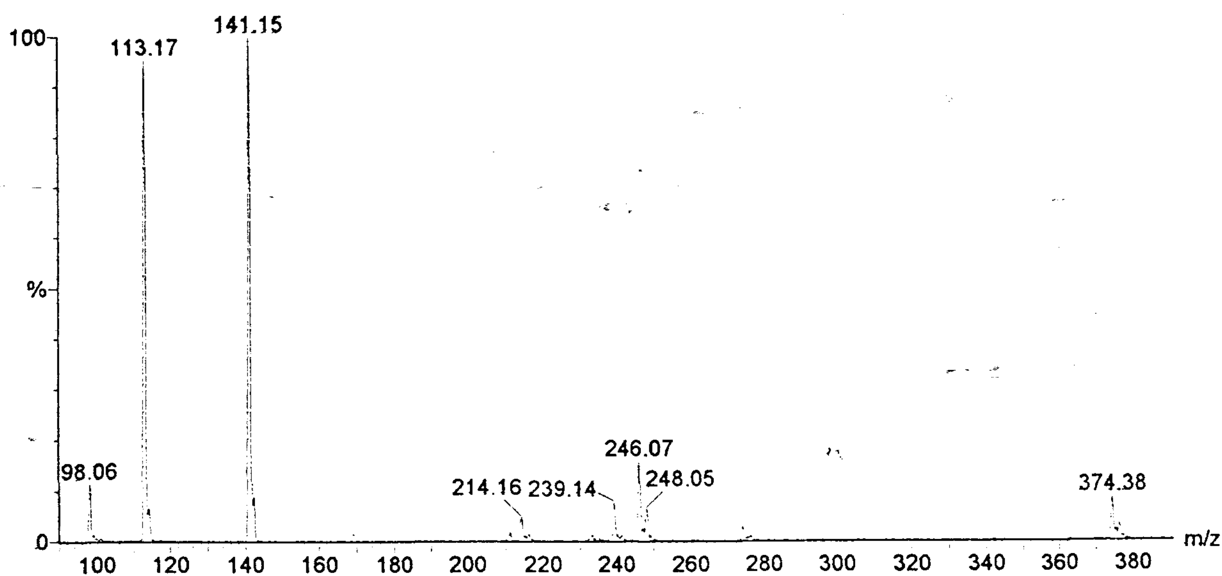


Figure 5.7 - LC-MS-MS spectrum of prochlorperazine, 39.

The product ion spectrum is a unique fingerprint for each compound and can thus be used for structural elucidation purposes as well as confirmation of the presence of a suspected compound. The LC-MS-MS product ion spectrum for the degradant at approximately 10.7 minutes, assigned by molecular mass information as dechloro-prochlorperazine **40** at *m/z* 340.27, is shown in Figure 5.8.

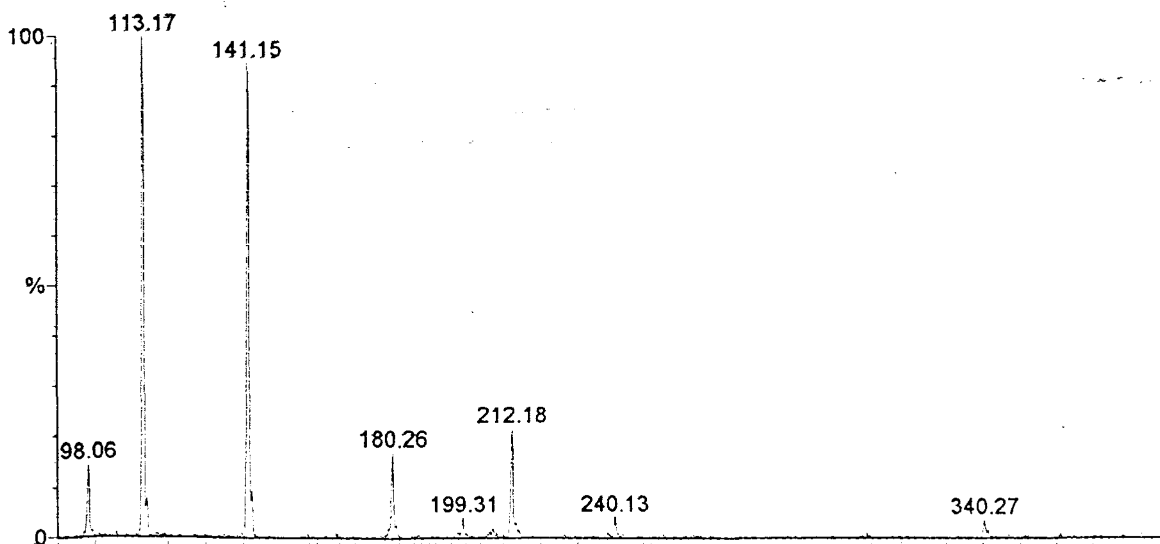


Figure 5.8 - LC-MS-MS spectrum for the dechloro-derivative, 40.

Photoreduction and photosubstitution of the chloro-derivatives are anticipated as a result of irradiation of solutions of the derivatives, owing to the photolabile nature of chlorine. This dechloro-derivative has a molecular mass 35 daltons less than the parent drug. Cleavage of the piperazine ring including the *N*-methyl substituent results in the formation of a product ion at *m/z* 240.13. Subsequent loss of the ethyl group from the side chain, and a further loss of the sulphur atom from the phenothiazine ring gives rise to product ions at *m/z* 212.18, and 180.26, respectively. Formation of the 2-hydroxy derivative, *m/z* 356.34, another commonly reported degradant of phenothiazine derivatives is as a result of photosubstitution. The product ion spectrum for the component eluting at 8.7 minutes, shows it to be the corresponding sulphoxide **41** at *m/z* 390.20, Figure 5.9, which is 16 daltons greater than the parent drug due to the addition of oxygen. The characteristic product ions at *m/z* 232, 139 and 113 are also evident in the mass spectrum of the prochlorperazine sulphoxide standard synthesised in chapter 4.

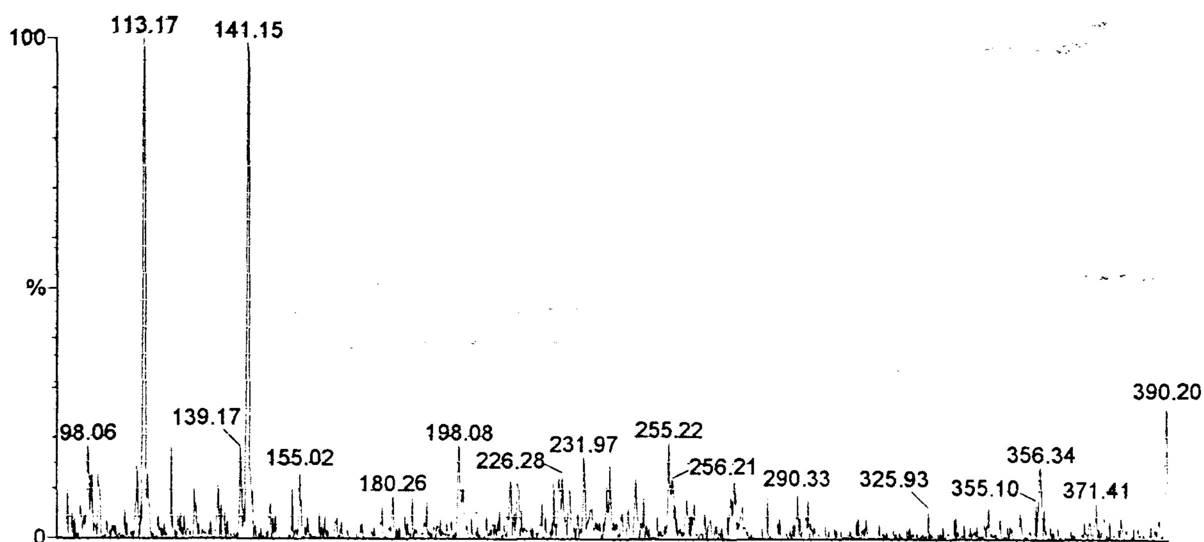


Figure 5.9 - LC-MS-MS spectrum of prochlorperazine sulphoxide, 41.

Dechlorination of **41**, results in the formation of a product ion at *m/z* 356.34 with subsequent cleavage of the oxygen atom from the sulphur group and loss of a methyl group from the piperazine side chain to form the product ion at *m/z* 325.93. Loss of 70 daltons $(C_4H_3N)^+$ is responsible for the formation of the product ion at *m/z* 255.22. Further cleavage of the propyl

group from the ring nitrogen atom results in a product ion at *m/z* 198.08.

