CHAPTER I

INTRODUCTION

Imidazole antimycotic drugs constituent an important class of drug that continue to expand. These antimycotics are currently used in a variety of pharmaceutical formulation such as tablets, cream, lotions, etc. at relatively low concentrations (1-2%). Imidazole derivative, such as clotrimazole and ketoconazole are widely used in the treatment of fungal infections. Their antifungal activities are the alteration of cellular membrane, resulting in increased membrane permeability, secondary metabolic effects and finally following by growth inhibition. Besides the fungistatic activity of the drug may result from interference with ergosterol synthesis. In field of medical, pharmaceutical and cosmetic sciences, cream is the semisolid emulsion which can be prepared either oil-in-water (o/w) or water-in-oil (w/o) emulsions.

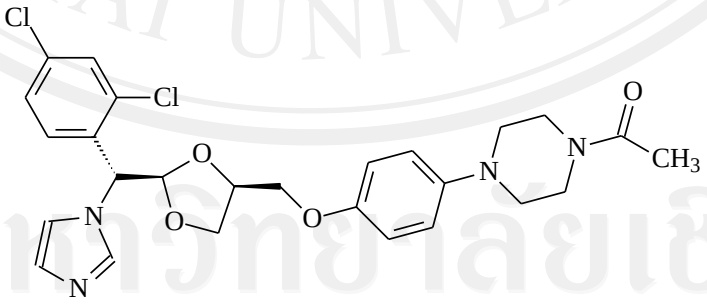

Figure 1.1 Structure of ketoconazole

Ketoconazole (cis-1-acetyl-4-[4-2-(2,4-di-chlorophenyl)-2-(1H-imidazole-1-yl methyl-1,3-dioxolan-4-yl] methoxy piperazine) is an imidazole antifungal agent. As with other imidazole, it has five-membered ring structure containing two nitrogen atoms with the structure shown in Figure 1.1. Ketoconazole is available in many dosage forms; cream, shampoo, solution, gel/jelly and foam. The side effects of ketoconazole are as follows; it can cause rash, itching, nausea, vomiting, abdominal pain, headache, dizziness, fatigue, impotence and blood count abnormalities. Rarely, ketoconazole has serious allergic reactions (anaphylaxis). Ketoconazole 1% shampoo is used to treat dandruff and ketoconazole 2% shampoo is used to treat "sun fungus". These shampoos may cause abnormal hair texture, scalp pustules (pimples), dry skin and itching. There may also be oiliness and dryness of the hair and scalp. Rarely, there may be some hair loss. Ketoconazole is the only member of the immidazole class that is currently used for treatment of systemic infections. Ketoconazole was more widely used before the development of newer, less toxic and more effective triazole compounds, fluconazole and itraconazole, its use has now been limited. It now appears as an alternative drug for specific indications. Ketoconazole works principally by inhibition of cytochrome P450 14a-demethylase. This enzyme is in the sterol biosynthesis pathway that leads from lanosterol to ergosterol [(1)]. The affinity of ketoconazole for fungal cell membranes is less compared to that of fluconazole and itraconazole. Ketoconazole has thus more potential to affect mammalian cell membranes and induce toxicity [(2)].

1.1 Development of High Performance Thin Layer Chromatographic Method for the Determination of Ketoconazole

1.1.1 Principles of High Performance Thin Layer Chromatography

The term high performance has, within the past year, been applied to three commercial TLC systems. The first is a commercial precoated TLC plate [3, 4]. The literature reports observed plate heights of 0.0012 cm and relatively short optimum solvent migration distances ($X_s \cong 5$ cm) suggest that these plates incorporate a small particle diameter (possibly ≅5 *um)* and a narrow particle size distribution in the layer. The published height values suggest that such plates should be capable of generating 5000 theoretical plates in a reasonable amount of time (perhaps 15 min or less). The disadvantage of these plates is that they require extreme care in sample application (literature values suggest 10 to 100 nL) if their potential is to be realized. This poses major problems in many real-life situations which require the analysis of dilute of complex solutions for trace components. Despite the great sensitivity which results from compact spots, such low load limits appear likely to severely tax the capabilities of current detection and quantitation techniques. The second system is a radial TLC system designed specifically for use with high performance plates [4] (Figure 1.2). The system allows controlled solvent delivery to the center of a plate mounted over an atmosphere whose composition can be controlled (Figure 1.3). Thus, this system allows the potential realization of the advantages of radial TLC, evaporative TLC, and high performance plates in combination.

