Chapter V

Development and Optimisation of a Novel Method Developed Ilaprazole by Ultra Performance Liquid Chromatography (UPLC)
5.1 INTRODUCTION

High performance liquid chromatography (HPLC) is a proven technique that has been used in laboratories worldwide, over the past 30-plus years. One of the primary drivers for the growth of this technique has been the evolution of packing materials used to effect separation. An underlying principle of the HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. The classic separation method is HPLC (High Performance Liquid Chromatography) with many advantages like robustness, ease of use, good selectivity and adjustable sensitivity. Its main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis [1-2] due to low diffusion coefficients in liquid phase, involving slow diffusion of analyte in the stationary phase. The Van Demeter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the HPLC system can operate only up to 400 bars. That is columns filled with particles of about 2μm are used with these systems to accelerate the analysis without loss of efficiency, while maintaining an acceptable loss of load. As particle size decreases to less than 2.5μm, there is a significant gain in efficiency and it doesn’t diminish at increased linear velocities or flow rates according to the common Van Demeter equation [3]. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which describe the relationship among the linear velocity flow rates known as Ultra Performance.

To improve the efficiency of the HPLC and separations, the following can be done which lead to the development of new chromatographic technique called UPLC.

a) Working at higher temperatures - allows high flow rates by reducing the viscosity of mobile phase which significantly reduces back pressure [4-5].

b) Using monolithic columns - contains polymerized porous support structure that provides lower flow resistances than conventional particle-packed columns [6-8].
UPLC refers to Ultra Performance Liquid Chromatography, which improves in three areas: [9-13].

1. Chromatographic resolution
2. Speed
3. Sensitive analysis

The UPLC uses fine particles and saves time and reduces solvent consumption. This new category of analytical separation science retains the practicality and principles of HPLC while increasing the overall interrelated attributes of speed, sensitivity and resolution. Today's pharmaceutical industries are looking for new vistas to cut cost and shorten time for development of drugs while at the same time improving the quality of their products and analytical laboratories are not exception to this trend. Speed allows a greater number of analyses to be performed in a shorter amount of time thereby increasing sample throughput and lab productivity. These are the benefits of faster analysis and hence the ultra performance liquid chromatography. A typical assay was transferred and optimized for UPLC system to achieve both higher sample analysis throughput and better assay sensitivity. Analysis of operation cost and sample throughput found UPLC cost advantageous over HPLC.

5.2. THEORY OF SEPARATIONS USING SMALLER PARTICLES

According to Van Demeter equation, smaller particles provide not only increased efficiency but also the ability to do work at an increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as the HPLC. In the fundamental resolution equation, resolution is proportional to the square root of $N$. [14]

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{a-1}{a}\right)^{\frac{k}{k+1}}$$

Where $N$ is number of theoretical plates, $a$ is selectivity factor and $k$ is mean retention factor. But since $N$ is inversely proportional to particle size ($d_p$) [14];
as the particle size is lowered by thrice, i.e. from 5 mm to 1.7 mm, \( N \) is increased by three. \( N \) is also inversely proportional to the square of the peak width.

\[
N \propto \frac{1}{p^2}
\]

This illustrates that the narrower the peaks are, the easier they are to separate from each other. Also peak height is inversely proportional to the peak width \( w \):

\[
H \propto \frac{1}{w}
\]

So as the particle size decreases to increase \( N \) and subsequently \( R_s \), an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient separations, desirable for many applications such as natural extracts, peptide maps etc. still another equation comes in to force.(fig. 5.1 and Tables 5.1 and 5.2). Van Demeter plot moves toward smaller particles [15].

\[
F_{opt} \propto \frac{1}{dp}
\]

As particle size decreases, the optimum flow rate \( (F_{opt}) \) to reach maximum \( N \) increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressure and a system is properly designed for the same. Higher resolution and efficiency can be levelled further when analysis's speed is the primary objective. Efficiency is proportional to column length and inversely proportional to the particle size.

\[
N \propto \frac{L}{dp}
\]

Therefore the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to smaller particles and shortening the column by one third, the separation is completed in 1/9th the time while maintaining resolution.
Fig. 5.1 Van Deemter plot illustrating the evolution of particle sizes over the last three decades

Table 5.1

<table>
<thead>
<tr>
<th>Resolution improvement</th>
<th>Speed improvement</th>
<th>Sensitivity improvement</th>
<th>Back Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 to 1.7 μm Particles</td>
<td>1.7X</td>
<td>3X</td>
<td>1.7X</td>
</tr>
<tr>
<td>3 to 1.7 μm Particles</td>
<td>1.3X</td>
<td>2X</td>
<td>1.3X</td>
</tr>
</tbody>
</table>

Table 5.2

<table>
<thead>
<tr>
<th>Resolution improvement</th>
<th>Speed improvement</th>
<th>Sensitivity improvement</th>
<th>Back Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 to 1.7 μm Particles</td>
<td>Same</td>
<td>9X</td>
<td>3X</td>
</tr>
<tr>
<td>3 to 1.7 μm Particles</td>
<td>Same</td>
<td>3X</td>
<td>2X</td>
</tr>
</tbody>
</table>

Therefore it is possible to increase throughput, and thus the speed of analysis without affecting the chromatographic chromatography, which can take advantage of the separation performance. The advent of UPLC has demanded the development of a new instrumental system for liquid chromatography, which can take advantage of the separation performance (by reducing dead volumes) and consistent with the pressures (about 8000 to 15,000 PSI, compared with 2500 to 5000 PSI in HPLC). Efficiency is proportional to column length and inversely
proportional to the particle size. Therefore, the column can be shortened by the same factor as the particle size.