Table 5.3 contains the relative intensities of degradants formed from prochlorperazine under the various light conditions expressed as a percentage.

Table 5.3 - Relative Intensities expressed as a % of Degradants from Prochlorperazine.

m/z	400 W - UV	30 W - UV	Sunlight	55 W Fluorescent / Diffuse Daylight
101 **(44)**	2.29	6.57	5.11	0.43
136 **(43)**	17.71	11.95	8.55	9.72
340 **(40)**	9.31	11.32	18.54	3.89
356 **(42)**	1.06	8.54	8.10	0.44
390 **(41)**	1.28	8.62	14.47	0.66
429	1.53	8.78	4.51	0.29
527	2.96	5.52	4.89	4.90

Perphenazine - Figure 5.10 illustrates the MS-MS product ion spectrum of perphenazine at *m/z* 404, a chloro-derivative, structurally similar to prochlorperazine. Fragmentation of the derivative to produce a product ion at *m/z* 246.07 corresponds to the cleavage of the ethyl piperazine side chain, occurring similarly in prochlorperazine. The presence of the β-hydroxyethyl group on the piperazine ring in the side chain of perphenazine is the principle difference between these two compounds. Diagnostic product ions, evident in all degradants of perphenazine, at *m/z* 171.10 $(C_9H_{19}N_2O)^+$, 143.13 $(C_7H_{15}N_2O)^+$, and 100.04 $(C_5H_{12}N_2)^+$ are representative of derivatives of the propyl piperazine side chain of perphenazine. The MS-MS spectra for product ions at *m/z* 386, 420, and 370 correspond to components eluting at 5.54, 6.42, and 7.21 minutes respectively. Structural elucidation (LC-MS-MS) identifies the formation of the dechloro sulphoxide, the sulphoxide and dechloro-derivative of perphenazine. The ions resulting from the fragmentations are identical to those observed for the same prochlorperazine degradants and are shown in Table 5.4.

Table 5.4 - Product Ions of Perphenazine Derivatives.

Proposed Structure	Derivative + H⁺			
Perphenazine sulphoxide	420	212.18	198.08	-*
Dechloro perphenazine sulphoxide	386	212.18	198.08	-*
Dechloro perphenazine	370	212.18	-*	180.01

* not evident

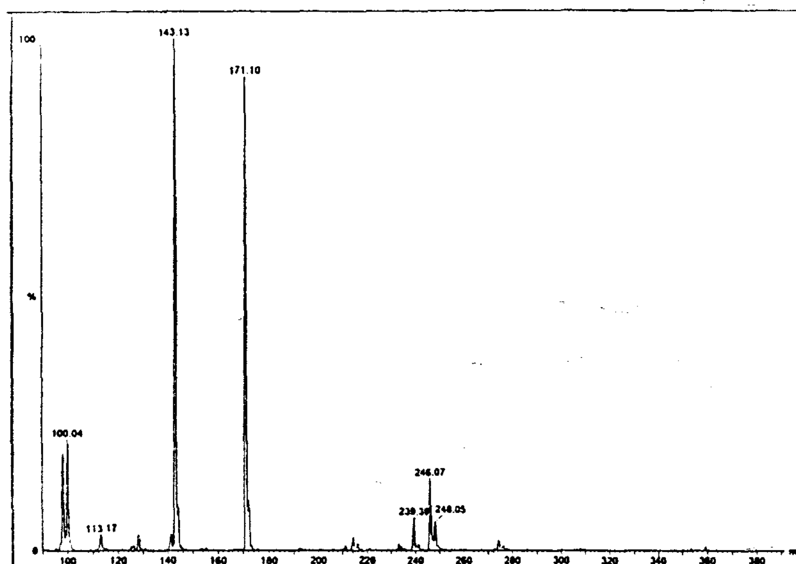


Figure 5.10 - LC-MS-MS spectrum of perphenazine.

Table 5.5 contains the relative intensities of the degradants from perphenazine, expressed as a percentage.

Table 5.5 - Relative Intensities expressed as a % of Degradants from Perphenazine.

m/z	400 W - UV	30 W - UV	Sunlight	55 W Fluorescent / Diffuse Daylight
136 **(50)**	13.37	10.26	14.23	10.64
370 **(46)**	26.85	23.70	33.95	18.90
389 **(49)**	5.42	4.42	9.02	3.41
402 **(47)**	6.55	6.37	7.56	5.31
420 **(48)**	2.35	3.69	-*	5.36
429	4.26	4.35	-*	3.08

* less than 0.1%

Figure 5.11 shows the proposed reaction pathway for the formation of the degradants of

perphenazine (stressed solutions).

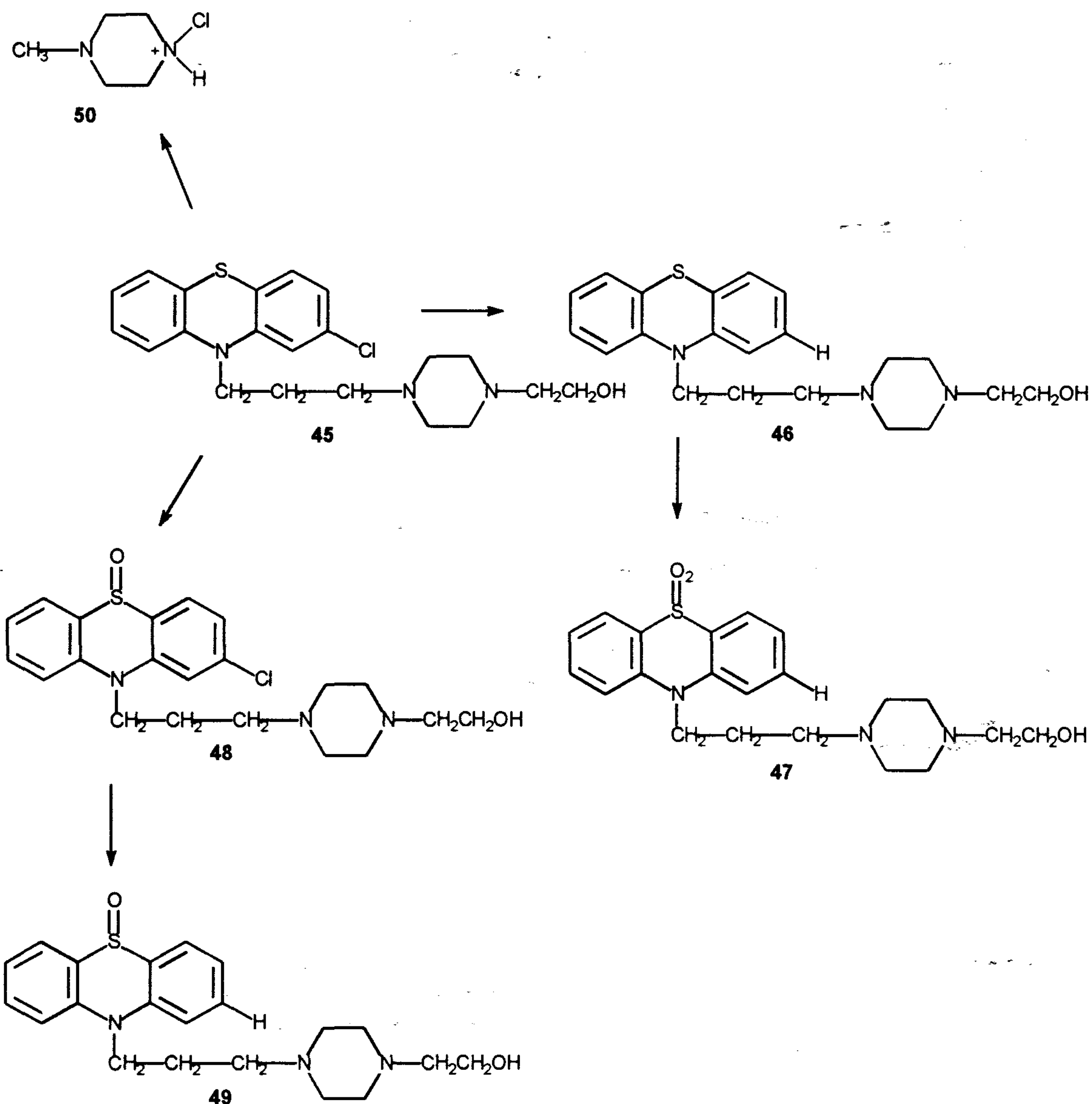


Figure 5.11 - Proposed formation of degradants from perphenazine.