Figure 1.2 CAMAG U-Kammer apparatus for radial high performance thin layer chromatography. CAMAG Inc., New Berlin, Wis.) [(4)]

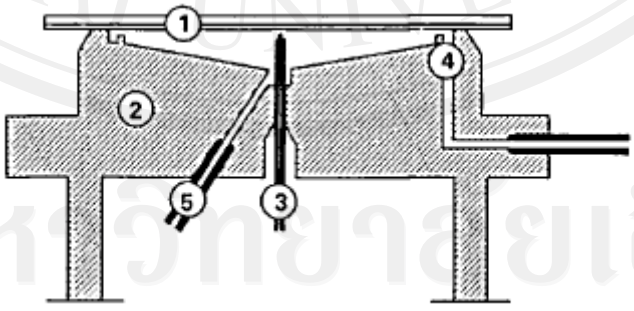

Figure 1.3 Cross-sectional diagram of CAMAG UKammer. The HPTLC plate (1), measuring 50 × 50 mm, rests with its layer facing downward on the U-chamber body. (2) Elution solvent is fed to the center of the plate via a platinum-iridium capillary (3)

of 0.2 mm internal diameter. Vapor phase, made up externally, may be passed through the chamber, in through the circular channel (4) and out through the center bore (5) before, during, and after chromatographic development. The direction of gas flow may also be reversed. [4]

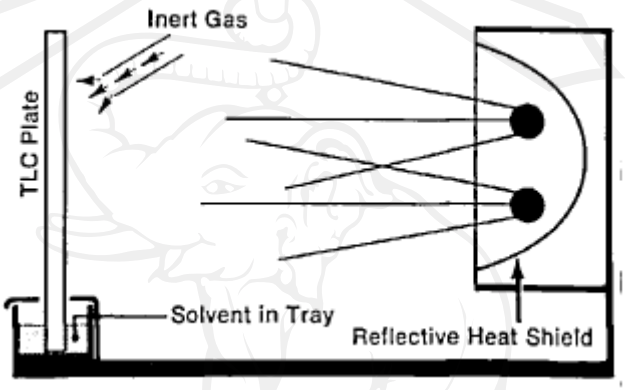

Figure 1.4 Schematic diagram of PMD developer. The thin layer plate is approximately 5 in. distant from and centered with respect to the radiator. [9]

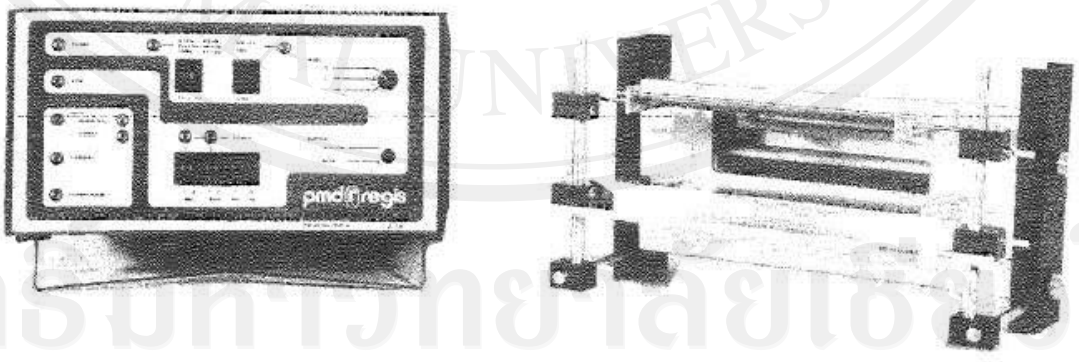

Figure 1.5 Programmer and developer for programmed multiple development (PMD). [10]

On the basis of the discussion of radial TLC and evaporative TLC presented above, we would expect this system to be capable of operating at equivalent plate heights of less than 0.001 cm. The restriction on solvent migration distance with this system (typically, less than 2.5 cm) limits the equivalent plate number to less than 5000. However, separations over such a short distance are rapid; the potential delivery is in the range of two to ten equivalent plates per second. In addition to producing high efficiency, the radial HPTLC system ("U-chamber") allows control of the solvent flow rate, but not the shape of the velocity gradient, up to the maximum capacity of the bed. The composition of the vapor in the atmosphere next to the layer can also be controlled and varied during the separation to increase selectivity in specific cases [5, 6]. Provision is also made for the control of plate temperature during development. Thus, the potential exists for obtaining further improvements in performance from the combination of aspects of evaporative TLC with radial TLC. The disadvantages of the system include those typically associated with radial chromatography (such as difficulties in quantitative analysis using conventional commercially available densitometers), as well as the limitation on sample loading capacity associated with the use of HPTLC plates.