5.3 INSTRUMENTATION OF UPLC

To truly take advantage of the increased speed, superior resolution and sensitivity afforded by small particles, instrument technology also had to keep pace. A completely new spic and span design with advanced technology in the pump, auto sampler, detector, data system, and service diagnostics was required. The ACQUITY UPLC system has been designed for low system and dwell volume to take full without loss of resolution. The application of UPLC resulted in the detection of additional drug metabolites, superior separation and improved spectral [16-17] advantage of low dispersion and small particle technology.

The lines of attack for fast LC method development are varied. Method development simulation softwares such as ACD™ [18], DryLab ™ [19] or Chromsword ™ [20] are valuable tools for optimizing and streamlining methods. Such softwares allow to increase the information obtained from a limited number of runs and to predict the best possible separation conditions and Table 5.3 shows the comparison of UPLC and HPLC characteristics.

Table 5.3
Comparison of UPLC and HPLC

<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th>HPLC</th>
<th>UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Size</td>
<td>3 to 5μm</td>
<td>Less than 2μm</td>
</tr>
<tr>
<td>Maximum Back Pressure</td>
<td>35-40 Mpa</td>
<td>103.5MPa</td>
</tr>
<tr>
<td>Analytical Column</td>
<td>Alltim a C18</td>
<td>Acquity UPLC BEH C18</td>
</tr>
<tr>
<td>Column Dimensions</td>
<td>150X3.2mm</td>
<td>50X2.1mm</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>30°C</td>
<td>65°C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>5μl (Std in100% MeOH)</td>
<td>2μl (Std in100%MeOH)</td>
</tr>
</tbody>
</table>
5.3.1 Pumping Systems

Achieving small particle and high peak capacity separations requires a greater pressure range than that achievable by today’s HPLC instrumentation. The calculated pressure drop at the optimum flow rate for maximum efficiency across a 15 cm long column packed with 1.7 μm particles is about 15,000 psi. Therefore, a pump capable of delivering solvent smoothly and reproducibly at these pressures, which can compensate for solvent compressibility and operate in both the gradient and isocratic separation modes, is pressure required. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. There are built-in solvent select valves to choose from one to four solvents. There is a 15,000 psi pressure limit (about 1000 bar) to take full advantage of the sub-2μm particles.

5.3.2 Sample Injection

In the UPLC, sample introduction is critical. Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from extreme pressure fluctuations, the injection process must be relatively pulse-free and the swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carry over are also required to increase sensitivity [21]. There are also direct injection approaches for biological samples. [22-23]

5.3.3 Sample Manager

The sample manager also incorporates several technology advancements. Using pressure assisted sample introduction, low dispersion is maintained through the injection process, and a series of transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and needle calibration sensor increases accuracy. Injection cycle time is 25 seconds without a wash and 60 sec with a dual wash used to further decrease carry over. A variety of micro titer plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional
sample organiser, the sample manager can inject from 0.1 to 22 micro titer plates. The sample manager also controls the column heater. Column temperatures up to 65°C can be attained. To minimize the sample dispersion, a “pivot out” design allows the column outlet to be placed in closer proximity to the source inlet of MS detector. [24]

5.3.4 UPLC Columns

The design and development of sub-2μm particles is a significant challenge and researchers have been very active in this area to capitalize on their advantages [25-26]. Although high efficiency non porous 1.5μm particles are commercially available, they suffer from low surface area, leading to poor loading capacity and retention. To maintain retention and capacity similar to HPLC, UPLC must use a novel porous particle that can withstand high pressures. Silica based particles have good mechanical strength, but suffer from a number of disadvantages. These include tailing of basic analytes and a limited pH range. Another alternative polymeric column can overcome pH limitations, but they have their own issues including low efficiencies and limited capacities.

In 2000, Waters introduced a first generation hybrid chemistry, called XTerra RP8, which combines the advantageous properties of both silica and polymeric columns – they are mechanically strong, with high efficiency and operate over an extended pH range. XTerra RP8 columns are produced using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups. However, in order to provide the kind of enhance mechanical stability UPLC requires, a second generation hybrid technology [27] was developed, called ACQUITY UPLC. ACQUITY 1.7μm particles bridge the methyl groups in the silica matrix as shown in fig.5.2, which enhances their mechanical stability.
Resolution is increased in a 1.7 μm particle packed column because efficiency is better. Separation of the components of a sample requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations such as.