Similar degradation pathways for the chloro-derivatives involve the formation of the dechloro-derivative, the respective sulphoxides and the development of a degradant with a molecular mass at *m/z* 429. It is evident from Tables 5.3 and 5.5 that solutions of perphenazine stressed under the various light sources are more susceptible to photoreduction and photosubstitution and thus have a greater tendency to undergo dechlorination as compared to prochlorperazine. Because of these similar patterns for the two chloro-derivatives, the

trifluoromethyl-derivatives, trifluoperazine and fluphenazine were examined for any similarities arising in the degradants from the photostability studies. These data are reported briefly.

Trifluoperazine and Fluphenazine - HPLC analysis indicated the presence of 8 unknown compounds in solutions of trifluoperazine stressed under the various light sources, the retention times of which are 3.47, 4.53, 4.55, 6.55, 6.58, 7.43, 8.71 and 10.85 minutes, with trifluoperazine eluting at approximately 11.23 minutes. These retention times are comparable to those illustrated in the TIC chromatograms. 5 Unknown compounds were detected in stressed solutions of fluphenazine. A similar pattern as observed for the chloro-derivatives is evident for the trifluoromethyl-derivatives as structures of the degradants retain much of the original parent compounds, trifluoperazine and fluphenazine.

The LC-MS-MS spectra for the unknown components at *m/z* 424, 440, and 384 for trifluoperazine and *m/z* 454, 470, and 414 for fluphenazine were used to elucidate the respective structures of the compounds, confirming the presence of the sulphoxide, sulphone and detrifluoro-sulphone for both trifluoromethyl-derivatives. While similarities exist between the derivatives, the additional peaks evident in stressed samples of trifluoperazine confirm the differences between these two compounds. These differences are explained by the respective LC-MS spectra, indicating molecular masses of 400, 508, and 416, for the unknown degradants of trifluoperazine.

LC-MS-MS spectra for both derivatives, illustrated a product ion, which resulted from the cleavage of the side chain from the parent compound, with a molecular mass of 136 for trifluoperazine and 105 for fluphenazine. Figure 5.12 proposes the reaction pathway for the formation of degradants from trifluoperazine and fluphenazine.

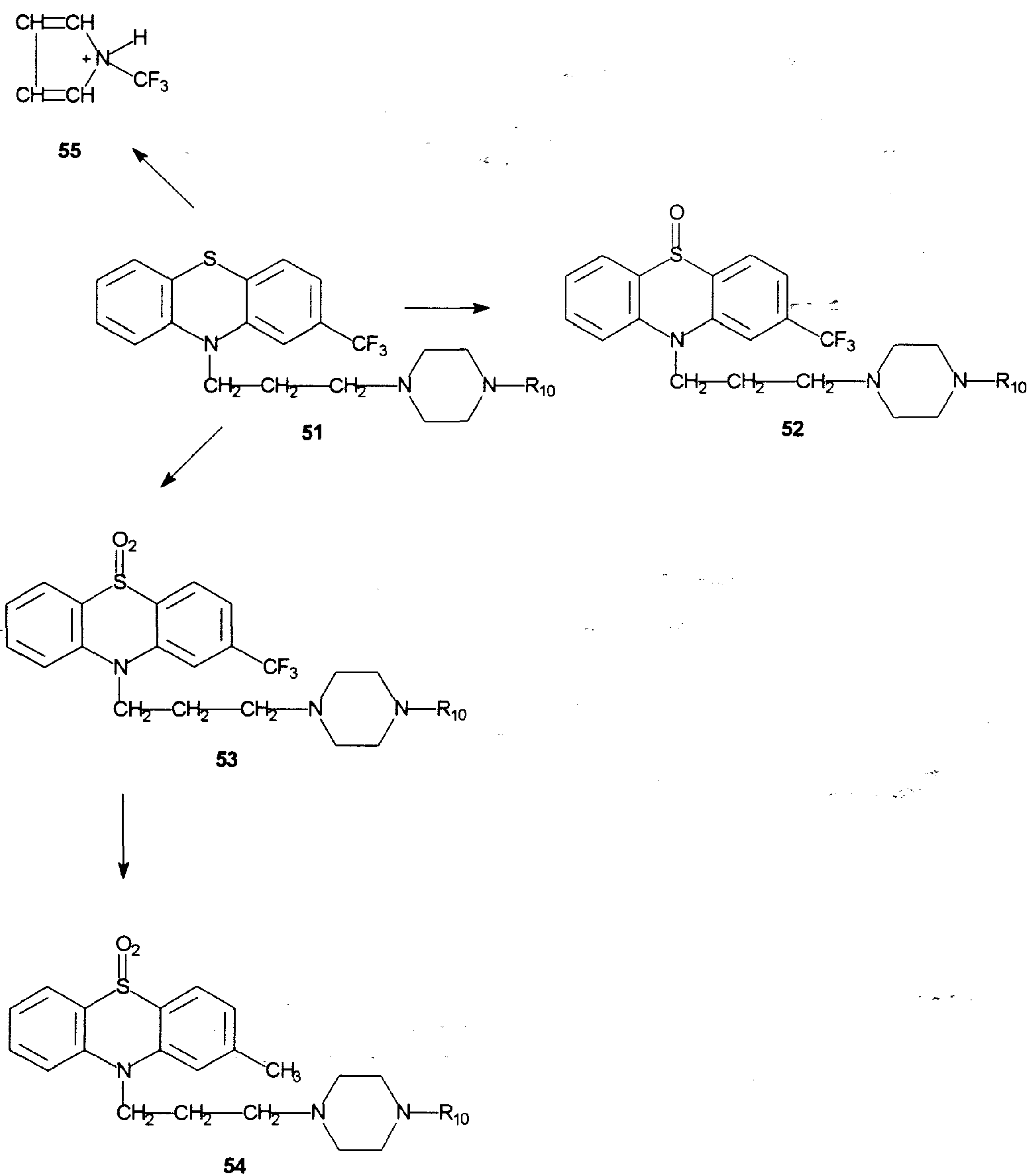


Figure 5.12 - Proposed pathway for the formation of degradants from trifluoperazine, where $R_{10} = CH_3$ and fluphenazine, where $R_{10} = CH_2CH_2OH$.

Table 5.6 - Relative Intensities expressed as a % of Degradants from Trifluoperazine and Fluphenazine.

Phenothiazine Derivative	*m/z*	400 W-UV	30 W-UV	Sunlight	55 W-Diffuse Daylight	Phenothiazine Derivative	*m/z*	400 W-UV	30 W-UV	Sunlight	55 W-Diffuse Daylight
Trifluoperazine	136 **(55)**	5.36	1.37	3.92	0.10	Fluphenazine	105	1.67	4.42	2.04	3.35
	384 **(54)**	54.82	33.04	41.33	16.9		136 **(55)**	73.59	34.67	44.45	14.50
	400	-*	5.47	-*	1.66		414 **(54)**	8.27	7.56	3.76	2.39
	416	4.31	-*	-*	-*		454 **(52)**	9.01	1.51	0.47	2.19
	424 **(52)**	0.31	2.73	3.09	-*		470 **(53)**	0.83	2.29	1.11	1.49
	440 **(53)**	-*	0.63	1.71	0.60						
	446	0.46	2.73	1.54	7.77						
	508	0.49	2.06	5.22	6.76						

* less than 0.1%

While similarities are evident with respect to the nature of the degradants formed in the stressed solutions of trifluoperazine and fluphenazine, the relative intensities of the degradants differ, Table 5.6. The relative intensity of the sulphone which has undergone cleavage of the trifluoro group from the 2-position is between 16 and 55% for trifluoperazine and is also the major degradant in the stressed samples. Although this degradant is present in solutions of fluphenazine (relative intensity 2.39 - 8.27%), the most significant degradant with a molecular mass of 136 displays a relative intensity of between 14 and 74%.

Common to all the stressed samples of the four derivatives is the occurrence of the corresponding sulphoxides, however there is considerable variance in the relative intensities of these degradants amongst the derivatives and this is due to the nature of the conditions of light exposure (Table 5.7).

Table 5.7 - Relative Intensities expressed as a % of Sulphoxides Formed.

Phenothiazine Derivative	400 W-UV	30 W-UV	Sunlight	55 W-Diffuse Daylight
Prochlorperazine	1.28	8.62	14.47	0.66
Perphenazine	2.35	3.69	-*	5.36
Trifluoperazine	0.31	2.73	3.09	-*
Fluphenazine	9.01	1.51	0.47	2.19

* Less than 0.1% level

LC-MS profiling and LC-MS-MS substructural analysis are valuable tools for rapidly obtaining the molecular mass and structural information concerning low-level impurities and degradants. Degradation products in the stressed samples were elucidated on the basis of their chromatographic retention times using standardized HPLC conditions, molecular mass information obtained from the mass spectrum acquired during LC-MS profiling, and the product ion spectrum acquired during LC-MS-MS analysis studies. The degradation products formed in the stressed solutions of the phenothiazine derivatives are due primarily to oxidation of the sulphur atom in the phenothiazine nucleus, as well as dehalogenation.

