Determination of Mesalamine Related Impurities in Mesalamine Delayed Release Tablets

Overview

The present chapter deals with the determination of mesalamine related impurities in mesalamine delayed release tablets using the developed and validated, stability indicating, RP-UPLC method.

6.1 LITERATURE REVIEW

The purity evaluation of mesalamine in drug product by determination of related substances would be a first step in examination of the safety and quality of the drug product. Chemical structures and UV spectra of mesalamine and its six impurities are shown in [Figure 6.1]. Mesalamine drug profile [1] and degradation mechanism in aqueous solution is reported [2]. Several article for mesalamine metabolism [3, 4] and its determination by HPLC [5-7] and HPLC-ESI-MS/MS [8] has been reported. Estimation of mesalamine and its metabolites in plasma and urine by HPLC [9-11] and by fluorescence detector [12] are also reported. Mesalamine HPLC determination in rectal tissue biopsies [13] and endoscopic intestinal biopsy in human has been reported [14]. Identification of unknown impurity in mesalamine is also reported [15, 16].

Mesalamine protects against colorectal cancer in inflammatory bowel disease [17]. HPLC determination of mesalamine and related impurities using ion-pairing reagent [18] and by simple liquid chromatography [19] has been reported. Mesalamine formulation determination by spectrophotometric [20], by HPLC and ultraviolate [21] and by differential pulse voltammetry is
reported [22]. Determination of Mesalamine and its related impurity by micellar electrokinetic capillary chromatography has been reported [23]. Determination of mesalamine related impurities by micellar electrokinetic chromatography with an ion-pair reagent is reported [24]. Mesalamine determination in pharmaceutical dosage forms by HPLC, DPPH and nitrosation is also reported [25].

Literatures survey reveals that the mesalamine drug substance is official in US Pharmacopeia [26] as well as in British Pharmacopeia [27]. Mesalamine extended release capsules [28] and mesalamine delayed-release tablets [29], formulation is also official in US Pharmacopoeia. In pharmacopoeia method (mesalamine delayed-release tablets), requirement of resolution (system suitability) in between salicylic acid, mesalamine and 3-aminosalicylic acid is not less than 2. This resolution requirement (system suitability) is very low for the low ppm solution. In this method total run time, solution stability, impurities RRT (related retention time) and its order of elution for all impurities also not mentioned. Also, mesalamine delayed-release tablet USP monograph having unknown single max limit of 0.5 % by the area percent method. Now a day’s determination of Genotoxic and Carcinogenic Impurities (may be available in formulation as an unknown) in drug substances and product is the new approach from the regulatory agency [30]. Mesalamine delay release formulation having a higher amount of drug substance (total daily intake 1.2 g/day), below the qualification threshold, no investigation is required (intake is up to 2g/day), although impurities at levels above 1000 ppm (or 1mg/day) are expected, at the least, to be identified [31]. Subsequent guidance from the U.S. Food and Drug Administration (USFDA) [30] conform that the ICH thresholds may not be acceptable for carcinogenic impurities (may be unknown in drug substances). To control and determination of the unknown impurity in drug product at lower level will help to reduce the risk of carcinogenic.

So, determination of related substances would be a first step in examination of the safety and quality of the drug product. As per new approach related impurities/degradation products/unknown impurities RRT and good amount of resolution between all related substances in the drug product
Determination of Mesalamine Related Impurities in Mesalamine Delayed Release Tablets

analytical method (with the low limit determination capability) is the first step to measure the quality of drug product.

UPLC is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 µm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceuticals and biomedical analysis [32-35]. In the present work, this technology has been applied to the method development and validation study of assay determination (AMB, CTZ, MP and PP) in liquid pharmaceutical formulation.

Mesalamine; Mesalazine; 5-Aminosalysilic acid; [5-ASA]

2,5-dihydroxybenzoic acid; [Impurity-A]

2-hydroxy-5-nitrobenzoic acid; 5-nitrosalicylic acid; [Impurity-B]
2-hydroxybenzoic acid; Salicylic acid; [Impurity-C]

[Figure 6.1 Chemical structures and UV spectra of mesalamine and its six impurities]
6.2 THE SCOPE AND OBJECTIVES OF PRESENT STUDY

Comprehensive literatures to measure the quality of mesalamine delayed-release product by HPLC have long run time, limited solution stability, less efficiency (N), higher limit for unknown single maximum impurity, lack of related retention time for all impurities and less resolution, as such there is lack of a suitable procedure for the quantification and estimation for them. Therefore, the aim of the present work is to develop and validate a simple, precise, accurate, short runtime and specific method for the quantification and separation of 5-ASA and its impurities/degradation product by reversed-phase UPLC method, in mesalamine delayed-release formulation. Thereafter, this method is validated according to the ICH guideline.

The objectives of the present work are as follow:

- Development of rapid, stability indicating RP-UPLC method for determination of mesalamine related impurities in mesalamine delayed release tablets formulation.
- Forced degradation study.
- To separates 5-ASA from its all seven (Imp-A, B, C, D, E, and Imp-F) known impurities and any unknown degradation product generated during forced degradation study.
- Validation for the developed method.