The former difficulty may well be temporary and trivial; it is unlikely that currently available densitometers could be used with sub-millimeter wide spots without degrading resolution. The latter disadvantage is typical of high performance chromatographic systems. The third type of HPTLC is programmed multiple development (PMD) [7, 8]. PMD is defined as the repeated development of a TLC plate with the same solvent in the same direction for gradually increasing distances;

between developments, the solvent is removed from the thin-layer bed by controlled evaporation while the plate remains in contact with the solvent reservoir.

This is accomplished either by heating the back of the TLC plate or by passing a stream of inert gas across the front surface of the bed (Figure 1.4). In either case, the result is an increase in the rate of evaporation from the plate surface until it exceeds the rate of solvent influx through the thin-layer bed. The solvent front then recedes until it reaches a steady-state position at which the entire solvent influx is evaporated from the remaining wetted area. Thus, PMD combines features of both multiple-development (in a sandwich chamber) and evaporative TLC (during solvent removal). In addition, a further spot reconcentration occurs as the receding solvent front passes through a spot during solvent removal [(9)]. The result is a decrease in spot width because the trailing edge of the spot is swept forward for a time while the leading edge is fixed in place. PMD instrumentation (Figure 1.5) allows a wide variety of development programs to be performed. A summary of typical program parameters is given in Table 1.1. A variation on the PMD principle uses selective evaporation from a narrow vertical zone of the thin-layer bed during solvent removal [(10)] (Figure 1.6). The result is a solvent flow from both sides of the bed toward the center line of this zone, perpendicular to the direction of solvent migration. This flow serves to re-concentrate spots laterally and results in dramatically increased sensitivity as compared to conventional TLC. The wide variety of possible PMD programs makes the formulation of explicit relationships between PMD parameters and performance difficult. However, apparent plate numbers have been shown to increase with increasing numbers of developments. Apparent plate numbers in excess of 100,000

have been reported for a program of 68 developments lasting 72 hr [11]. Although only crude models for PMD spot behavior presently exist, can make a rough prediction of performance by combining alternate periods of incremental multiple development and evaporative TLC with a receding solvent front [12]. Results for various initial spot widths from such a model are shown in Figure 1.7. The optimum resolution predicted corresponds to an equivalent plate height of 0.0007 cm. Figure 1.7 suggests and published data [13] support the belief that the advantages of PMD over conventional TLC become more pronounced as sample loading and initial spot size are increased; this feature is shared with other forms of multiple development. In addition, PMD shares with multiple development the independence of final spot position from origin location [13, 14]. The mechanism of this spot alignment has been demonstrated to be the same as the spot re-concentration mechanism discussed above [15]. The theory and practice of PMD have been reviewed [11, 16, 17].

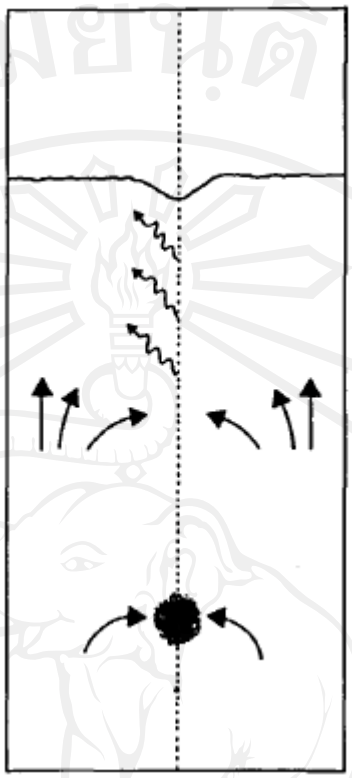

Figure 1.6 Centered PMD. Selective evaporation along the chromatogram (vertical) center line, accomplished by heat or a nitrogen stream, induces horizontal solvent flow toward the center of the bed. This flow concentrates spots and counters horizontal spreading. [10]