Solid

Semi-Preparative Studies

Several detection systems were investigated in an attempt to assess their application to semi-preparative HPLC, including UV, RI and diode array. UV detection has limited application to compounds which absorb between 200 - 400 nm. The highly coloured nature of the stressed samples however, encouraged further investigation into the use of RI detection. Diode array detection was chosen primarily due to the conflicting results achieved on LC analysis in that the samples were coloured but showed minimal degradation, therefore an evaluation of peak purity was essential.

The need for components of the mobile phase to be volatile to provide an isolated pure sample suitable for further analysis by NMR excluded the use of the mobile phase developed for the analytical studies. The mobile phase developed for the LC-MS analysis of the stressed solutions was considered and with polarity alterations to optimize separation, the final chromatographic conditions were achieved. The mobile phase was methanol: 30 mM ammonium acetate (50:50 v/v) at a flow rate of 1.0 mL per minute.

Diode array detection of the two fractions collected from the stressed solid fluphenazine sample (7 months stressed under 30 W Philips UV lamp) from the semi-preparative HPLC system suggested them to be pure, however, NMR and MS analysis of the fractions indicated the presence of more than one compound in each fraction.

The samples from semi-preparative analysis were dissolved in water and analyzed by MS, thus the addition of an equal volume of acetonitrile and some formic acid, to obtain a final concentration of 50% acetonitrile and 0.1% formic acid was necessary. A volume of 10 µL was injected and a carrying solvent of 50% acetonitrile with 0.1% formic acid at a flow rate of 15 µL per minute was used. The capillary voltage was 3.5 kV with the source temperature

of 80 °C and the cone voltage 35 or 50 V. For fragmentation the samples were introduced into the ESMS by continuous infusion using a syringe pump at 5 μL per minute. Data were acquired in the MCA mode.

Fraction 1, on comparison with a standard of known retention time, was assumed to be the parent compound, however no such molecular ion was detected on MS with peaks at *m/z* 455, 425, and 390 present. Similarities between the peaks at *m/z* 455 and 425 were evident on fragmentation, while differences were also apparent. The ion at *m/z* 266, which is characteristic of fluphenazine occurs in both. Product ions at *m/z* 144 and 172, diagnostic of fluphenazine were only evident on fragmentation of the ion at *m/z* 455. Similarities were observed between the ions at *m/z* 425 and 390. A product ion with a molecular mass of 70 was detected at *m/z* 455, 425 and 390.

Fraction 2 owing to its lower concentration gave a greater interference on analysis, particularly at low masses and lower cone voltage. Similarities observed between the spectra of fractions 1 and 2 are the ions at *m/z* 425, 195, 309, and at a cone voltage of 50 V, both fractions appeared to contain an ion at *m/z* 148. Fraction 2 is reported to contain a small peak at *m/z* 438 which upon fragmentation exhibits fragment ions at *m/z* 144 and 172, diagnostic of fluphenazine. The molecular ion at *m/z* 415 is unique to fraction 2, fragmentation of which suggests it to be a fluphenazine derivative. Other peaks unique to fraction 2 are at *m/z* 248, 166, and 125. Fragments at *m/z* 166 and 125 are related as the ion at *m/z* 125 is in fact a fragment of 166. Furthermore, the difference between the fragments which is approximately 40 daltons, suggests an adduct of acetonitrile.

It can therefore be concluded that the fractions collected from the HPLC semi-preparative analysis of fluphenazine do not each contain a single compound, suggesting the necessity for the use of LC-MS for the structural elucidation of the four derivatives present in the solid

samples of the four derivatives.

LC-MS Studies

The LC-MS procedure for the determination of degradants from liquid studies was applied to the characterization of the unknown compound(s) present in the stressed solid states. *In vitro* investigations by Gaertner, Breyer and Liomin report the major metabolic pathways of the phenothiazine derivatives to be: *N*-dealkylation, *N*-oxidation, sulphoxidation and hydroxylation of the aromatic ring system. It is reported that the rate of these *in vitro* transformations is dependent on the concentration of the substrates, and that the formation of the sulphoxides has been proved to be a slow reaction *in vitro.*[25]

Prochlorperazine and Trifluoperazine

In spite of previous reports,[25] sulphoxidation is identified as the principal degradation pathway for stressed samples of prochlorperazine and trifluoperazine, giving rise to the 5-sulphoxide in the presence of the respective parent compound. Previous HPLC analysis of stressed solid samples of prochlorperazine and trifluoperazine suggested the presence of the respective sulphoxides due to comparable retention times. The analysis of these samples spiked with the standards (sulphoxides) synthesised in chapter 4 indicated the presence of a single compound, confirmed by diode array determinations. LC-MS-MS data for the unknown compounds eluting at approximately 7.8 and 7.4 minutes with molecular ions at *m/z* 390 and 424, for prochlorperazine and trifluoperazine respectively, confirmed the presence of the sulphoxides. The relative intensity (%) data obtained from these studies indicates the sulphoxide for prochlorperazine to be 1.93%, while the sulphoxide for trifluoperazine had a relative intensity of less than 0.1%, thus not requiring examination or identification according to ICH regulations.[74]

Fluphenazine

Figure 5.13 shows the TIC chromatogram of the stressed solid state of fluphenazine to illustrate the resolution of chromatographic peaks of fluphenazine at 7.17 minutes and its degradants. Figure 5.14 illustrates the LC-MS chromatograms of each unknown degradant, with molecular ions at *m/z* 414, 454 and 494 corresponding to the peaks eluting at approximately 4.0, 5.0 and 8.3 minutes.

N-dealkylation, sulphoxidation, and dehalogenation with sulphone formation are the suggested pathways responsible for the formation of the 3 unknown compounds, Figure 5.15.