6.3 MESALAMINE

Mesalamine (5-aminosalicylic acid, 5-ASA), the therapeutically active moiety of sulfasalazine [36-38] is routinely employed in the treatment of inflammatory bowel disease, that is ulcerative colitis and Crohn’s disease. Various types of formulations are available for the mesalamine [39]. Orally administrated mesalamine is rapidly and almost completely absorbed from the small intestine [40-42]. Formulations able to deliver the intact drug to the lower intestine are nowadays successfully used [43, 44]. Mesalamine [Figure 6.2] molecular weight is 153.135 g/mol with molecular formula C7H7NO3. Its dissociation constant (pKa) 15.48 [45] is reported. Its melting point is 283°C [45].
Figure 6.2  Chemical structure of Mesalamine (5-ASA)]

Indications
For the treatment of active ulcerative proctitis.

Pharmacodynamics
Mesalazine (INN, BAN), also known as Mesalamine (USAN) or 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug used to treat inflammation of the digestive tract (Crohn's disease) and mild to moderate ulcerative colitis. Mesalazine is a bowel-specific aminosalicylate drug that is metabolized in the gut and has its predominant actions there, thereby having fewer systemic side effects. As a derivative of salicylic acid, 5-ASA is also an antioxidant that traps free radicals, which are potentially damaging by-products of metabolism.

Mechanism of action
Although the mechanism of action of mesalazine is not fully understood, it appears to be topical rather than systemic. Mucosal production of arachidonic acid metabolites, both through the cyclooxygenase pathways, i.e., prostanoids, and through the lipoxygenase pathways, i.e., leukotrienes and hydroxyeicosatetraenoic acids, is increased in patients with chronic inflammatory bowel disease, and it is possible that mesalazine diminishes inflammation by blocking cyclooxygenase and inhibiting prostaglandin production in the colon.

Absorption
20 to 30% absorbed following oral administration. 10 to 35% absorbed from the colon (rectal suppository) - extent of absorption is determined by the length of time the drug is retained in the colon.
Protein binding

About 80% of N-Ac-5-ASA is bound to plasma proteins, whereas 40% of mesalamine is protein bound.

Metabolism

Rapidly and extensively metabolized, mainly to N-acetyl-5-ASA (Ac-5-ASA) in the intestinal mucosal wall and the liver. Ac-5-ASA is further acetylated (deactivated) in at least 2 sites, the colonic epithelium and the liver.

Route of elimination

Approximately 28% of the mesalamine in Asacol tablets is absorbed after oral ingestion, leaving the remainder available for topical action and excretion in the feces. It is excreted mainly by the kidney as N-acetyl-5-aminosalicylic acid.

Half life

The mean elimination half-life is 5 hours for 5-ASA and six hours for N-acetyl-5-ASA following the initial dose. At steady state, the mean elimination half-life is seven hours for both 5-ASA and N-acetyl-5-ASA.

Toxicity

Oral, mouse: LD$_{50}$ = 3370 mg/kg; Oral, rat: LD$_{50}$ = 2800 mg/kg; Skin, rabbit: LD$_{50}$ = >5 gm/kg. There have been no documented reports of serious toxicity in man resulting from massive overdosing with mesalamine. Under ordinary circumstances, mesalazine absorption from the colon is limited.

Affected organisms

Humans and other mammals.
6.4 EXPERIMENTAL

6.4.1 Materials and reagents

Mesalamine delayed-release tablets, placebo of mesalamine tablets, Mesalamine [5-ASA] in-house reference standard, impurity-A (purity 90.0 %), impurity-B (purity 99.9 %), impurity-C (purity 99.7 %), impurity-D (purity 93.1 %), impurity-E (purity 99.8 %) and impurity-F (purity 99.7 %) are provided by Dr. Reddy’s laboratories Ltd., Hyderabad. HPLC grade acetonitrile and methanol has been obtained from J.T.Baker (NJ, USA). HPLC grade 1-octane sulphonic acid sodium salt has been obtained from RANKEM (RFCL Ltd., Delhi). Di-potassium hydrogen orthophosphate purified, potassium dihydrogen orthophosphate purified, GR grade orhtophosphoric acid and GR grade hydrochloric acid have been obtained from Merck (Mumbai, India). 0.2 µm nylon 66 membrane filter and 0.2 µm nylon syringe filter has been manufactured by Pall life science limited (India). 0.2 µm PVDF syringe filter is manufactured by Millipore (India). High purity water is generated by using Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

6.4.2 Equipments

Acquity UPLC™ system (Waters, Milford, USA), consisting of a binary solvent manager, sample manager and PDA (photo diode array) detector. System control, data collection and data processing are accomplished using Waters Empower™-2 chromatography data software. Cintex digital water bath is used for specificity study. Photo stability studies are carried out in a photo-stability chamber (SUNTEST XLS+, ATLAS, Germany). Thermal stability study is performed in a dry air oven (Cintex, Mumbai, India).

6.4.3 Preparation of mobile phase and gradient program

Buffer preparation

pH 2.2 buffer preparation: 1.36 gm of potassium dihydrogen orthophosphate and 5.5 gm of 1-octane sulphonic acid sodium salt is dissolved in 890 mL of Milli-Q water. The pH of this solution
is adjusted to 2.2 with orthophosphoric acid and then filtered through 0.2 µm nylon 66 membrane filter.

**pH 6.0 buffer preparation:** 1.74 gm of dipotassium hydrogen orthophosphate and 5.5 gm of 1-octane sulphonic acid sodium salt is dissolved in 890 mL of Milli-Q water. The pH of this solution is adjusted to 6.0 with orthophosphoric acid and then filtered through 0.2 µm nylon 66 membrane filter.