- ACQUITY UPLC BEH T M C18 and C8 (straight chain alkyl columns)
- ACQUITY UPLC BEH Shield RP 18 (embedded polar group column)
- ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl) [28]
- ACQUITY UPLC BEH Amide columns (trifunctionally bonded amide phase)

Each column of chemistry provides a different combination and hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes.
53.4.1 ACQUITY UPLC BEH Column Chemistries

ACQUITY UPLC BEH T M C18 and C8 Columns

These are considered as the universal columns of choice for most UPLC separations by providing the widest pH range. They incorporate trifunctional ligand bonding chemistries which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7μm BEH particle to deliver the widest usable pH operating range.

Fig. 5.3 ACQUITY UPLC BEH Column Chemistries

53.4.2 ACQUITY UPLC BEH Shield R18 Columns

These are designed to provide selectivities that complement the ACQUITY UPLC BEH T M C18 and C8 Columns.

53.4.3 ACQUITY UPLC BEH Phenyl Columns

These utilize a trifunctional C6 alkyl ethyl between the phenyl ring and the silyl functionality.

53.4.4 ACQUITY UPLC BEH Amide columns

BEH particle technology, in combination with a trifunctionally bonded amide phase, provides exceptional column life time, thus improving assay robustness. BEH Amide columns facilitate the use of a wide range of phase pH 2 – 11 to
facilitate the exceptional retention of polar analytes spanning a wide range in polarity, structural moiety and PkA.

Ligand combined with the same proprietary end capping processes as the ACQUITY UPLC BEH T M C18 and C8 columns provides long column lifetimes and excellent peak shape. This unique combination creates a new dimension in selectivity allowing a quick match to the existing HPLC column.

Packing a 1.7μm particle in reproducible and rugged columns was also a challenge that needs to be overcome. The column hardware required a smoother interior surface and the end fruits were re-designed to retain the small particles and resist clogging. Packed bed uniformity is also critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations. All ACQUITY columns also include the eCord microchip technology that captures the manufacturing information for each column, including the quality control tests and certificates of analysis. When used in the Waters ACQUITY UPLC system, the eCord database can also be updated with real time method information, such as the number of injections, or pressure information, to maintain a complete column history.

An internal dimension (ID) of 2.1 mm column is used. For maximum resolution, choose a 100 mm length and for faster analysis, and higher sample throughput, choose 50 mm Column. Half-height peak widths of less than one second are obtained with 1.7μm particles, which gives significant challenges for the detector. In order to integrate an analyte peak accurately and reproducibly, the detector sampling rate must be high enough to capture enough data points across the peak. The detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2-3 times higher than HPLC separations, depending on the detection technique. MS detection is significantly enhanced by UPLC and increased peak concentrations with reduced chromatographic dispersion at lower flow rates promote increased source ionization efficiencies.
5.3.5 Detectors

For UPLC detection, the tuneable UV/Visible detector is used which includes new electronics and firmware to support Inter communications at the high data rates. Conventional absorbance-based optical detectors are concentration sensitive detectors, and for UPLC use, the flow cell volume would have to be reduced in standard UV/Visible detectors to maintain concentration and signal. According to Beer's Law, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends. A reduction in cross-section means the light path is reduced and transmission drops with increasing noise. Therefore, if a conventional HPLC flow cell were used, UPLC sensitivity would be compromised. The ACQUITY Tunable UV/Visible detector cell consists of a light guided flow cell equivalent to an optical fibre. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10mm flow cell path length with a volume of only 500nL. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems [29].

5.4 ADVANTAGES AND DISADVANTAGES OF UPLC

The UPLC has several advantages and disadvantages

5.4.1 Advantages

The UPLC has the following advantages

➢ Decreases run time and increases sensitivity.
➢ Provides the selectivity, sensitivity, and dynamic range of LC analysis
➢ Maintaining resolution performance.
➢ Expands scope of Multi residue Methods
➢ UPLC's fast resolving power quickly quantifies related and unrelated compounds
➢ Faster analysis through the use of a novel separation material of very fine particle size
➢ Operation cost is reduced
➢ Less solvent consumption
> Reduces process cycle times, so that more product can be produced with existing resources
> Increases sample throughput and enables manufacturers to produce more material that consistently meet or exceeds the product specifications, potentially eliminating variability, failed batches, or the need to re-work material.
> Delivers real-time analysis in step with manufacturing processes and
> Assures end-product quality, including final release testing.

5.4.2 Disadvantages

The UPLC has the following disadvantages
> Due to increased pressure requires more maintenance and reduces the life of the columns of this type. So far performance similar or even higher has been demonstrated by using stationary phases of size around 2 μm without the adverse effects of high pressure and
> In addition, the phases of less than 2 μm are generally non-regenerable and thus have limited use [30-31].