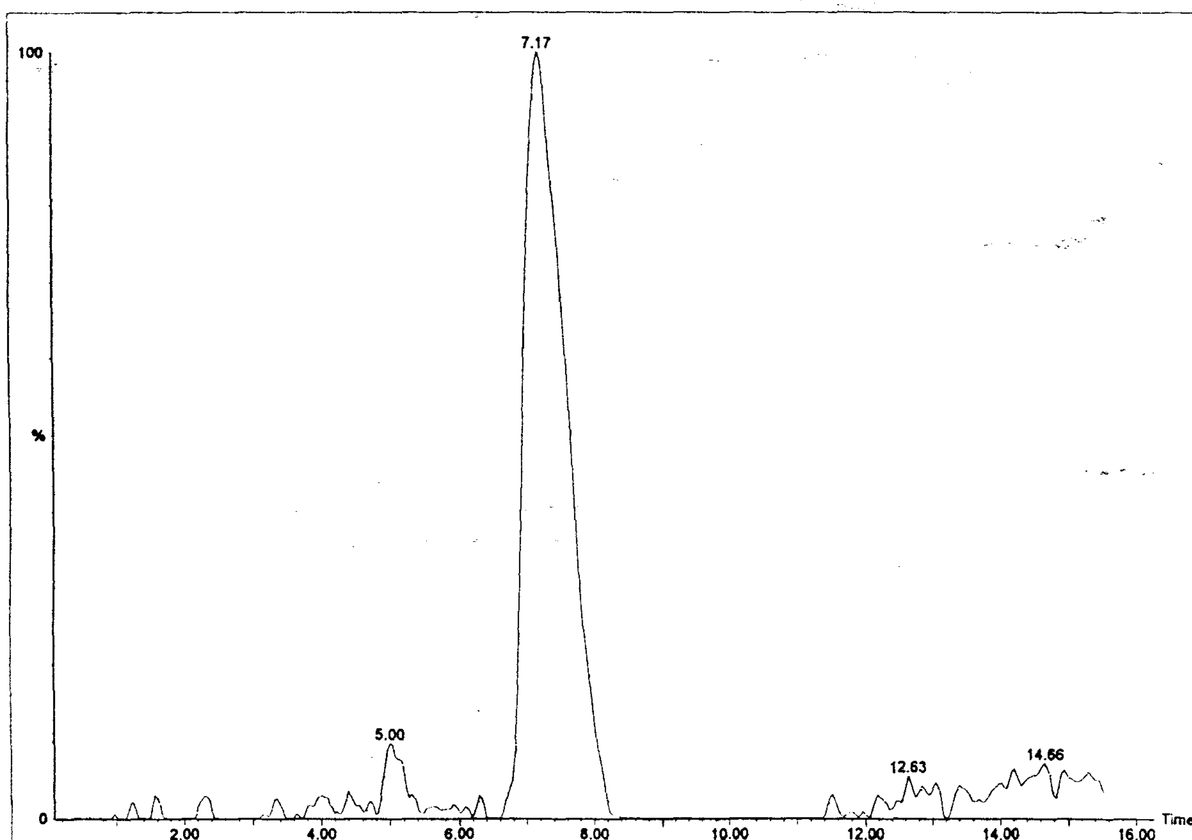
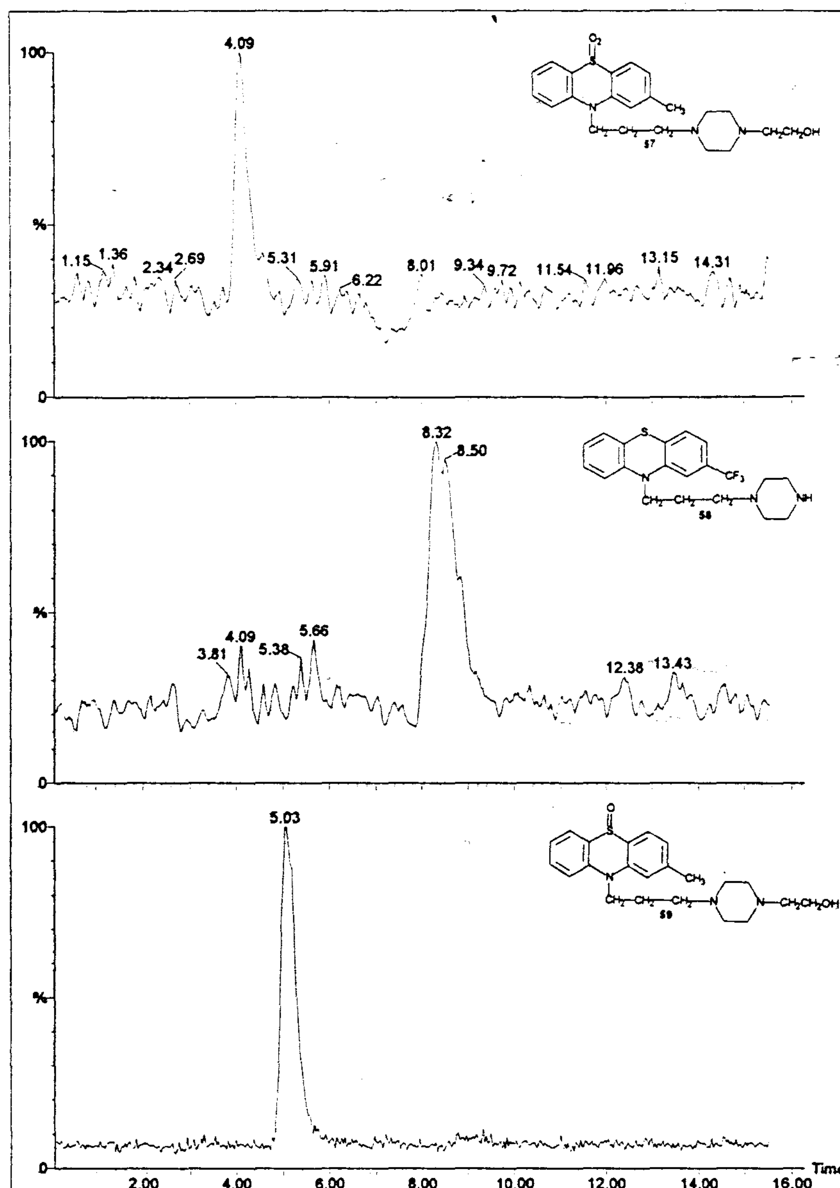


Figure 5.13 - Total Ion Current chromatogram of a stressed solid sample of fluphenazine.



5.14 - LC-MS chromatograms for the degradants from fluphenazine.

Removal of the β-hydroxyethyl group is unique to the stressed solid state of fluphenazine, while sulphoxidation, and sulphone formation with the removal of the trifluoro group is similar to stressed solutions of the derivative. Literature reports indicate the methyl-derivatives, prochlorperazine and trifluoperazine to be more susceptible to *N*-dealkylation as opposed to the β-hydroxyethyl derivatives, a phenomenon not observed in these studies.[25] Analytical studies however suggest this to be a minor degradation pathway for fluphenazine as the *N*-dealkylated derivative is reported to be present in a quantity of less than 0.1%.

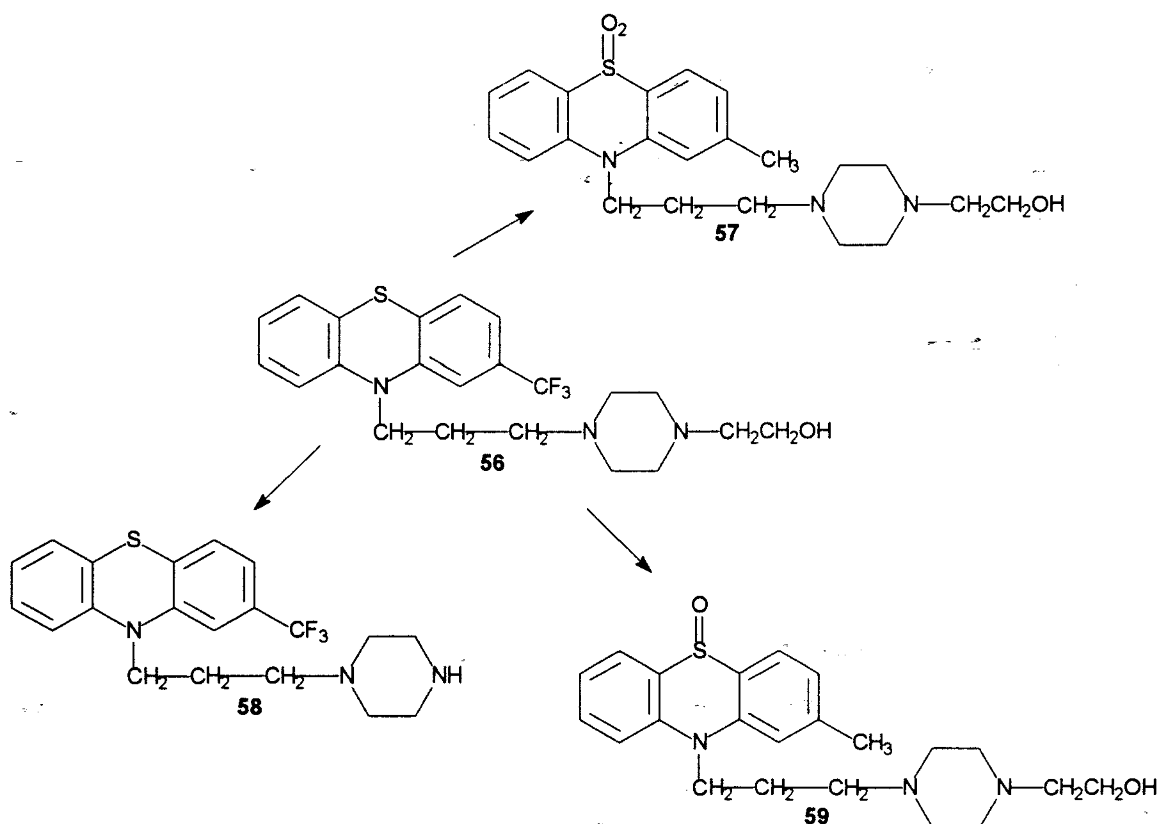


Figure 5.15 - Proposed pathway for the formation of degradants from a stressed solid sample of fluphenazine.

Figure 5.16 illustrates the MS-MS product ion spectrum of the compound eluting at approximately 8.32 minutes, which is significantly less polar than the parent compound. *N*-dealkylation to result in the loss of the β-hydroxyethyl group from the parent compound forms this fragment ion at *m/z* 393.69. Further fragmentation yields a product ion at *m/z* 278.84 which corresponds to the cleavage of the nitrogen atom from the piperazine side chain, and loss of the sulphur atom from the phenothiazine nucleus. The product ion at *m/z* 216.82 representing a side chain derivative including the trifluoromethyl group is evident in the MS-MS spectra of all the degradants.