**Note:** Bench top stability at room temperature for the both buffer preparation is found stable with respect to pH and visual clarity up to 48h.

Mobile Phase-A (MP-A): Buffer pH 2.2.

Mobile phase-B (MP-B): Mixture of buffer pH 6.0, methanol and acetonitrile in the ratio of 890:80:30 (v/v/v) respectively.

### Table 6.1 Gradients program for elution

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (mL/min)</th>
<th>% MP-A</th>
<th>% MP-B</th>
<th>Gradient curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.7</td>
<td>90</td>
<td>10</td>
<td>Isocratic</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>90</td>
<td>10</td>
<td>Isocratic</td>
</tr>
<tr>
<td>13</td>
<td>0.7</td>
<td>10</td>
<td>90</td>
<td>Linear</td>
</tr>
<tr>
<td>13.1</td>
<td>0.7</td>
<td>90</td>
<td>10</td>
<td>Isocratic</td>
</tr>
<tr>
<td>15</td>
<td>0.7</td>
<td>90</td>
<td>10</td>
<td>Equilibration</td>
</tr>
</tbody>
</table>

MP-A and MP-B is filtered through 0.22 µm nylon membrane filter and degassed under vacuum prior to use.

### 6.4.4 Diluent preparation

**Diluent-1:** 1N hydrochloric acid is used.

**Diluent-2:** 1.36 gm of potassium dihydrogen orthophosphate and 2.2 gm of 1-octane sulphonic acid sodium salt are dissolved in 890 mL of Milli-Q water. The pH of this solution is adjusted to
2.2 with orthophosphoric acid. Adjusted pH (2.2) buffer, methanol and acetonitrile are mixed well in the ratio of 890:80:30 (v/v/v) respectively.

### 6.4.5 System suitability solution preparation

1 mg of 2-hydroxy benzoic acid (salicylic acid), 1 mg of 5-ASA and 1 mg of 3-amino salicylic acid standard is taken in 100 mL volumetric flask. About 6 mL of diluent-1 is added to this volumetric flask and sonicated in an ultrasonic bath for 10 min. This solution is then diluted up to the mark with diluent-2, mixed well. Three millilitres of this solution is transferred into 10 mL volumetric flask and diluted up to the mark with diluent-2, mixed well.

### 6.4.6 Standard solution preparation

About 32 mg of 5-ASA working standard is taken into 100 mL volumetric flask, then added 6 mL of diluent-1 and dissolved it by 10 minutes sonication. This solution is then diluted to the mark with diluent-2, mixed well. Two millilitres of this solution is transferred into 200 mL volumetric flask and diluted up to the mark with diluent-2, mixed well.

### 6.4.7 Sample solution preparation

Twenty tablets are crushed to fine powder. An accurately weighed portion of the powder equivalent to 400 mg of mesalamine is taken into 250 mL volumetric flask. About 15 mL of diluent-1 is added to this volumetric flask and sonicated in an ultrasonic bath for 10 minutes. This solution is then diluted up to the mark with diluent-2, mixed well. It is then filtered through 0.2 µm PVDF syringe filter and the filtrate is collected after discarding first few millilitres.

### 6.4.8 Placebo solution preparation

Twenty tablets of placebo are crushed to fine powder. An accurately weighed portion of the placebo powder equivalent to 400 mg of mesalamine is taken into 250 mL volumetric flask. About 15 mL of diluent-1 is added to this volumetric flask and sonicated in an ultrasonic bath for 10 minutes.
This solution is then diluted up to the mark with diluent-2, mixed well. It is then filtered through 0.2 µm PVDF syringe filter and the filtrate is collected after discarding first few millilitres.

### 6.4.9 Chromatographic conditions

The chromatographic condition is optimised using Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm) column. The finally selected and optimized conditions are as follows: injection volume 7 µL, gradient elution [Table 6.1], at a flow rate of 0.7 mL/min at 40°C (column oven) temperature, detection wavelength 220 nm. The stress degraded samples and the solution stability samples are analyzed using a PDA detector covering the range of 200-400nm.

### 6.4.10 Method Validation

The method described herein has been validated for related substance determination in mesalamine delayed-release tablets formulation.

#### 6.4.10.1 System Suitability

System suitability parameters are measured so as to verify the system performance. In the system suitability solution chromatogram resolution between salicylic acid, mesalamine and 3-aminosalicylic acid is measured. The similarity factor for the peak of mesalamine in duplicate standard preparation is measured. In the standard preparation theoretical plates and tailing factor for mesalamine peak is measured. Percentage related standard deviation for the peak areas of mesalamine for six replicate (standard solution) injections is also measured. All these system suitability parameters cover the system, method and column performance.

#### 6.4.10.2 Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [46, 47]. Forced degradation studies are performed to demonstrate selectivity and stability indicating capability of the proposed method. The sample solution is exposed to acidic condition [2N HCl (5 mL), 60°C, 6h], alkaline condition [1N NaOH (5 mL), 60°C, 1h], strong
oxidizing [6 % H₂O₂ (5 mL), bench top for 2h], hydrolysis [water, 60°C, 2h]. The powdered sample of tablets is exposed to photolytic [1.2 million Lux hours] and dry heat [105°C, 12h] degradation conditions. Also, placebo of the tablets is exposed to above all stress conditions to identify the source of degradation peak. The entire exposed samples are analyzed by the proposed method with PDA detector.