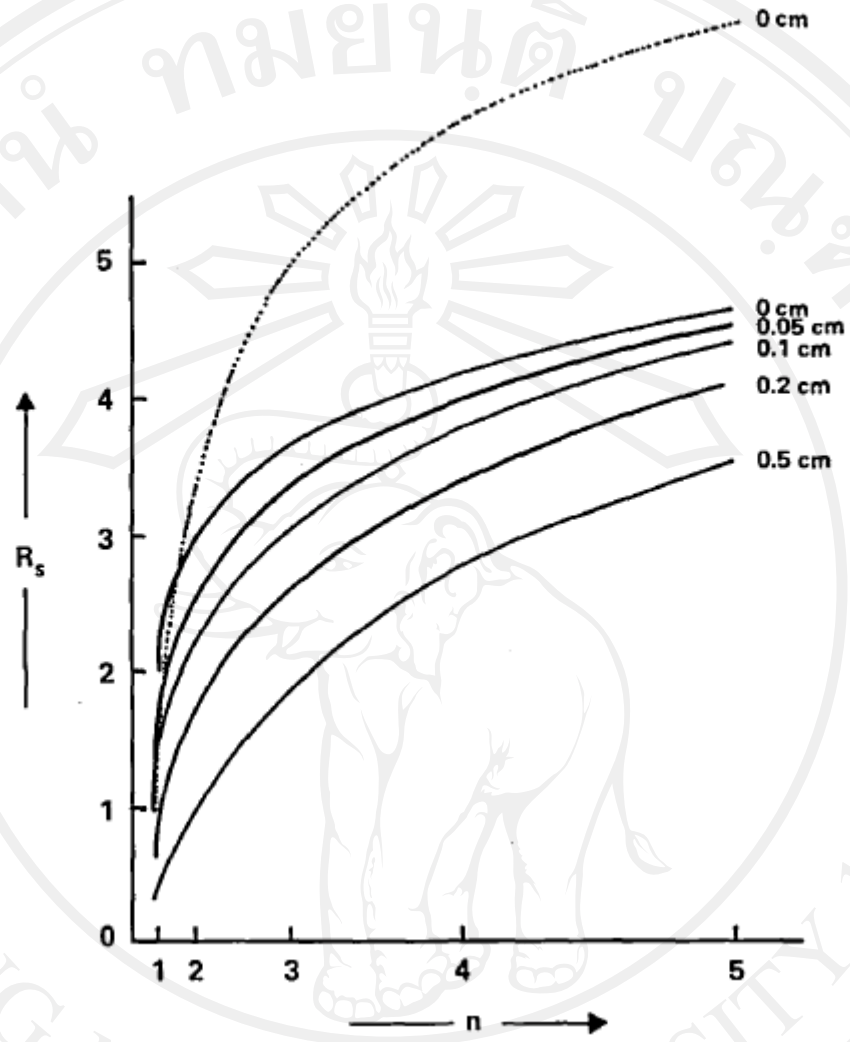

Figure 1.7 Influence of initial spot width (W_o) on spot behavior in programmed multiple development (PMD); resolution (R_s) between two spots of R = 0.20 and 0.21 as a function of the number of developments (n). The development program follows the pattern of IMD. Heavy emphasis on PMD solvent removal (dotted line) and light emphasis on PMD solvent removal (solid lines) are assumed. [13]

Table 1.1 PMD Program Parameters

PMD Program Parameters

Parameter	Symbol	Limits	Function
Cycles	n	1–99	Sets the number of developments and dryings in the program
Mode	mode	1, 2, or 3	Establishes the dependence of development time (T_n) on cycle number (n), mode 1, $T_n \propto n$; mode 2, $T_n \propto n(n+1)/2$; mode 3, $T_n \propto n^2$
Solvent removal	F or S	–	F, fixed (constant) drying time between developments; S, scheduled (proportional to development time) drying time between developments
Advance time	t_a	10–100 sec	Sets time for first development; subsequent development times are multiples of t_a
Advance power	P_a	0–12.5%	Sets the power level of the IR heaters (100% = 400 W) during development
Removal time	t_r	0–100 sec	Sets time for drying after the first development; drying time after subsequent developments is either equal to fixed (F) or a multiple of t_r scheduled (S)
Removal power	P_r	0–100% (F) or 0–50% (S)	Sets power level of the IR heaters during drying
Preheat time	t_{pr}	0–100 sec	Sets a preconditioning time before the first development, during which the heater is controlled by the removal power level.
Interim power	P_{int}	0–100%	Sets the power level of the IR heaters before, after, and during pauses in the program

Note: These parameters are entered into the PMD programmer by means of thumbwheel switches on the front panel. They determine the sequence of development and drying which constitutes a PMD program.