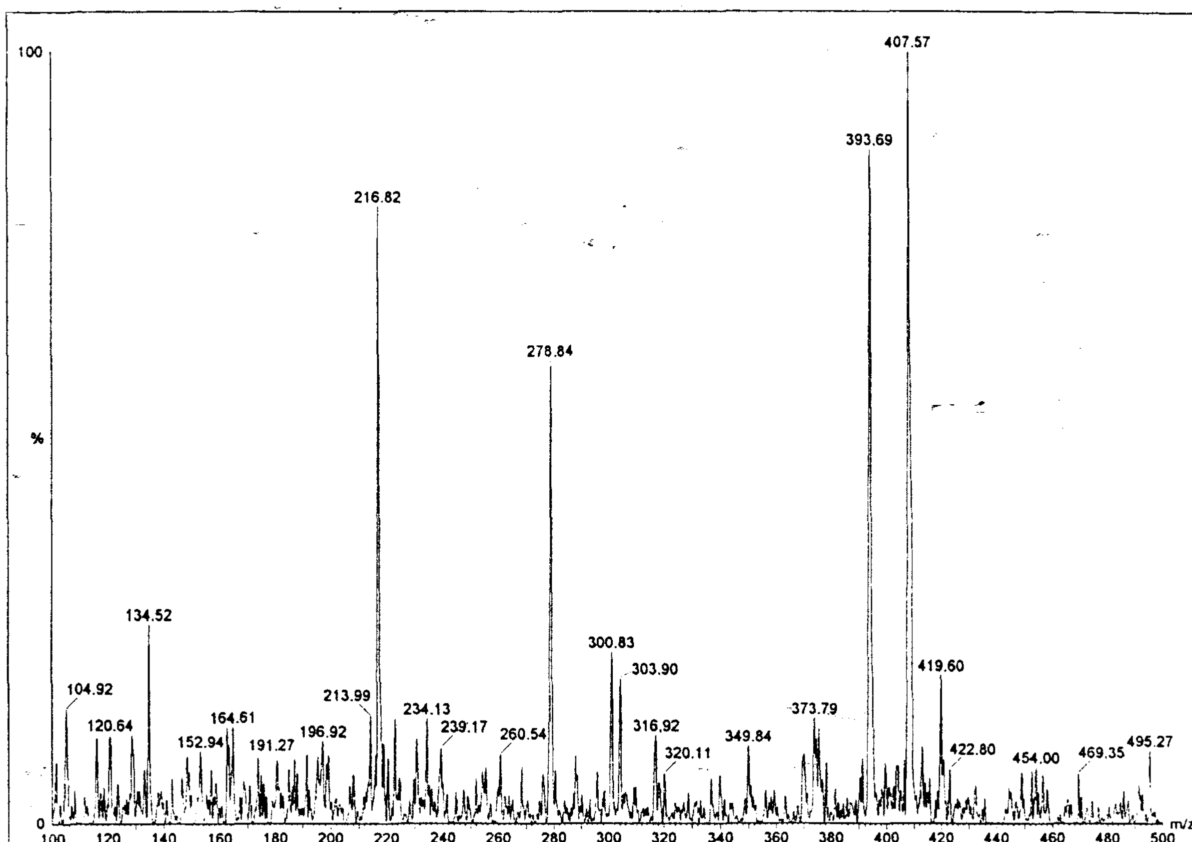


Figure 5.16 - LC-MS-MS spectrum for the degradant eluting at approximately 8.32 minutes, 58.

The LC-MS-MS spectrum for the degradant at *m/z* 413.71 (3.37% relative intensity), eluting at approximately 4.09 minutes confirms the presence of the sulphone with the loss of the 2-trifluoro group from the phenothiazine nucleus, Figure 5.17. Fragmentation of this derivative to form a product ion at *m/z* 305.87 corresponds to the loss of the sulphur atom from the phenothiazine nucleus, two oxygen atoms and the methoxy group from the piperazine side chain. Further fragmentation resulting in the loss of 28 daltons from the piperazine side chain forms the product ion at *m/z* 278.60, similar to the fragment observed for the *N*-dealkyl derivative. The product ion at *m/z* 216.82 is evident in the spectrum.

Fragmentation of the degradant at *m/z* 454 (20.23% relative intensity), characterized as the sulphoxide, yields only the common product ion at *m/z* 216.82.

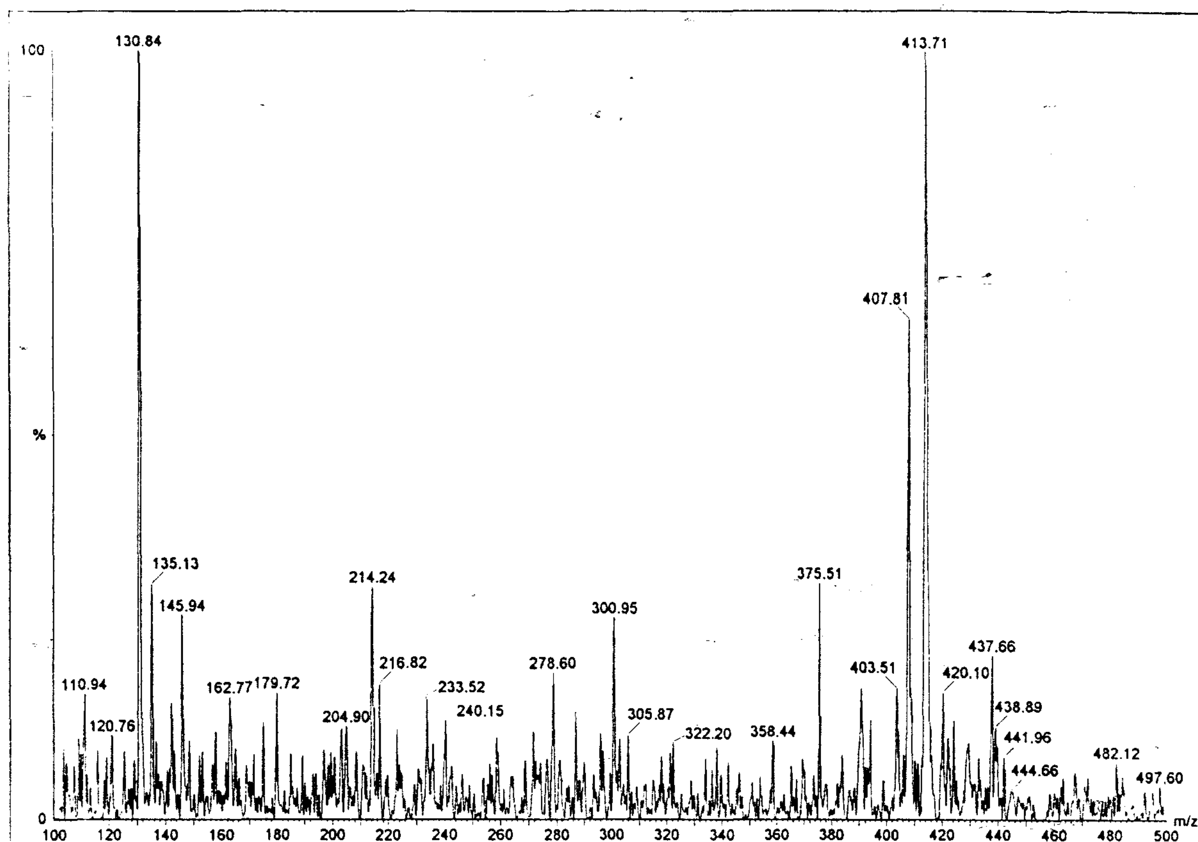


Figure 5.17 - LC-MS-MS spectrum of the degradant at *m/z* 413.71.

Similarities exist between the MS data from the semi-preparative fractions collected and the LC-MS data for solid samples of fluphenazine, while significant differences are also evident. Fraction 1 analyzed by MS indicated the presence of compounds at *m/z* 455, 425 and 390, while LC-MS data for the stressed solid sample of fluphenazine, identified degradants at *m/z* 454 and 394 which upon structural elucidation are the sulphoxide and *N*-dealkylated derivatives of fluphenazine. Reports of the MS data obtained from the semi-preparative HPLC fractions, identify two ions unique to fraction 2 at *m/z* 415 and 248, while ions at *m/z* 425, 195, 309, and 148 were also reported to be present in fraction 2. The ion at *m/z* 414 was characterized by LC-MS-MS as the sulphone with the loss of the 2-trifluoro group from the phenothiazine nucleus.

Perphenazine

The TIC chromatogram obtained for the stressed solid sample of perphenazine illustrates good resolution between the parent compound and the degradants. LC-MS data of the stressed sample of perphenazine indicate the presence of 3 degradants eluting at 6.08, 6.89 and 7.17 minutes respectively, while the parent compound displays a retention time of 7.06 minutes. All degradants however occurred at levels lower than 0.1% and thus do not require further examination and identification. However, structural elucidation has been attempted.