6.4.10.3 Precision

The precision of the related substances method is verified by repeatability and by intermediate precision. Precision is investigated using sample preparation procedure for six real samples (with spiked impurities in known concentration level) of tablets and analyzing by proposed method. Intermediate precision study is performed with different column, different instrument, and different day by another analyst. Precision is also performed at LOQ (in placebo), at 100 % and 150 % of specification limit level. The mean of percentage impurity (n=6) and the percentage relative standard deviation is also calculated for all substances.

6.4.10.4 Accuracy

To confirm the accuracy of the proposed method, recovery experiments are carried out by standard addition technique. Four different levels (LOQ, 50 %, 100 % and 150 %) of impurities standards are added to pre-analyzed tablet samples in triplicate. Four different levels (LOQ, 50 %, 100 % and 150 %) of mesalamine standard are added to pre-analyzed placebo samples in triplicate. The percentage recoveries of mesalamine and impurities at each level and each triplicate are determined. The mean of percentage recoveries is calculated.

6.4.10.5 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of mesalamine and all six impurities are determined (in placebo solution) by using signal to noise approach as defined in International Conference on Harmonization (ICH) guideline [46, 47]. Increasingly dilute solution of drug and each impurity is injected into the chromatograph and signal to noise (S/N) ratio is calculated at each concentration.
6.4.10.6  **Linearity**

Linearity is demonstrated from LOQ to 200% of working concentration by using minimum seven calibration levels for the mesalamine compound and all impurity standards. The method of linear regression is used for data evaluation. Peak area of compound is plotted against respective concentrations. Linearity is described by regression equation, correlation coefficient and Y-intercept bias.

6.4.10.7  **Robustness**

The robustness is a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions is studies by testing influence of small changes in flow rate (±0.05 mL/min), change in column oven temperature (38°C to 42°C) and change in pH of M.P.-A (pH 2.1 to pH 2.3). Measured the system suitability criteria for all the above experiment and compared the related retention time for all the impurities with the initial RRT.

6.4.10.8  **Solution stability**

Stability of standard and sample solution is established by storage of sample solution (duplicate preparation) and standard solution at ambient temperature for 24h. Sample solution stability is demonstrated by spiking impurities standards in pre-analyzed tablet sample. Standard and sample solutions are re-analyzed after 12h and after 24h. For sample solution, percentage difference in impurities is calculated against fresh injected sample solution. Percentage RSD is calculated for standard preparation.

6.4.10.9  **Filter compatibility**

Filter compatibility is performed for nylon 0.2 µm syringe filter (Pall Life sciences) and PVDF 0.2 µm syringe filter (Millipore). To confirm the filter compatibility in proposed method, filtration recovery experiments are carried out by sample filtration technique. The working concentration level impurities standard is added to pre-analyzed tablet sample in duplicate. Spiked impurities
samples is filter through both syringe filter and percentage difference is calculated against centrifuged sample.

6.5 RESULTS AND DISCUSSION

6.5.1 Method Development and Optimization

The important criteria for development of RP-UPLC method for determination of mesalamine related substances in delayed-release tablets are: the method should be able to determine all impurities of the drug in single run with the good amount of resolution and it should be accurate, reproducible, robust, stability indicating, free from interference (blank/ placebo/ other unknown degradation product) and straightforward enough for routine use in quality control laboratory.

To develop the stability indicating method, first the retention behaviour of these all compounds with change in percentage of organic solvent (acetonitrile and methanol) and with change in buffer substances and change in pH of buffer is studied on Waters Acquity BEH C18 column (50 mm x 2.1 mm, 1.7 µm). 1-octane sulphonic acid ion pair reagent is used in buffer preparation to improve the resolution and avoid the other substances co elution at same retention time in RP chromatography. The buffer pH 2.2 for MP-A is found more appropriate for robust resolution, peak shape and RRT performance of all the interested substances. The final gradient run is chosen with regards to the peak resolution and analysis time as well. The gradient program is given in Table 6.1. The flow rate of 0.7 mL/min is optimized with regard to the back pressure and analysis time as well. Diluents concentration is optimized to improve the solution stability and peak shape. Detection wavelength 220nm is selected for mesalamine and its related substances due to higher detector response at this wavelength. Thus, determination of impurities is possible in single run. Working concentration and RRT for all related substances are mentioned in Table 6.2.
Table 6.2 Name of the impurities, working concentration (in % and µg/mL) and related retention time with respect to 5-ASA are as follow.

<table>
<thead>
<tr>
<th>Impurities</th>
<th>Working concentration</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp-A</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Imp-B</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Imp-C</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>5-ASA</td>
<td>1600</td>
<td>100</td>
</tr>
<tr>
<td>Imp-D</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Imp-E</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Imp-F</td>
<td>3.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

6.5.2 Analytical Parameters and Validation

After satisfactory development of method it is subjected to method validation as per ICH guideline [46]. The method is validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (system suitability, accuracy, precision, linearity, robustness, ruggedness, solution stability, LOD and LOQ, filter compatibility and stability indicating capability).

6.5.2.1 System suitability

The percentage RSD of mesalamine area count of six replicate injections is below 2.0 %. Low values of % RSD of replicate injections indicate that the system is precise. Result of other system suitability parameters such as resolution, theoretical plates, tailing factor and similarity factor (between two standard preparations) are presented in Table 6.3. As seen from this data, the acceptable system suitability parameters would be: related standard deviation of replicate injections is not more than 2.0%, resolution between salicylic acid and 5-ASA is not less than 3.0, resolution between 5-ASA and 3-aminosalicylic acid is not less than 6.0, theoretical plates for 5-ASA is not less than 10000, tailing factor for 5-ASA is not more than 1.5 and similarity factor (between two standard preparations) is not less than 0.95 and not more than 1.05. Results of system suitability
parameters from different studies are presented in Table 6.3. Overlay chromatograms of replicate standard injection are presented in Figure 6.3.