5.5 APPLICATIONS OF UPLC

5.5.1 Analysis of natural products and traditional herbal medicine

The UPLC is widely used for analysis of natural products and herbal medicines for traditional herbal medicines to provide evidence-based validation of their effectiveness as medicines and to establish safety parameters for their production. The UPLC provides high-quality separations and detection capabilities to identify active compounds in highly complex samples that results from natural products and traditional herbal medicines. Metabonomics-based analysis, using UPLC, exact mass MS and Marker Lynx Software data processing for multivariate statistical analysis, can help quickly and accurately characterize these medicines and also their effect on human metabolism. Preparative-scale fractionation and purification is used along with classic quantitative bioanalytical tools used in drug development.
5.5.2 Identification of Metabolite

Biotransformation of new chemical entities (NCE) is necessary for drug discovery. When a compound reaches the development stage, metabolite identification becomes a regulated process. It is of the utmost importance for lab to successfully detect and identify all circulating metabolites of a candidate drug. Discovery studies are generally carried out in vitro to identify major metabolites so that metabolic weak spots on the drug candidate molecule can be recognized and protected by changing the compound structure. Key for analysts in metabolite identification is maintaining high sample throughput and providing results to medicinal chemists as soon as they are available. The UPLC/MS/MS addresses the complex analytical requirements of biomarker discovery by offering unmatched sensitivity, resolution, dynamic range, and mass accuracy.

5.5.3 Study of Metabonomics/Metabolomics

Metabonomics studies are carried out in labs to accelerate the development of new medicines. The ability to compare and contrast large sample groups provides insight into the biochemical changes that occur when a biological system is exposed to a new chemical entity (NCE). Metabonomics provides a rapid and robust method for detecting these changes, improves understanding of potential toxicity and allows monitoring the efficacy. The correct implementation of metabonomic and metabolomic information helps similar discovery, development and manufacturing processes in the biotechnology and chemical industry companies. With these studies, scientists are better able to visualize and identify fundamental differences in sample sets. The UPLC/MS System combines the benefits of UPLC analyses, high resolution exact mass MS and specialized application managers to rapidly generate and interpret information-rich data, allowing rapid and informed decisions to be made.

5.5.4 Bioanalysis/Bioequivalence Studies

For pharmacokinetic, toxicity and bioequivalence studies, quantitation of a drug in biological samples is an important part of development programs. The drugs are generally of low molecular weight and are tested during both preclinical and clinical studies. Several biological matrices are used for quantitative bioanalysis, the most common being blood, plasma and urine. The primary
technique for quantitative bioanalysis studies, scientists are better able to visualize and identify LC/MS/MS. The sensitivity and selectivity of UPLC/MS/MS at low detection levels generates accurate and reliable data that can be used for a variety of different purposes, including statistical pharmacokinetics (PK) analysis. Developing a robust and compliant LC/MS/MS assay has traditionally been the domain of very experienced analysts. The UPLC/MS/MS helps in the processes of method development for bioanalysis into logical steps for MS, LC, and sample preparation. Quantitative bioanalysis is also an integral part of bioequivalence studies, which are used to determine if new formulations of existing drugs allow the compound to reach the bloodstream at a similar rate and exposure level as the original formulation. UPLC/MS/MS solutions are proven to increase efficiency, productivity, and profitability for bioequivalence laboratories. Applications of UPLC/MS/MS in bioequivalence and bioanalysis include UPLC/MS/MS, LC and MS instruments and software combine in a sophisticated and integrated system for bioanalysis and bioequivalence studies, providing unprecedented performance and compliance support.

- UPLC/MS/MS delivers excellent chromatographic resolution and sensitivity MS delivers simultaneous full-scan MS and multiple reaction monitoring (MRM) MS data with high sensitivity to address matrix monitoring.
- UPLC Sample Organizer maximizes efficiency by accommodating large numbers of samples in a temperature-controlled environment, ensuring maximum throughput. Increase the sensitivity of analyses, quality of data including lower limits of quantitation (LLOQ), and productivity of laboratory by coupling the UPLC System's efficient separations with fast acquisition rates of tandem quadrupole MS systems.
- Easily acquire, quantify and report full system data in a compliant environment using security-based data collection software.
- Ensure the highest quality results and reliable system operation in regulated environment.
5.5.5 DISSOLUTION TESTING

For quality control and release in drug manufacturing, dissolution testing is dire essential in the formulation, development and production process. In sustained-release dosage formulations, testing higher potency drugs is particularly important where dissolution can be the rate-limiting step in medicine delivery. The dissolution profile is used to demonstrate reliability and batch-to-batch uniformity of the active ingredient. Additionally, newer and more potent formulations require increased analytical sensitivity. The UPLC provides precise and reliable automated online sample acquisition. It automates dissolution testing, from pill drop to test start, through data acquisition and analysis of sample aliquots, to the management of test result publication and distribution.

5.5.6 MANUFACTURING/QA/QC

Identity, purity, quality, safety and efficacy are the important factors to be considered while manufacturing a drug product. The successful production of quality pharmaceutical products requires that raw materials meet purity specifications and manufacturing processes proceed as designed. Continued monitoring of material stability is also a component of quality assurance and control. UPLC is used for the highly regulated quantitative analyses performed in QA/QC laboratories. The supply of consistent and high quality consumable products plays a pivotal role in a registered analytical method. The need for consistency over the lifetime of a drug product which could be in excess of 30 years is essential in order to avoid method revalidation and associated production delays.