The LC-MS-MS spectrum for the compound at *m/z* 369.86 confirms the presence of the dechloro-derivative, Figure 5.18. Fragmentation of this derivative shows identical product ions to those observed for the *N*-dealkylated derivative of fluphenazine at *m/z* 393.69, suggesting a pattern of dehalogenation and *N*-dealkylation for the β-hydroxyethyl derivatives. Product ions at *m/z* 316, 300, 278 and 216 are similar for both β-hydroxyethyl derivatives; fluphenazine and perphenazine, and correspond to the initial loss of the methoxy group from the piperazine side chain with further fragmentation of the piperazine side chain and the removal of the sulphur atom from the phenothiazine nucleus being responsible for the product ions at *m/z* 316.80, 300.71, and 278.72.

No indication of the development of the respective sulphoxide was obtained from the LC-MS study on the stressed solid sample of perphenazine.

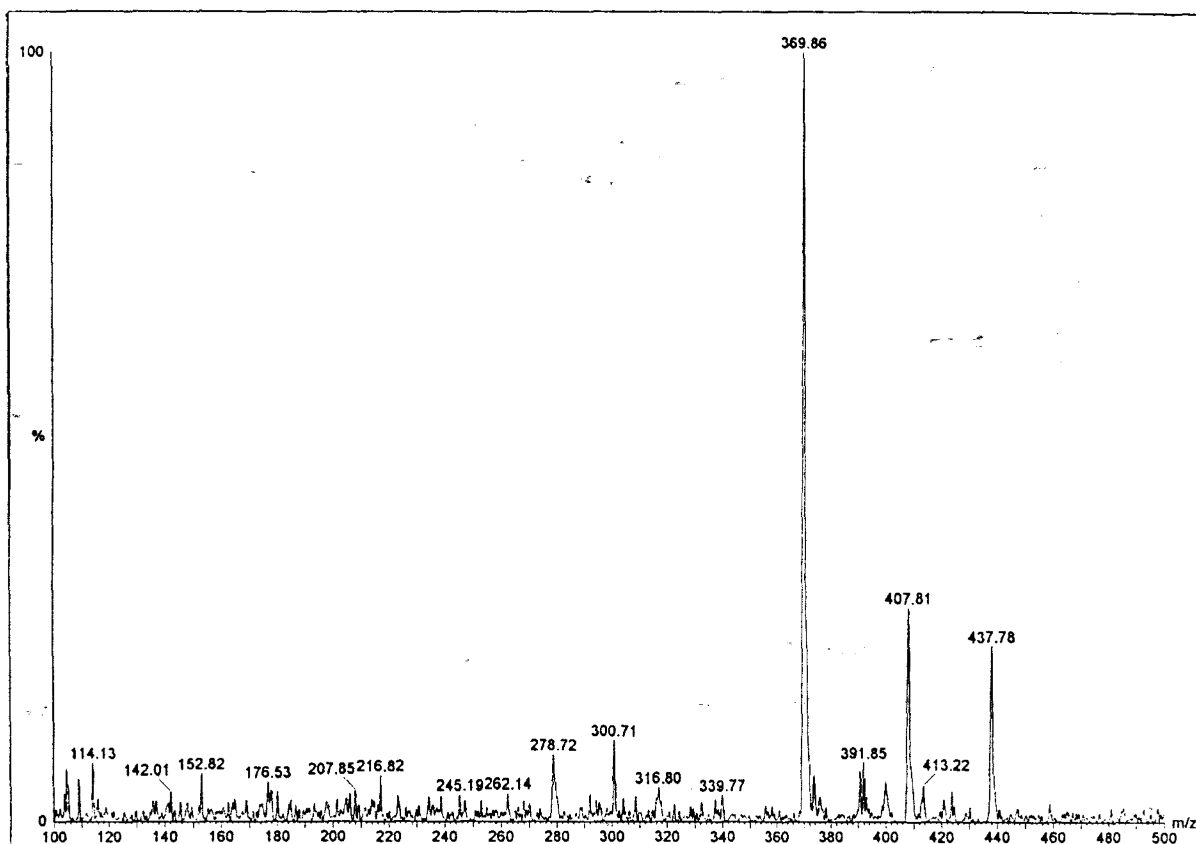


Figure 5.18 - LC-MS-MS spectrum for the compound at *m/z* 369.86.

5.4 Conclusion

LC-MS is a useful tool for rapidly obtaining detailed structural information on low-level degradants observed in HPLC analyses of samples. Furthermore chromatographic resolution of co-eluting or unresolved components is not essential to obtain product ion data for structural analysis, due to the mass-resolving capability of the mass spectrometry, thus this strategy has significant implications for industry, as impurities at greater than the 0.1% levels need to be examined and identified.

The TIC chromatograms obtained from LC-MS illustrate essentially the same resolution and retention times for degradants as observed in the analytical LC analyses, which enables the quantitation with some degree of accuracy of the levels at which the degradants are occurring

in the stressed samples. Attempts were made at structural elucidation based on the fragmentation patterns (LC-MS-MS spectra) of the degradants, however for molecular ions at *m/z* 429, 446, 400, 508 and 527 present in solution studies there is some degree of uncertainty with respect to the structures of the degradants. The following however is proposed.

The product ion at *m/z* 429 is evident in stressed solutions of the chloro-derivatives. The differences in the molecular mass between the product ion at *m/z* 429 for that of perphenazine and prochlorperazine are 25 and 55 daltons respectively. While a difference of 30 daltons exists between perphenazine and prochlorperazine, thus a difference of essentially 25 daltons is responsible for the product ion at *m/z* 429. The LC-MS-MS spectrum for this ion, suggests substructural differences between the degradant and the two chloro-derivatives. It is thus proposed that the product ion at *m/z* 429 is a sodium adduct of perphenazine, however in the case of prochlorperazine, a solvent addition product (MeOH) is evident in conjunction with the sodium adduct. A similar effect is observed in solution samples of trifluoperazine, where a product ion at *m/z* 446, is indicative of a sodium adduct of the sulphoxide.
The product ions at *m/z* 527 and 508 evident in stressed solutions of prochlorperazine and trifluoperazine, due to the high molecular mass are thought to be dimers of the respective derivatives, however, at this stage there is no conclusive evidence in support of this suggestion.

Similar degradation pathways exist for the chloro-derivatives with dehalogenation and subsequent photoreduction and photosubstitution most prominent, with sulphoxidation of the parent compounds of secondary importance. Perphenazine appears to be more susceptible to dehalogenation with levels of between 18.90 and 26.85% for the dechloro-derivative, while prochlorperazine reports significantly lower levels of between 3.89 and 18.54%.

Sulphoxidation is evident throughout the solutions studies in levels greater than 0.1%, for all four piperazine-substituted derivatives, with the exception of solutions of trifluoperazine stressed under diffuse daylight / fluorescent light 55 W and solutions of perphenazine exposed to sunlight. However, stressed solutions of perphenazine demonstrate the formation of the sulphoxide in the absence of the chlorine group at the 2-position on the phenothiazine nucleus, with levels higher than those observed for sulphoxidation of the parent compound itself. This phenomenon is not apparent in stressed solutions of prochlorperazine.

Photostability studies of the four derivatives indicate a less complex pattern of degradation for the solid state as opposed to the solution studies. In the solid state studies, trifluoperazine and prochlorperazine give rise to a similar degradation pattern yielding only to the respective sulphoxides, as a result of exposure to ultraviolet irradiation, while the stressed sample of fluphenazine is reported to degrade primarily to the corresponding sulphoxide (20.23%), with the loss of the trifluoro group from the sulphone and *N*-dealkylation as additional pathways. In the solid state, perphenazine appears to be the most stable derivative under these conditions with no significant levels of impurities resulting after 7 months of exposure to the 30 W UV source.

It can thus be concluded that sulphoxidation appears to be a common route of degradation for the four piperazine-substituted derivatives on exposure to light, while evidence of dehalogenation, sulphonation, hydroxylation and dealkylation are amongst the other degradation pathways observed in the photostudies. These results concur with the routes of degradation which have been well documented in previously reported *in vitro* studies.[25] However the potential for further investigations into the possible development of the 7-hydroxy derivative and *N*-oxide in stressed samples of the derivatives exists, while isolation by semi-preparative HPLC and structural elucidation by IR, NMR, MS etc. remains the ultimate goal.