Table 6.3  System suitability results (precision, intermediate precision and robustness)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Resolution between Imp-C and 5-ASA</th>
<th>Resolution between 5-ASA and Imp-D</th>
<th>Theoretical plates for 5-ASA</th>
<th>Tailing factor for 5-ASA</th>
<th>Similarity factor between two Standard</th>
<th>% RSD of Standard Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>3.89</td>
<td>6.21</td>
<td>15416</td>
<td>1.25</td>
<td>0.97</td>
<td>0.5</td>
</tr>
<tr>
<td>Intermediate Precision</td>
<td>4.11</td>
<td>6.89</td>
<td>14434</td>
<td>0.85</td>
<td>1.02</td>
<td>0.9</td>
</tr>
<tr>
<td>At 0.65 mL/min flow rate</td>
<td>3.73</td>
<td>6.54</td>
<td>14821</td>
<td>0.81</td>
<td>1.03</td>
<td>1.1</td>
</tr>
<tr>
<td>At 0.75 mL/min flow rate</td>
<td>3.74</td>
<td>6.57</td>
<td>14438</td>
<td>0.82</td>
<td>0.99</td>
<td>0.7</td>
</tr>
<tr>
<td>At 38°C Column oven temp.</td>
<td>3.74</td>
<td>6.52</td>
<td>15403</td>
<td>0.83</td>
<td>1.01</td>
<td>0.5</td>
</tr>
<tr>
<td>At 42°C Column oven temp.</td>
<td>3.79</td>
<td>6.58</td>
<td>14262</td>
<td>0.80</td>
<td>1.03</td>
<td>0.9</td>
</tr>
<tr>
<td>MP-A pH 2.1</td>
<td>3.82</td>
<td>6.63</td>
<td>14985</td>
<td>0.81</td>
<td>0.98</td>
<td>0.8</td>
</tr>
<tr>
<td>MP-A pH 2.3</td>
<td>3.83</td>
<td>6.60</td>
<td>15050</td>
<td>0.81</td>
<td>0.97</td>
<td>0.7</td>
</tr>
</tbody>
</table>

[Figure 6.3  Overlaid chromatograms of replicate standard injections]
6.5.2.2 **Specificity**

Typical overlaid chromatograms are presented in Figure 6.4 and 6.5, which shows separation of individual compounds and also shows that there is no any interferences at the RT (retention time) of individual compound due to blank and placebo. Spectral purity of all related substances are presented in Figure 6.6. Chromatograms of base and peroxide degraded tablet samples are presented in Figure 6.7 and 6.8 respectively. Mesalamine is found to be stable under acid [Figure 6.9], humidity [Figure 6.10], heat [Figure 6.11] and photolytic [Figure 6.12] degradation condition. Further, spectra of unknown degradation products in tablet sample are similar to that of unknown degradation products of individual standards eluting at respective retention time. Also spectra of known impurities in degraded tablet sample are similar to its respective impurity standard substance, indicating that there is no co-elution of unknown degradation peak at retention times of respective known impurities. Peak due to mesalamine is investigated for spectral purity in the chromatogram of all exposed samples and found spectrally pure. The max plot of chromatograms degradation samples is also checked to ensure that no degradation peak is missed due to use of wavelength of 220 nm. Therefore, the method is specific and suitable for routine work. The results of forced degradation study are given in Table 6.4, which indicate the spectral specificity of method.
[Figure 6.4  Overlaid chromatograms of placebo and spiked impurities with its 3D and purity plot]

[Figure 6.5  Overlaid chromatograms of blank, placebo and system suitability solution]

Impurity-A  

Impurity-B
Determination of Mesalamine Related Impurities in Mesalamine Delayed Release Tablets

[Figure 6.6 Spectral purity of impurity standards]

[Figure 6.7 Chromatogram of alkali degraded tablet sample]
Figure 6.8  Chromatogram of peroxide degraded tablet sample

Figure 6.9  Chromatogram of acid degraded tablet sample

Figure 6.10  Chromatogram of water hydrolyzed degraded tablet sample
Determination of Mesalamine Related Impurities in Mesalamine Delayed Release Tablets

**Table 6.4** Forced degradation data and purity results of mesalamine (5-ASA)

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>5-ASA Purity Flag</th>
<th>Total % degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolysis [2N HCl, 60°C for 6h]</td>
<td>No</td>
<td>0.25 %</td>
</tr>
<tr>
<td>Base hydrolysis [1N NaOH, 60°C for 1h]</td>
<td>No</td>
<td>5.05 %</td>
</tr>
<tr>
<td>6 % H₂O₂ solution bench top for 2h</td>
<td>No</td>
<td>4.30 %</td>
</tr>
<tr>
<td>Photolytic [1.2 Million Lux hours]</td>
<td>No</td>
<td>0.27 %</td>
</tr>
<tr>
<td>Dry heat [105°C, 12h]</td>
<td>No</td>
<td>0.29 %</td>
</tr>
<tr>
<td>Hydrolysis [Water, 60°C, 2h]</td>
<td>No</td>
<td>0.26 %</td>
</tr>
</tbody>
</table>

$\$=For waters UPLC system,
Purity flag: No, which indicates that purity angle is less than purity threshold and
Purity flag: Yes, which indicates that purity angle is more than purity threshold
6.5.2.3 Precision

Precision (at LOQ, 100 % and 150 %) results are shown in Table 6.5 along with intermediate precision data. Low values of RSD, indicates that the method is precise. Specimen chromatograms of precision study at LOQ and at 100 % level are presented in Figure 6.13 and 6.14 respectively. Precision and intermediate precision study data indicate that method is enough precise for its intended use.