5.5.7 IMPURITY PROFILING

For the drug development and formulation process, profiling, detecting and quantifying drug substances and their impurities in raw materials and final product testing is an essential part. Impurity profiling requires high-resolution chromatography capable of reliably and reproducibly separating and detecting all of the known impurities of the active compound. Also critical is the ability to accurately measure low-level impurities at the same time as the higher concentration active pharmaceutical component. This activity, however, can be complicated by the presence of excipients in the sample, often resulting in long
HPLC analysis times to achieve sufficient resolution. To characterize impurities, it is often necessary to perform several analytical runs to obtain the necessary MS and MS/MS data. The UPLC combines with exact mass LC/MS, which by operating with alternating low- and high collision energies, known as MS, has been successfully employed for the identification of drug and endogenous metabolites. The rapid switching of the collision cell energy produces both precursor and product ions of all of the analytes in the sample while maintaining a sufficient number of data points across the peak for reliable quantification. The sensitivity and flexibility of exact mass time-of-flight mass spectrometry with alternating collision cell energies, combined with the high resolving power of the UPLC system, allows for the rapid profiling and identification of impurities.

5.5.8 COMPUTER LIBRARY MAINTENANCE

Confirming the identity and purity of a candidate pharmaceutical is critical to effectively screening chemical libraries that contain vast types of small molecules across a range of biological targets. Chemists need to be sure they have synthesized the expected compound. The use of the fast scanning MS along with the throughput of the UPLC System’s remote status monitoring software allows chemists to obtain high-quality comprehensive data about their compounds in the shortest possible timeframes. This combined with intelligent open access software, allows making informed decisions faster, and better supporting the needs of the modern drug discovery process.

5.6 ANTI-ULCER DRUGS

Proton pump inhibitors [32] such as lnaprazole, omeprazole, lansoprazole, pantoprazole sodium and rabeprazole are extensively used for therapeutic control of acid-related disorders including gastroesophageal reflux disease and Zollinger-Ellison syndrome and for peptic-ulcer disease caused by stress (stress-related erosive syndrome), nonsteroidal antiinflammatory drugs and Helicobacter pylori infection [33-36]. These compounds share a common structural motif contributed by a substituted pyridylmethylsulfinylbenzimidazole (Fig.5.4). Inhibition of gastric acid secretion by these compounds is considered as one of the important steps to control the disorders [37]. Proton pump inhibitors inhibit acid secretion by irreversibly interacting with the H⁺-K⁺-ATPase, the terminal proton pump of
the parietal cell [38, 39]. In the acid space of the secreting parietal cell or in the vicinity of the enzyme, these compounds are converted to thiophilic sulfenamide or sulfenic acid, which reacts mainly with the Cys-813 residue in the catalytic subunit of the H⁺-K⁺-ATPase, which is critical for enzyme inactivation. Although omeprazole, the primary member of the proton pump inhibitors, has been extensively used to control these disorders, lansoprazole, the second member of the substituted benzimidazole containing a trifluoromethoxy group, has also been used more recently.

![Chemical structures of proton pump inhibitors](image)

**Fig. 5.4 Chemical structure of proton pump inhibitors**

The role of acid in gastroduodenal pathogenesis has been extensively studied. Although gastric ulcer patients show normal or reduced level of acid secretion, duodenal ulcer patients usually secrete more acid [40, 41]. In fact, “no acid, no ulcer” is the dictum for duodenal ulcer. Because 30 per cent of patients having duodenal ulcer and very few patients with gastric ulcer are hyperchlorohydric, clearly factors other than acid are involved in the pathogenesis of gastroduodenal ulcer. Although the secreted acid itself is not sufficient for ulcer formation, its corrosive property and increased peptic activity is sufficient to aggravate the ulcer. Even the normal rate of acid secretion may cause ulceration in the breached mucosa when some gastroprotective factors are lost. Hence, acid suppression by omeprazole is a common practice to control gastroduodenal lesions. Suppression of intragastric acid also helps in the healing of ulcer [42].

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animals, the role of acid in gastric lesions has been studied using some animal models such as stress or nonsteroidal anti-inflammatory drug-induced gastric ulcer. Stress itself inhibits gastric acid secretion through a central nervous reflex mechanism [43]. Restraint cold stress or restraint water immersion stress induces gastric lesions, which are associated with a decreased or normal level of acid secretion [44, 45]. Because restraint or water immersion stress significantly decreases acid secretion induced by pylorus ligation, acid plays a minor role in stress ulcer. Administration of antacids to neutralize secreted acid does not protect stress ulcer [46], suggesting that factors other than acid are involved in ulcer formation. However, in indomethacin-induced gastric damage, acidity may be increased because of decreased biosynthesis of prostaglandin [47, 48]. Because acidity as high as 0.6 M HCl can experimentally produce gastric lesions [49], mild irritants like 0.35 M HCl prevents gastric damage caused by stronger necrotizing agent through "adaptive cytoprotection" mediated by increased formation of prostaglandin [50].