CHAPTER 6: CONCLUSION

The propyl piperazine-substituted phenothiazines are an important subclass of these neuroleptic drugs exhibiting increased potency over the prototype chlorpromazine, a member of the aliphatic subclass. The susceptibility of the phenothiazine drugs to photodegradation has been reported, including mention of the fact that 2-chloro phenothiazines give rise to the dechlorinated product, a dimer and the corresponding sulphoxide on irradiation.[36] These findings implicating the chlorine substituent in photodegradations are confirmed by Pawelczyk et al. who report that trifluoperazine is more stable than prochlorperazine under various light conditions.[41, 42] This, together with various regulatory authorities requirements for stability profiles, isolation and identification of potential degradants and the ICH forum's, attempts not only to require structural elucidation of degradants present in concentrations of > 0.1%,[74] but also to standardize conditions for light stability studies. These requirements provided a motivation for this study to investigate the photostability of prochlorperazine, perphenazine, trifluoperazine and fluphenazine with a view to evaluating not only the effect of the R_2 substituent on the phenothiazine nucleus as reported by Pawelczyk et al. but also the R_{10} substituent on the piperazine ring on the photostability of these phenothiazines.[41] Since both the R_2 and R_{10} substituents influence the pharmacological activity of these compounds the question is raised as to whether these substituents introduced to increase activity compromise their stability.

Since the mechanism of action is explained in terms of interaction with the dopamine receptor,[3, 4] fluphenazine can be assumed to display the greatest neuroleptic potency due to the increased affinity of the β-hydroxyethyl side chain for the trifluoromethyl substituent as compared to the other derivatives. This is supported by the fact that the 2-substituent affects the lipophilicity of the molecule with the trifluoromethyl group making a greater contribution and thus enhancing penetration of the CNS.[10] The trifluoromethyl-derivative's greater surface

activity due to these hydrophobic effects and increased pharmacological activity over the chloro-derivatives, suggests a relationship between phenothiazine absorption and hydrophobicity. The ranking of the four derivatives in decreasing order of activity to be fluphenazine > trifluoperazine > perphenazine > prochlorperazine explains the major role played by the R_2 as opposed to the R_{10} substituent.

Since the phenothiazines have been associated with photosensitivity effects in individuals exposed to light with resulting photooxidation,[27] it is important to clarify which of the substituent groups is responsible for these effects. The photolability of the Cl and accompanying generation of free radicals has been established and the resulting photopolymer reported to be membrane active implicated in these photosensitivity reactions.[34] Further, irradiation of an aqueous solution of a chloro-derivative liberating HCl with a decrease in pH and formation of the membrane active 2-hydroxy derivative, substantiates the role of the chloro-derivatives in photosensitivity reactions, as opposed to their trifluoromethyl-derivatives which are essentially non-photosensitizing.[6]

The possibility of common *in vivo* and *in vitro* metabolites and decomposition products has been confirmed by *in vitro* metabolism of perazine occurring *via* *N*-oxidation, *N*-hydroxylation, *N*-demethylation, sulphoxidation and aromatic hydroxylation[18] and the decomposition products of chlorpromazine being the *N*-oxide, sulphoxide, hydroxypromazine, promazine, dimers and polymers.[36]

Reported stability studies on the phenothiazines in aqueous solutions, buffers and methanol and the involvement of a variety of light conditions, accounts for movement on the part of the ICH forum towards standardizing these conditions.[74] Although different amounts of the degradants are obtained under these various conditions there are similarities with respect to the nature of the degradants, providing information on the degradation pathway. Since even

in methanol, the presence of the photoreduced and solvent addition or photosubstituted product gives some indication that the mechanism of degradation involves fission of the carbon-chlorine bond.[31, 36]

Method validation of the HPLC method developed to analyze the photostudies is to ensure that performance and reliability meet the requirements of the intended analytical application. Although this was undertaken in this study to satisfy the linearity, accuracy and precision, selectivity, LOD and LOQ and ruggedness parameters, a complete validation should include degradants and synthetic precursors. Therefore at this stage the method validation is not comprehensive even though there is some knowledge of the susceptibility of the derivative to degradation. This knowledge is applied to the ruggedness parameters where the stability of solutions for injection was evaluated and instability of the chloro-derivative noted with a 26-fold decrease in stability observed over that of the trifluoromethyl-derivatives. However as Hokanson has pointed out, the process is dynamic with the ability to expand as additional information becomes available.[54] The fact that additional information has become available at the end of this study which together with the need to isolate the degradants, characterized by LC-MS, using semi-preparative HPLC has provided for further possible expansion of the method validation.

However, the method validation has overcome some of the limitations of the application of kinetics[62, 65] to the preliminary rate studies in chapter 3 in that linearity over the concentration range required in these studies is confirmed by correlation coefficients of $\geq$ 0.98, precision and accuracy requirements especially at the lower concentration limits satisfied and an LOQ of 3 - 4 x 10^{-4} mg/mL determined. Because the validation procedure does not include degradants and the method is used to quantify the percentage degradation in the stability studies by measuring the drug peak height, the importance of the photodiode array detection to ensure the integrity of these peaks cannot be over emphasised. This highlights the importance of the

selectivity parameter in this study where peak integrity of all four derivatives was confirmed under heat, light, oxygen and acid and base conditions. The highest degree of consistency has been seen in the application of validation parameters to HPLC with linearity, accuracy and precision, LOD and LOQ and solution stability most used, providing some correlation with the parameters used in this study.[49]

In addition to the limitation of the method when applying kinetics to rate studies, variations in light intensity and spectral character must be taken into account to allow certain comparisons to be made.[65] In these preliminary light studies in solution an aqueous medium at a controlled pH was used and only comparisons between the derivatives within a particular light condition were made and rate constants calculated. Although light intensity (lux) was measured in sunlight and fluorescent / diffuse daylight, in order to make comparisons between the 30 W and 400 W UV sources and for experiments performed at different times the application of actinometry is essential. These preliminary solutions studies showed perphenazine to be the least stable of all the derivatives.

With the main oxidative product of the phenothiazines being the monosulphoxide[33] and their *in vivo* and *in vitro* accumulations to higher concentrations than the parent compounds, an emphasis is placed on the importance of the synthesis of these compounds to provide standards to aid in their identification and characterization in chapter 5. Various synthetic methods were proposed[21, 24, 45, 47, 69, 70] but taking into account the different solubilities of these four derivatives no one method was suitable. Therefore adaptation of the Owens method[7] was accomplished and all four sulphoxides characterized (identity and purity) by mp, HPLC, TLC, UV, IR, NMR, MS, accurate mass and elemental analyses. Although this method is specific for the sulphoxides in view of the presence of the sulphones on irradiation of trifluoperazine and fluphenazine it is suggested that the oxidation profiles of these derivatives are studied in order to provide other oxidised products as standards for comparison.

The ICH forum regulations specifying the examination and identification of impurities > 0.1% led to attempts of isolation of degradants from various photostudies using semi-preparative HPLC. These many attempts were unsuccessful due to an inability to adjust the mobile phase to exclude non-volatile buffer components and the ion-pairing reagent and maintain adequate separation. Since the use of LC-MS has been well documented in structural elucidation, it was applied to a detailed solution and a preliminary solid state photostudy.[74, 75, 76, 77]

In the solution study of the chloro-derivatives, prochlorperazine and perphenazine, formation of the dechloro-derivative (photoreduction) and the sulphoxide occurred under all light conditions in varying amounts with dechlorination proving to be the major pathway in both cases but especially with respect to perphenazine. The presence of the 2-hydroxy derivative in the prochlorperazine solution reported to be membrane active confirms these derivatives involvement in adverse effects.

For the trifluoromethyl-derivatives, trifluoperazine and fluphenazine in solution, replacement of the $-CF_3$ with a $-CH_3$ sulphoxide and sulphone formation are seen to occur. When considering all four derivatives under the light conditions employed, the prochlorperazine sulphoxide was observed in the highest percentage (14.42) in sunlight.

In the preliminary solid state studies conducted under a UV source (30 W Philips) over a period of seven months, sulphoxidation appeared to be the major degradation pathway except for perphenazine where the dechloro-derivative was observed but at < 0.1%. The order of stability appears to be reversed here with the trifluoromethyl-derivatives less stable than the chloro-derivatives. Further studies are therefore necessary to provide conclusive evidence of this fact.

The pseudo first-order rate constants for the solution stability studies indicate the chloro-

derivatives (and specifically perphenazine) to be less stable than the trifluoromethyl-derivatives and although Pawelczyk et al. have confirmed these results with both their thermal and photolytic degradation studies on prochlorperazine and trifluoperazine this study extends their considerations of the effect of the R_2 to include the R_{10} substituent.[41, 42, 57] Results indicate the order of stability (30 W Philips UV) to be trifluoperazine > fluphenazine > prochlorperazine > perphenazine confirming the R_2 and not the R_{10} substituent to be the major determinant of stability. It can be concluded that the design of these derivatives to include the trifluoromethyl group to improve activity is justified. Although it has been seen to be the minor determinant of stability, the inclusion of the β-hydroxyethyl group, as opposed to the methyl group in the side chain to improve activity, is questionable in terms of its effect on stability.

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