Table 6.5 Precision (LOQ, 100 % and 150 %) and intermediate precision results

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Precision at LOQ</th>
<th>Precision at 100 %</th>
<th>Precision at 150 %</th>
<th>Intermediate Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Imp# % RSD*</td>
<td>% Imp# % RSD*</td>
<td>% Imp# % RSD*</td>
<td>% Imp# % RSD*</td>
</tr>
<tr>
<td>Imp-A</td>
<td>0.005 1.68</td>
<td>0.204 0.40</td>
<td>0.293 0.31</td>
<td>0.207 0.26</td>
</tr>
<tr>
<td>Imp-B</td>
<td>0.005 1.59</td>
<td>0.206 0.06</td>
<td>0.301 0.41</td>
<td>0.203 0.43</td>
</tr>
<tr>
<td>Imp-C</td>
<td>0.018 1.63</td>
<td>0.196 0.14</td>
<td>0.309 0.92</td>
<td>0.208 0.75</td>
</tr>
<tr>
<td>Imp-D</td>
<td>0.019 1.78</td>
<td>0.200 1.22</td>
<td>0.295 0.88</td>
<td>0.212 0.50</td>
</tr>
<tr>
<td>Imp-E</td>
<td>0.020 0.50</td>
<td>0.208 0.28</td>
<td>0.311 0.49</td>
<td>0.200 0.43</td>
</tr>
<tr>
<td>Imp-F</td>
<td>0.018 0.38</td>
<td>0.202 0.74</td>
<td>0.281 1.48</td>
<td>0.180 0.47</td>
</tr>
</tbody>
</table>

# Average of six determinations;  * Determined on six values;  
@ Demonstrated by spiking known impurities into sample

[Figure 6.13 Specimen chromatogram of precision study at LOQ]
6.5.2.4 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method compared with the true values. To confirm the accuracy of the proposed method, recovery experiments are carried out by standard addition technique. The amount recovered is within ±10% of amount added, which indicates that the method is accurate and also there is no interference due to excipients present in tablets. The results of recoveries for 5-ASA and impurities are shown in Table 6.6. Overlay chromatograms of accuracy are presented in Figure 6.15.

Table 6.6 Accuracy results

<table>
<thead>
<tr>
<th>Substances</th>
<th>Mean % Recovery (With triplicate determination at each level)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At LOQ&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Imp-A</td>
<td>105.5</td>
</tr>
<tr>
<td>Imp-B</td>
<td>102.4</td>
</tr>
<tr>
<td>Imp-C</td>
<td>97.4</td>
</tr>
<tr>
<td>5-ASA</td>
<td>95.9</td>
</tr>
<tr>
<td>Imp-D</td>
<td>104.3</td>
</tr>
<tr>
<td>Imp-E</td>
<td>101.1</td>
</tr>
<tr>
<td>Imp-F</td>
<td>103.9</td>
</tr>
</tbody>
</table>

<sup>*</sup> Average of three determinations
6.5.2.5 LOD and LOQ

The concentration (in %) with signal to noise ratio of at least 3 is taken as LOD and concentration with signal to noise of at least 10 is taken as LOQ, which meets the criteria defined by ICH guidance. The LOD and LOQ results of all substances are presented in Table 6.7. Precision result at LOQ is also presented in Table 6.5.

Table 6.7 Limit of detection and limit of quantification

<table>
<thead>
<tr>
<th>Substances</th>
<th>In % (w.r.t. working con.)</th>
<th>LOD</th>
<th>LOQ</th>
<th>Signal to Noise Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp-A</td>
<td>0.0018</td>
<td>0.006</td>
<td>2.9</td>
<td>10.4</td>
</tr>
<tr>
<td>Imp-B</td>
<td>0.0018</td>
<td>0.006</td>
<td>3.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Imp-C</td>
<td>0.006</td>
<td>0.020</td>
<td>2.7</td>
<td>10.0</td>
</tr>
<tr>
<td>5-ASA</td>
<td>0.006</td>
<td>0.020</td>
<td>2.4</td>
<td>9.7</td>
</tr>
<tr>
<td>Imp-D</td>
<td>0.006</td>
<td>0.020</td>
<td>3.0</td>
<td>9.9</td>
</tr>
<tr>
<td>Imp-E</td>
<td>0.006</td>
<td>0.020</td>
<td>3.3</td>
<td>13.5</td>
</tr>
<tr>
<td>Imp-F</td>
<td>0.006</td>
<td>0.020</td>
<td>3.1</td>
<td>10.2</td>
</tr>
</tbody>
</table>
6.5.2.6   Linearity

The response is found linear for all substances from LOQ to 200 % of working concentration. This test is performed on seven different levels of each substance, which gave us a good confidence on analytical method with respect to linear range. For the all substances correlation coefficient is greater than 0.999. Correlation coefficients, Y-intercept bias and linearity equations for mesalamine and impurities are presented in Table 6.8. Overlay chromatograms of different linearity levels is also presented in Figure 6.16. Compounds linearity curve are presented in Figure 6.17-6.23.