It is now generally agreed that gastric lesions dwelled when the delicate balance between some gastroprotective and aggressive factors is lost. Although the cellular and molecular bases of gastric mucosal defense against gastrodamaging factors are known [51], the mechanism of mucosal damage by the aggressive factors is not fully clear today. Stress [52, 53], nonsteroidal antiinflammatory drugs [54], and H. pylori [55] cause mucosal damage through a number of mechanisms, of which some reactive oxygen species (ROS) such as \( \text{O}_2^\cdot \) and \( \text{OH}^\cdot \) are now considered to be one of the major causative factors for mucosal lesions through oxidative damage [56-65]. Lipid peroxidation, an important parameter for \( \text{OH}^\cdot \)-induced oxidative damage of membrane, is increased in gastric lesions caused by ethanol [66], indomethacin, and water immersion stress [67]. Increased lipid peroxidation, increased protein oxidation, and decreased glutathione level are also evident in restraint cold stress-induced gastric lesions as a result of oxidative damage caused by the significant generation of \( \text{OH}^\cdot \) [68]. Hydroxyl radical-mediated oxidative damage of membrane lipid and protein and depletion of glutathione have also recently been reported in human gastric ulcer.
5.7 ILAPRAZOLE

Ilaprazole (IY-81149) is a new proton-pump inhibitor (PPI) not previously studied in human patients with ulcer disease. This study evaluated and compared it with a reference PPI, omeprazole, in the treatment of gastric and duodenal ulcers. PPIs have been used therapeutically for many years, and shown great efficacy in accelerating ulcer healing. Currently researches are focused on more potent PPIs. Some preclinical studies have shown that ilaprazole might be such a new substitute. Ilaprazole-[(4-methoxy-3-methyl-pyridin-2-yl)methylsulfinyl]-6-pyrrol-1-yl-1H-benzoimidazole, Figure 5.5, a substituted benzimidazole, is a new candidate drug that is an H⁺/K⁺-ATPase inhibitor designed for the treatment of gastric ulcers [69, 70]. Ilaprazole was under development by IIYang Pharmacy Co (Seoul, Korea) and has been proven by a series of animal studies to be a potent and safety antiulcer agent and the major one being ilaprazole sulfone. Recently, a new metabolite of ilaprazole, ilaprazole thiol ether and chloro was identified (Figure 5.6) an improved LC-MS/MS method for quantitative determination of ilaprazole and its metabolites in human plasma [71, 72]. A validation was performed in accordance with current guidelines [73, 74] Two hundred and twelve gastric ulcer patients (median age 53.3 years) and 306 duodenal ulcer patients (median age 49.7 years) were recruited more over 71.8 and 85% of gastric and duodenal ulcer patients respectively were taken and completed the study. Ulcers were successfully healed in 64.29, 67.14, and 63.89% of gastric ulcer patients and 78.85, 83.65, and 78.57% of duodenal ulcer patients after treatment with 20 mg omeprazole, 5 mg ilaprazole and 10 mg ilaprazole respectively. Most patients (>90%) became asymptomatic after treatment. At the dosages administered, both drugs exhibited similar efficacy and a similar safety profile [75].

A novel method developed ilaprazole Ultra Performance Liquid Chromatography (UPLC). is used to treat drug for in the treatment of duodenal ulcers and provide some characteristics of the dose-response relationship for later studies. The gradient LC method employs solutions A and B as mobile phase. A contains a Milli Q water and solution B contains prepare a mixture Acetonitrile, water, and triethylamine in the ratio of 160:40:01 adjust pH to 7.0 with orthophosphoric acid, Filter through 0.22 µm membrane filter and the diluents of 0.1N sodium hydroxide solution and methanol in the ratio of 75:25 v/v. The
Chromatography was performed on Aquity UPLC BEH Shield RP 18 (100 X 2.1 mm), 1.7μm and detector of UV at 237nm and 210 nm, 1.40μL as an Injection volume. A new metabolite of ilaprazole, ilaprazole thiol ether and chloro was identified (Fig.5.5). Accuracy satisfactory by per cent recovery obtained in the range of 94.9 – 105.9, the linearity results for ilaprazole and metabolites in the specified concentration Calibration curves were linear with a coefficient of variation (r) not less than 0.99. An accelerated degradation study on ilaprazole as following conditions Hydrolytic and Oxidative degradation, Thermal degradation, Humidity degradation, Photolytic degradation, Forced degradation studies (Acid, Alkali, Peroxide Thermal, Humidity and Photolytic).The proposed method was found to be specificity, linearity, and precision, intermediate precision, and accuracy, stability in analytical solution and robustness. The validation was performed according to the current requirements as laid down in the ICH guidelines.

![Ilaprazole](image1)

![Ilaprazole sulfone](image2)

![Ilaprazole thiol ether](image3)

![Ilaprazole chloro](image4)

*Fig. 5.5 Structures of Ilaprazole and Impurities*
5.8 EXPERIMENTAL

5.8.1 Reagents and chemicals

HPLC grade Acetonitrile, Methanol, Triethylamine and Ortho phosphoric acid were obtained from Rankem, Ranbaxy Fine Chemical Limited, New Delhi, India. All other chemical of analytical grade were procured from local sources unless specified.