1.2 Development of Ion Pair Liquid Chromatographic Method for the Determination of Ketoconazole

1.2.1 Principles of Ion Pair Chromatography

1.2.1.1 Separation Mechanism

Ion-exchange selectivity is mediated by both the mobile and stationary phases. In contrast, the selectivity of an ion pair separation is determined primarily by the mobile phase. The two major components of the aqueous mobile phase are the ion pair reagent and the organic solvent; the type and concentration of each component can be varied to achieve the desired separation. The ion pair reagent is a large ionic molecule that carries a charge opposite of the analyte of interest. It usually has both a hydrophobic region to interact with the stationary phase and a charged region to interact with the analyte. Stationary phases used for ion-pair are neutral, hydrophobic resins such as polystyrene/divinylbenzene (PS/DVB) or bonded silica. A single stationary phase can be used for either anion or cation analysis. Although the retention mechanism for ion-pair chromatography is not fully understood, three major theories have been proposed:

- Ion pair formation
- Dynamic ion exchange
- Ion interaction

In the first model, the analyte and the ion pair reagent form a neutral "pair", which then partitions between the mobile phase and the stationary phase. Retention can be controlled by varying the concentration of organic solvent in the mobile phase,

as in classic reversed-phase chromatography. The dynamic ion-exchange model maintains that the hydrophobic portion of the ion pair reagent adsorbs to the hydrophobic stationary phase to form a dynamic ion exchange surface. The analyte is retained on this surface, as it would be in classic IC. Using this scenario, solvents used in the mobile phase can be used to impede interaction of the ion-pair reagent with the stationary phase, thereby altering the "capacity" of the column. Figure 1.8 summarizes the major interactions described in these two theories. In this example, a cationic analyte (i.e., C+) is being separated using an acetonitrile/water mobile phase containing octanesulfonate as the ion-pair reagent. A PS/DVB column acts as the stationary phase. The cations are retained by a combination of their interaction with the octanesulfonate that is adsorbed to the stationary phase (the hydrophobic environment), and by their interaction with the octanesulfonate ions in the mobile phase (the hydrophilic environment). When "paired" with the octanesulfonate in solution, the cations are able to partition between the mobile and stationary phases. Note that the acetonitrile in the mobile phase is also adsorbed to the stationary phase, thereby lowering the effective capacity of the column.

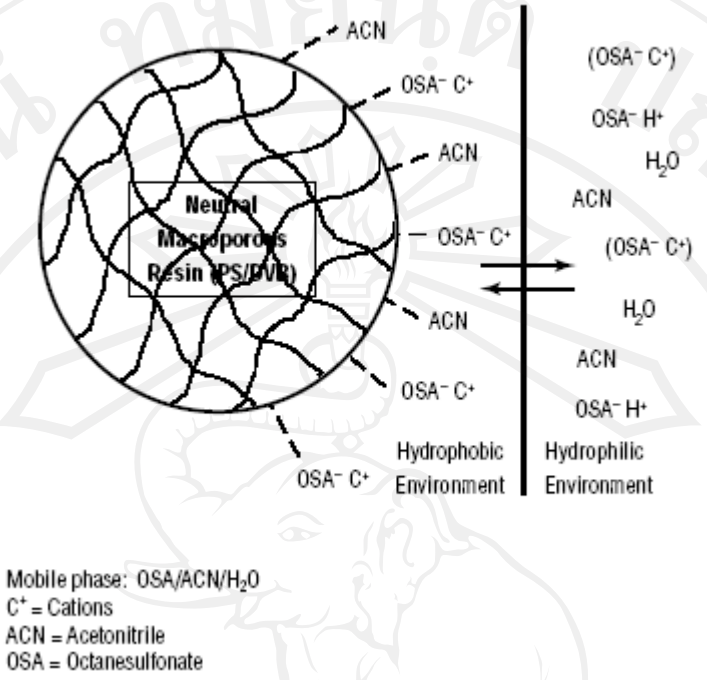

Figure 1.8 Cation ion pairing interactions [18]