[Figure 6.16   Overlaid specimen chromatograms of linearity study (LOQ to 200 %)]
Table 6.8  Linearity results for 5-ASA and impurities

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linearity range (µg/mL)</th>
<th>Correlation Coefficient ($r^2$)</th>
<th>Linearity (Equation)</th>
<th>Y- Intercept bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp-A</td>
<td>0.096 to 6.4</td>
<td>0.9997</td>
<td>$y = 44304(x) - 1747.9$</td>
<td>-1.243</td>
</tr>
<tr>
<td>Imp-B</td>
<td>0.096 to 6.4</td>
<td>0.9999</td>
<td>$y = 40668(x) - 1425.4$</td>
<td>-1.104</td>
</tr>
<tr>
<td>Imp-C</td>
<td>0.32 to 6.4</td>
<td>0.9997</td>
<td>$y = 18256(x) - 747.63$</td>
<td>-1.285</td>
</tr>
<tr>
<td>5-ASA</td>
<td>0.32 to 6.4</td>
<td>0.9999</td>
<td>$y = 21814(x) - 856.65$</td>
<td>-1.231</td>
</tr>
<tr>
<td>Imp-D</td>
<td>0.32 to 6.4</td>
<td>0.9997</td>
<td>$y = 21414(x) + 484.48$</td>
<td>0.696</td>
</tr>
<tr>
<td>Imp-E</td>
<td>0.32 to 6.4</td>
<td>0.9999</td>
<td>$y = 45988(x) - 301.78$</td>
<td>-0.204</td>
</tr>
<tr>
<td>Imp-F</td>
<td>0.32 to 6.4</td>
<td>0.9997</td>
<td>$y = 30299(x) - 657.93$</td>
<td>-0.675</td>
</tr>
</tbody>
</table>

[Figure 6.17  Linearity of mesalamine]

[Figure 6.18  Linearity of impurity-A]
[Figure 6.19  Linearity of impurity-B]

**Impurity-B**

\[ y = 40668x - 1425.4 \]
\[ R^2 = 0.9999 \]

[Figure 6.20  Linearity of impurity-C]

**Impurity-C**

\[ y = 18256x - 747.63 \]
\[ R^2 = 0.9997 \]

[Figure 6.21  Linearity of impurity-D]

**Impurity-D**

\[ y = 21414x + 484.48 \]
\[ R^2 = 0.9997 \]
6.5.2.7 Robustness

No significant effect is observed on system suitability parameters such as resolution, theoretical plates, tailing factor, similarity factor and RSD of respective components, when small but deliberate changes are made to chromatographic conditions. The results are presented in Table 3 along with system suitability parameters of normal methodology. No significant effect is observed on related retention time (RRT) of all impurities, when deliberate changes are made to chromatographic conditions. The impurities RRT variation results are presented and compared with normal methodology result, which is presented in Table 6.9. Thus, the method is found to be robust with respect to variability in variable conditions.
### Table 6.9 Robustness results

<table>
<thead>
<tr>
<th>Impurities</th>
<th>Initial</th>
<th>Flow rate per minutes</th>
<th>Column oven temperature</th>
<th>MP-A pH variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.65mL</td>
<td>0.75mL</td>
<td>38°C</td>
</tr>
<tr>
<td>Imp-A</td>
<td>0.134</td>
<td>0.139</td>
<td>0.139</td>
<td>0.139</td>
</tr>
<tr>
<td>Imp-B</td>
<td>0.334</td>
<td>0.348</td>
<td>0.348</td>
<td>0.348</td>
</tr>
<tr>
<td>Imp-C</td>
<td>0.914</td>
<td>0.915</td>
<td>0.915</td>
<td>0.915</td>
</tr>
<tr>
<td>5-ASA</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Imp-D</td>
<td>1.320</td>
<td>1.302</td>
<td>1.302</td>
<td>1.302</td>
</tr>
<tr>
<td>Imp-E</td>
<td>1.666</td>
<td>1.631</td>
<td>1.631</td>
<td>1.631</td>
</tr>
<tr>
<td>Imp-F</td>
<td>1.904</td>
<td>1.855</td>
<td>1.855</td>
<td>1.854</td>
</tr>
</tbody>
</table>

#### 6.5.2.8 Solution stability

Solution stability is performed and determined with duplicate spike sample preparation. Percentage difference in all impurities is calculated with respect to freshly injected sample solution. Sample solution does not show any appreciable change in all impurities value when stored at ambient temperature up to 24h, which is presented in Table 6.10. Standard solution did not show any unknown peak during this 24h study and also full fill the requirement of % RSD, which is also presented in Table 6.11.