5.8.2 Instrumentation and Chromatographic Conditions:

The Chromatography was performed on Aiquity UPLC BEH Shield RP 18 (100 X 2.1 mm), 1.7µm and detector of UV at 237nm and 210 nm, 1.40µL as an Injection volume

5.8.3 Mass spectrometer conditions

The API4000 triple quadruple mass spectrometer was operated under the positive electrospray ionization mode (ESI"). The mass spectrometer was tuned by infusion of ilaprazole and its two metabolites (1.0 µg/mL) and omeprazole (1.0 µg/mL) in the mobile phase at a flow rate of 10 µL/min with a syringe pump (Harvard Apparatus, South Natick, MA, USA). The tandem mass spectrometer was tuned to monitor the m/z 351.2 → m/z 168.1 for ilaprazole thiol ether and m/z 401.1 → m/z 184.0 for Chloro impurity respectively using positive electrospray ionization. The spectra and the proposed patterns of ilaprazole, ilaprazole sulfone, ilaprazole thiol ether and omeprazole fragmentation, as well as a summary of the adjusted MS conditions and the compound-specific MS-parameters are presented in Fig.5.6 and Table 5.4.

Fig. 5.6 Mass spectrum of ilaprazole thiol ether and ilaprazole chloro
<table>
<thead>
<tr>
<th>Mass spectrometer</th>
<th>API 4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interface</td>
<td>Electrospray</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive</td>
</tr>
<tr>
<td>Scan type</td>
<td>MRM</td>
</tr>
<tr>
<td>Resolution</td>
<td>Q1-unit resolution</td>
</tr>
<tr>
<td>Curtain gas (CUR)</td>
<td>Q3-unit resolution</td>
</tr>
<tr>
<td>Collision gas (CAD)</td>
<td>6</td>
</tr>
<tr>
<td>IonSpray voltage (IS)</td>
<td>4500</td>
</tr>
<tr>
<td>Temperature (TEM)</td>
<td>500 °C</td>
</tr>
<tr>
<td>Ion source gas 1 (GS 1)</td>
<td>45</td>
</tr>
<tr>
<td>Ion source gas 2 (GS 2)</td>
<td>65</td>
</tr>
<tr>
<td>Solvent split ratio</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 5.4 Ms-Parameters of Ilaprazole Chloro Impurity

5.9 RESULTS AND DISCUSSION

Reversed phase UPLC was proposed as a suitable method for ilaprazole. Chromatographic system consisted of an Aquity UPLC BEH Shield RP 18 (100 X 2.1 mm), 1.7 μm and detector of UV at 237 nm and 210 nm. 1.40 μL as an Injection volume.

5.9.1 Reference solution

Accurately weighted and transferred about 5 mg each of ilaprazole sulphone impurity, ilaprazole sulphide impurity, ilaprazole chloro impurity and ilaprazole standards into a 100 mL volumetric flask, dissolved in and diluted to volume with diluent. Further diluted 1.0 mL of this solution to 50 mL with diluent.

5.9.2 Standard preparation

Weighed and transferred accurately about 35.0 mg of ilaprazole into 50 mL volumetric flask. Added to it 30 mL of acetonitrile and sonicated to dissolve it completely and dilute to volume with the acetonitrile and mix. Diluted 2 mL of above solution to 50 mL volumetric flask, dilute to volume with diluent and mix. Again diluted 3 mL of above solution to 50 mL with diluents.
5.9.3 Method Validation

The objective of the validation procedure was to assess precision, Specificity, accuracy, linearity range and LOD & LOQ.

5.9.3.1 Precision

The system precision was performed by analysing system suitability standard solution six times. Results of Peak area of the API and the impurities were recorded. The peak area variation observed for Ilaprazole and impurities was less than 5.0%. The results comply with the acceptance criteria and indicating acceptable precision of the system. The percentage relative standard deviation of Peak area of six replicate injections for each impurity ≤ 5.0. The Precision of the method was determined by analyzing a sample of Ilaprazole solution spiked with impurities at 100% of the specification limit of six replicate sample preparations. The percentage relative standard deviation of recovery obtained for each impurity less than or equal to 5.0. Prior to this, system suitability parameters were calculated by injecting the system suitability solution. The %RSD was found to be 3.01.

5.9.3.2 Specificity

Each known impurity and Ilaprazole solutions were prepared individually at a concentration of 0.10 mg/ml and a solution of all known impurities spiked with Ilaprazole was also prepared. A test solution of Ilaprazole, solutions of impurities Ilaprazole sulfone, sulfide and chloro impurity and solutions of the Ilaprazole spiked with the impurities. The good selectivity of the method is apparent and Fig. 5.7 and Fig. 5.8 represent a blank chromatogram.
5.9.3.3 Accuracy

The accuracy of the method was determined using four solutions containing Ilaprazole spiked with the impurities at approximately LOQ, 25%, 50%, 100% and 150% of the working strength of API. Percentage of recovery obtained is in the range of 100.4 – 100.6 at 80.0% to 120.0%.