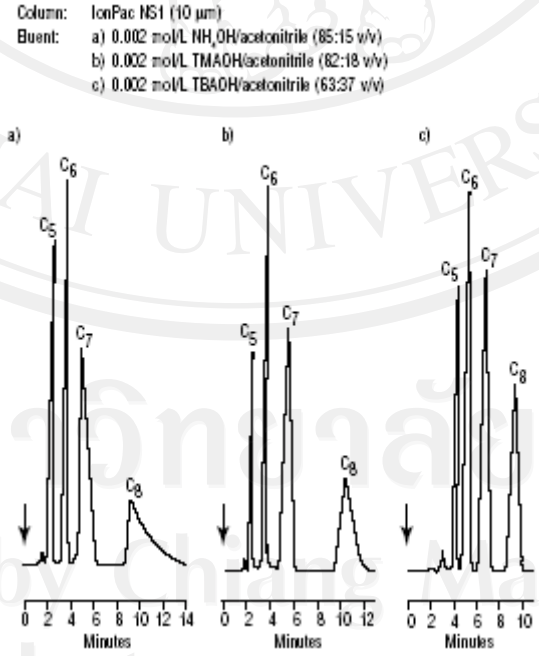

Figure 1.9 Effects of ion pair reagent hydrophobicity and solvent concentration [19]

A third model describes an electrical double layer that is formed when the ion pair reagent permeates the stationary phase, carrying with it an associated counter ion. Retention of the analyte ion in this model is dependent upon a combination of factors, including those described in the first two models. References 18 and 19 are recommended for a more in-depth discussion of ion pair mechanisms.

1.2.1.2 Ion Pair Reagents

There are two simple rules when choosing the appropriate ion pair reagent. The first rule is that hydrophilic ions are best separated using a hydrophobic ion pair reagent, and that hydrophobic analytes are best separated with a hydrophilic ion pair reagent. Common pairing reagents are listed in the order of increasing hydrophobicity:

- ***For anion analysis***, ion pair reagents include ammonium and tetramethyl-, tetraethyl-, tetrapropyl-, and tetrabutylammonium. Each of these must be in the hydroxide form to be suppressed for conductivity detection.

- ***For cation analysis***, ion pair reagents include hydrochloric, perchloric, and perfluorocarboxylic acids and pentane-, hexane-, heptane-, and octane sulfonic acids. Perfluorocarboxylic acids have the advantage of low conductance and are available in extremely high purity; they are useful for separating very hydrophobic cations. Cation-pairing reagents must be in the acid (hydronium ion) form to be suppressed for conductivity detection. The second rule for choosing the appropriate ion pair reagent is that smaller pairing reagents usually result in the best separation, since the structure and properties of the analyte will have a greater contribution to the analyte-reagent complex partitions in the system.

1.2.1.3 Reagent Concentration

Because the effective capacity of the column is determined mainly by the ion pair reagent, retention of the analyte will increase as the concentration of reagent is increased. However, electrostatic repulsion of reagent molecules on the stationary phase surface will ultimately limit the degree to which the capacity of the column can be increased. The concentration of the ion pair reagent is also limited by the capacity of the suppressor. Typical working concentration ranges are 0.5-20 mM [(18)].

1.2.1.4 Organic Modifiers

Organic solvents are used to decrease retention times and to modify the selectivity of the separation. These modifiers work in the following two ways: (1) by competing with the pairing reagent for the stationary phase, thus decreasing the effective capacity of the column; and (2) by decreasing the polarity of the mobile phase, which affects partitioning of the analyte-reagent pair into the hydrophobic environment (Figure 1.8). The optimum organic solvent concentration for a given separation depends to a degree on the hydrophobicity of the ion pair reagent. Figure 1.9 demonstrates that as the hydrophobicity of the ion pair reagent increases, more organic solvent is needed to keep the run time to approximately 10 minutes. In this example, pairing the alkylsulfonate analytes with ammonium requires only 15% acetonitrile in the mobile phase, but 37% acetonitrile is required with tetrabutylammonium as the ion pair reagent. Peak shapes for the more highly retained components are significantly improved, however, with the higher concentration of organic solvent. Keep in mind that increasing the concentration of organic solvent will eventually compromise conductivity detection limits. If this becomes a problem,

switching to a less hydrophobic ion pair reagent is advisable. Acetonitrile and methanol are by far the most common organic solvents used in MPIC. Higher concentrations of methanol are necessary to achieve results comparable to acetonitrile, but methanol has hydrogen-bonding properties which are sometimes useful.