### Table 6.10 Solution stability results (test preparation)

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Initial Sample</th>
<th>Sample After 12 hours</th>
<th>Sample After 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imp. in %</td>
<td>Imp. in %</td>
<td>Difference in %</td>
</tr>
<tr>
<td></td>
<td>Sam-1</td>
<td>Sam-2</td>
<td>Sam-1</td>
</tr>
<tr>
<td>Imp-A</td>
<td>0.207</td>
<td>0.208</td>
<td>0.205</td>
</tr>
<tr>
<td>Imp-B</td>
<td>0.210</td>
<td>0.210</td>
<td>0.206</td>
</tr>
<tr>
<td>Imp-C</td>
<td>0.200</td>
<td>0.200</td>
<td>0.203</td>
</tr>
<tr>
<td>Imp-D</td>
<td>0.207</td>
<td>0.205</td>
<td>0.210</td>
</tr>
<tr>
<td>Imp-E</td>
<td>0.212</td>
<td>0.212</td>
<td>0.208</td>
</tr>
<tr>
<td>Imp-F</td>
<td>0.204</td>
<td>0.206</td>
<td>0.205</td>
</tr>
</tbody>
</table>
Table 6.11  Solution stability results (standard preparation)

<table>
<thead>
<tr>
<th>I.D.</th>
<th>Initial</th>
<th>After 12 hrs.</th>
<th>After 24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area</td>
<td>% RSD</td>
<td>Area</td>
</tr>
<tr>
<td>Mesalamine</td>
<td>66552.8</td>
<td>0.60</td>
<td>66710.8</td>
</tr>
</tbody>
</table>

# - Determined on five replicate injections
$ - Determined on five initial and 12h standard injections (RSD of six Inj.)
@ - Determined on five initial, one 12h and one 24h standard injections (RSD of seven Inj.)

6.5.2.9  Filter compatibility

Filter compatibility with 0.2 µm syringe filter of PVDF and nylon filter is determined with duplicate sample preparation. Filtered sample solution does not show any significant changes in impurities percentage with respect to centrifuge samples impurities percentage. Difference in all impurities percentage results is presented in Table 6.12. In displayed result difference in % of impurities is not observed more than 0.002 %, which indicates that both syringe filter having a good compatibility with sample solution. Overlaid specimen chromatograms of filter compatibility study are presented in Figure 6.24.

Table 6.12  Filter compatibility results

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Centrifuged</th>
<th>PVDF Syringe filter 0.2µ (Millipore)</th>
<th>Nylon Syringe filter 0.2µ (Pall Life Sciences)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imp. in %</td>
<td>Imp. in %</td>
<td>Difference in %</td>
</tr>
<tr>
<td>Sam-1</td>
<td>Sam-2</td>
<td>Sam-1</td>
<td>Sam-2</td>
</tr>
<tr>
<td>Imp-A</td>
<td>0.205</td>
<td>0.204</td>
<td>0.205</td>
</tr>
<tr>
<td>Imp-B</td>
<td>0.206</td>
<td>0.207</td>
<td>0.207</td>
</tr>
<tr>
<td>Imp-C</td>
<td>0.203</td>
<td>0.204</td>
<td>0.203</td>
</tr>
<tr>
<td>Imp-D</td>
<td>0.210</td>
<td>0.210</td>
<td>0.209</td>
</tr>
<tr>
<td>Imp-E</td>
<td>0.208</td>
<td>0.209</td>
<td>0.210</td>
</tr>
<tr>
<td>Imp-F</td>
<td>0.205</td>
<td>0.205</td>
<td>0.203</td>
</tr>
</tbody>
</table>
6.6 CALCULATION FORMULA

6.6.1 Impurity (% w/w)

Calculate % impurity present in the finished product formulation using the following formula:

\[
\text{Impurity (\% w/w)} = \frac{C_{\text{std}} \times R_s \times 10,000 \times 1}{C_s \times R_{\text{std}} \times \text{RRF}}
\]
Where,

\[ C_{\text{std}} = \text{Concentration of standard solution in mg/mL} \]
\[ C_s = \text{Concentration of sample solution in mg/mL} \]
\[ R_s = \text{Compound peak response obtained from the sample preparation} \]
\[ R_{\text{std}} = \text{Compound peak response (mean peak area) obtained from the standard preparation} \]
\[ \text{RRF} = \text{Compound related response factor} \]

6.6.2 Relative standard deviation (% RSD)

It is expressed by the following formula and calculated using Microsoft excel program in a computer.

\[
\text{Related Standard Deviation (\%)} = \frac{\text{SD} \times 100}{\bar{X}}
\]

Where,

\[ \text{SD} = \text{Standard deviation of measurements} \]
\[ \bar{X} = \text{Mean value of measurements} \]

6.6.3 Accuracy (% Recovery)

It is calculated using the following equation:

\[
\% \text{ Recovery} = \frac{\text{Amount of substance found (mg)} \times 100}{\text{Amount of substance added (mg)}}
\]

6.7 CONCLUSION

A novel RP-UPLC method is successfully developed and validated for simultaneous determination of all six impurities from mesalamine delayed-release formulation. The total run time is 15 minutes, within which drug and their impurities/degradation products are well separated with each other. Method validation results have proven that the method is selective, precise, accurate, linear, rugged, robust and stability indicating with low LOD and LOQ. This method can be successfully applied
Determination of Mesalamine Related Impurities in Mesalamine Delayed Release Tablets

for the routine analysis as well as stability study of mesalamine delayed-release drug product. Overall, the method provides high throughput solution for determination of all related impurities in mesalamine delayed-release formulation with excellent selectivity, precision and accuracy.

Note: Intellectual Property Management (IPM) clearance number for the present research work is: PUB-00037-10.
6.8 REFERENCES


[31] A recent FDA guideline (July 2009) on impurities in generic drugs would effectively reduce the qualification threshold to 1000 ppm or 1mg/day for new impurities which were not present in the reference listed drugs.
Determination of Mesalamine Related Impurities in Mesalamine Delayed Release Tablets