5.9.3.4 Linearity and Range

The linearity of the method was demonstrated for Ilaprazole solutions ranging from LOQ 20%, 40%, 80%, 100%, 120% and 150%. Results obtained are shown in Table 5.5. The linearity results for Ilaprazole and impurities in the specified concentration range were found satisfactory, with a correlation coefficient greater than 0.99.
Table 5.5

Linearity method of Ilaprazole

<table>
<thead>
<tr>
<th>Component</th>
<th>Slope</th>
<th>Intercept</th>
<th>Correlation coefficient (R)</th>
<th>$R^2$</th>
<th>Intercept value w.r.to 100% conc.std response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilaprazole</td>
<td>35373637.75</td>
<td>697.147</td>
<td>0.9990</td>
<td>0.9979</td>
<td>1.79</td>
</tr>
<tr>
<td>Ilaprazole sulphide</td>
<td>29608419.44</td>
<td>-586.101</td>
<td>0.9999</td>
<td>0.9999</td>
<td>-0.50</td>
</tr>
<tr>
<td>Ilaprazole thiol ether</td>
<td>30311345.27</td>
<td>375.534</td>
<td>1.0000</td>
<td>0.9999</td>
<td>1.23</td>
</tr>
<tr>
<td>Ilaprazole chloro</td>
<td>66859263.97</td>
<td>-884.515</td>
<td>0.9996</td>
<td>0.9992</td>
<td>-1.34</td>
</tr>
</tbody>
</table>

5.9.3.5 System Suitability

Prepared the reference solution and the solution were analyzed six times as per the method described.

5.9.3.6 Limit of detection and Limit of quantification

The limit of detection (LOD) is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. The result obtained for each impurity is listed in Table 5.6. The limit of detection values obtained for each impurity was within the acceptance criteria. Signal to noise ratio about 3:1 and the detection less than 0.15%.

Limit of Quantification (LOQ) values were determined from the same experiment as mentioned in the limit of detection section. The LOQ values obtained are presented in Table 5.7. Signal to noise ratio is about 10:1 and the quantification limit is to be less than level of specification preferably much less.
Table 5.6
Limit of detection (LOD) for Ilaprazole and impurities

<table>
<thead>
<tr>
<th>Component</th>
<th>%Impurity w.r.to working strength</th>
<th>Concentration (mg/ml)</th>
<th>Signal to noise</th>
<th>LOD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilaprazole</td>
<td>0.0011</td>
<td>0.0000221</td>
<td>3.5:1</td>
<td>0.001</td>
</tr>
<tr>
<td>Ilaprazole sulphide</td>
<td>0.0014</td>
<td>0.0000280</td>
<td>2.7:1</td>
<td>0.001</td>
</tr>
<tr>
<td>Ilaprazole thiol ether</td>
<td>0.0014</td>
<td>0.0000275</td>
<td>3.1:1</td>
<td>0.001</td>
</tr>
<tr>
<td>Ilaprazole chloro</td>
<td>0.0008</td>
<td>0.0000151</td>
<td>3.5:1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 5.7
Limit of Quantitation for Ilaprazole and impurities

<table>
<thead>
<tr>
<th>Component</th>
<th>%Impurity w.r.to working strength</th>
<th>Signal to noise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilaprazole</td>
<td>0.004</td>
<td>9.7:1</td>
</tr>
<tr>
<td>Ilaprazole sulphide</td>
<td>0.005</td>
<td>9.5:1</td>
</tr>
<tr>
<td>Ilaprazole thiol ether</td>
<td>0.005</td>
<td>10.0:1</td>
</tr>
<tr>
<td>Ilaprazole chloro</td>
<td>0.003</td>
<td>9.9:1</td>
</tr>
</tbody>
</table>

5.9.3.7 Robustness

System suitability followed by a sample analysis was run to assess if these changes had a significant effect on the chromatography. A sample of Ilaprazole spiked with known impurities was analyzed for verifying the level of impurities at each variation. The retention time of all the impurities including Ilaprazole were affected by slight variation in the flow, pH and column temperature, however the system suitability criteria for the method were fulfilled. The number of theoretical plates for Ilaprazole peak not less than 3000. The resolution between the peaks
due to intermediate and Ilaprazole are not less than 2.0. The tailing factor for Ilaprazole peak is not more than 2.0.

5.9.3.8 Solution stability

A solution of Ilaprazole spiked with the impurities and the standard solution stability were kept at room temperature (24-26°C) as well as in the refrigerator at 2-8°C. The solution stability was monitored at different intervals (Initial, 24 hours and 48 hours). No significant variation in the percentage of impurities was observed up to 48 hours at 2-8°C for reference solution and sample solution. The level of unknown impurity was found to increase in the sample solution stored at room temperature. It is recommended to keep the solutions at 2-8°C for analysis. Record the results and assign the stability of the solution based on the experimental data. For a stable solution, the individual impurity values to be within ±0.03 of the original value and the total impurities to be within ±0.10 of the original value.

CONCLUSION

An ultra performance liquid chromatography method was developed and validated for the determination of the related substances for Ilaprazole. The proposed method was found to be good results for specificity, linearity, precision, intermediate precision, and accuracy, stability in analytical solution, robustness and degradation studies. Therefore the method is suitable for its intended use for commercialization.
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