1.2.1.5 pH Effects

When it is necessary to adjust the pH of the mobile phase, choosing an acid or base that is compatible with suppressed conductivity detection is critical. For instance, when multivalent anions are too highly retained, lowering the pH will decrease their ionization and thus their interaction with the ion-pair reagent. Boric acid is a good choice because, although it is not suppressed, it does not raise the background conductivity of the mobile phase significantly. The pH of the system can also be manipulated to enhance detection.

1.2.1.6 Other Mobile Phase Additives

Carbonate is commonly used to improve peak shape and reduce retention of multivalent anions [17]. A dramatic decrease in k' for divalents relative to monovalents is observed with increased carbonate concentration. This is consistent with the ion-exchange retention model. Classical IC theory predicts that the slope of log k' versus log [eluent ion concentration] (the eluent ion being carbonate in this case) for divalents should be twice that for monovalents. Typical values for carbonate concentrations range from 0.1 mM to 1 mM Na_2CO_3.

1.2.2 Literature Reviews

The official method normally involves titration in non-aqueous solvent [20, 21]. Various analytical methods were reported for the determination of ketoconazole. They are spectrophotometric methods [22-29], spectrofluorimetric methods [28], high performance liquid chromatography [30-44], stripping voltammetric and polarographic method [45] and capillary zone electrophoresis [46], high performance thin layer chromatography [47, 48].

Previous HPLC and HPTLC methods for the determination of ketoconazole

Method	Methodology	Reference
HPLC	The method was based on the formation of yellow orange complexes between iron(III) chloride and ketoconazole drugs. The optimum reaction conditions, spectral characteristics, conditional stability constants and composition of the water soluble complexes have been established.	F.M. Abou-Attia, *et. al.* [30]
HPLC	After extraction of the drug from plasma, the compound was separated by HPLC using a reversed-phase column and detected by UV light at 205 nm.	K.B. Alton [31]
HPLC	Separation of the drug from endogenous substances was achieved by solid-phase extraction followed by reversed-phase chromatography on a Novapak C_{18} column using a mobile phase of methanol-acetonitrile-0.02 M phosphate buffer (pH 6.8) (35:30:35).	C.M. Riley *et. al.* [32]
HPLC	Reversed-phase HPLC on different column packing materials (Hypersil C-18, Spherisorb-CN, Chromspher-B) was used to obtain selective separations of imidazole antimycotic drugs with UV detection.	A.M. Di Pietra *et. al.* [33]

Method	Methodology	Reference
HPLC	An HPLC method using fluorescence detection was developed for the determination of ketoconazole in human plasma. The mobile phase comprised 0.05 M disodium hydrogen orthophosphate and acetonitrile (50:50, v/v) adjusted to pH 6. Analysis was run at a flow-rate of 1.5 mL min^{-1} with the detector operating at an excitation wavelength of 260 nm and an emission wavelength of 375 nm.	K.H. Yuen *et. al.* [34]
HPLC	An HPLC method was described using octadecylsilica (3 µm) with an acetonitrile phosphate buffer mobile phase containing diethylamine which was capable of separating ketoconazole from four related compounds and from excipients in tablets, cream and shampoo.	A.S. Low *et. al.* [35]
HPLC	The separation of ketoconazole enantiomers by subcritical-fluid chromatography using an amylose-based column was described. The best results in terms of resolution and analysis time were obtained using 30% ethanol (containing 0.1% triethylamine and 0.1% trifluoroacetic acid), a pressure of 300 bar, a temperature of 35 ^{o}C and a flow-rate of 3 mL min^{-1}	J.L. Bernal *et. al.* [36]
HPLC	The sample extraction was achieved by a single solvent extraction involving a mixture of acetonitrile/butyl chloride (1:4). Ketoconazole was separated on a column packed with Inertsil ODS-80A and a mobile phase composed of triethylamine/ammonium hydroxide/THF/water/acetonitrile (1:1:25:450:502). The column effluent was monitored at a wavelength of 206 nm with a detector range set at 0.5.	P. Bruijn *et. al.* [37]

Method	Methodology	Reference
HPLC	An HPLC method has been developed for the simultaneous analysis of ketoconazole in rat plasma and hepatocyte culture. Analysis was performed using a 5 μm Symmetry C18 column (15 cm × 4.6 mm. i.d.) and isocratic elution with 25mM-potassium dihydrogen phosphate buffer of pH 4.5/acetonitrile (1:1) as mobile phase at a flow rate of 1 mL min^{-1}. Photodiode-array detection was used to monitor at 231 nm.	S. Bajad *et. al.* [38]
HPLC	An HPLC system that allows the determination of ketoconazole. The finally selected isocratic system consisted of an Interchrom Nucleosil (250 × 4.6 mm, 5 μm) C_8 column and a mobile phase containing acetonitrile–phosphate buffer 0.025 M, pH 4.0, 45/55 (v/v). Ketoconazole could immediately be determined at 250 nm after injection of diluted shampoo.	Y. Vander Heyden *et. al.* [39]
HPLC	The LC separation was carried out at room temperature and the detection wavelength was set at 225 nm. The chiral columns employed were Chiralpak AD 250 × 4.6 mm, and Chiralcel OD 250 × 4.6 mm.	J.L. Bernal *et. al.* [40]
HPLC	Chromatographic separation was carried out at ambient temperature on a μ-Bondapak™ C_{18} (25 cm × 4.6 mm, i.d.). The compounds were separated isocratically with a mobile phase consisting of a mixture of acetonitrile-25 mM trishydroxymethyl aminomethane in phosphate buffer (pH 7), 55:45, v/v). The pH of the binary solvent mixture was finally adjusted to 7.0 with *o*-phosphoric acid. The flow rate was 2 mL min^{-1}. The injection volume was 20 μL and UV-detection at 260 nm.	E.M. Abdel-Moety *et. al.* [41]

Method	Methodology	Reference
HPLC	The mobile phase contains a mixture 45:55 (v/v) of acetonitrile and 0.01 M $NaH_2PO_4 \cdot H_2O$ aqueous solution, adjusted to pH 4.0 with H_3PO_4 1 M solution. Analyses were performed at a flow rate of 1 mL min^{-1} and at a detection wavelength of 250 nm for ketoconazole. The HPLC conditions with the two short columns were kept the same as for the long column except for the injection volume, which was 20 μL	A. Nguyen Minh Nguyet *et. al.* [42]
HPLC	The mobile phase contains a mixture 45:55 (v/v) of acetonitrile and 0.01 M NaH_2PO_4 solution, the adjusted to pH 4.0 with H_3PO_4 1 M solution. Analyses were performed at a flow-rate of 1 mL min^{-1} and using a detection wavelength of 250 nm. The HPLC conditions with the two short columns were kept the same as for the long one, except for the injection volume, which was 20 μL with the long but only 5 μL with the short columns.	A. Nguyen Minh Nguyet *et. al.* [43]
HPLC	A reversed-phase Hypersil BDS-C_{18} column (250 mm × 4.6 mm, 5 μm) equipped with a precolumn Hypersil BDS-C_{18} (10 mm × 4 mm, 5 μm) was used. The mobile phase was composed of methanol, water and diethylamine 74:26:0.1 (v/v/v) and its flow rate was 1 mL min^{-1}. Absorption was measured at 240 nm	M.V. Vertzoni *et. al.* [44]
HPTLC	Ketoconazole were extracted with a mixture of equal volumes of chloroform and isopropyl alcohol and were separated by TLC on a precoated silica gel F_{254} plate with a solvent system of n-hexane-chloroform-methanol-diethylamine (50+40+10+1, v/v). The separated azoles were quantitated by scanning densitometry at 220 nm.	U. Roychowdhury *et. al.* [47]

Method	Methodology	Reference
HPTLC	The determination of ketoconazole in different pharmaceutical formulations (capsules, solutions, emulsions and creams). Different HPTLC layers, mobile phases and methods of detection were used. The methods were validated and compared.	D. D. Agbaba *et. al.* [48]

Very few high performance thin layer chromatographic methods (HPTLC) were reported in the literature and there is no report on the determination of this drug in shampoo formulation. The HPTLC and IPLC methods can be used for identification and for control of batch-to-batch consistency in the stability testing of drug and for purposes of control throughout the entire manufacturing process of drugs, as well as quality control of the finished product. It has the advantages of being sensitive, selective, rapid, accurate and reproducible.

1.3 The Aim of Research

The aims of this research work can be summarized as follows:

1.3.1 To develop and validate a HPTLC method for separation and determination of ketoconazole in pharmaceutical formulations

1.3.2 To develop and validate an ion pair liquid chromatographic (IPLC) method for separation and determination of ketoconazole in pharmaceutical formulations