Overview

The present chapter deals with the low level (high sensitive) determination of Aniline/Genotoxic impurity in Mesalamine delayed release tablets using the developed and validated, stability indicating, RP-UPLC method.

5.1 LITERATURE REVIEW

Higher sample throughput with more information per sample may decrease the time to market, an important driving force in today’s pharmaceutical industry [1-3]. 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. In the present work, this technology has been applied to the method development and validation study of aniline determination in mesalamine delayed-release dosage form.

Aniline is the critical genotoxic impurity to be considered for mesalamine drug substance and its drug product. Moreover, recently the US-FDA has also published draft guidance for ‘the Genotoxic and Carcinogenic impurities in drug substances and drug products’ [4]. This emphasises the importance of controlling aniline impurity in drug substances and in drug product as well.
A genotoxic activity of aniline and its metabolites and their relationship to the carcinogenicity of aniline in the spleen of rats is reported [5] by Ernst and Bernd. Potent genotoxicity of aminophenylnorharman, formed from aniline in the liver of gpt delta transgenic mouse is reported [6] by Ken-ichi Masumura et al. Carcinogenicity of aminophenylnorharman, formed from aniline in F344 rats had been reported [7] by Toshihiko Kawamori et al. A few analytical methods has also been reported for quantification of aniline and these are: by spectrometric [8], by azo-dye formation [9], by fluorescence [10, 11], by alternating-current oscilopolarographic titration [12], by NIR spectroscopy [13], by GC [14], by HPLC [15, 16] and by LCMS [17, 18].

Aniline determination in mesalamine drug substance by GC is official in US Pharmacopeia [19] and by HPLC is official in British Pharmacopeia [20].

5.2 THE SCOPE AND OBJECTIVES OF PRESENT STUDY

The comprehensive literature survey revealed that the HPLC methods used for determination of quality of mesalamine drug substance have low sensitivity, high LOQ and long run time. Moreover, these reported methods lack the suitable procedure for quantification and estimation of aniline. According to our literature survey, no UPLC method has yet been reported for the determination and quantification of aniline from mesalamine delayed-release tablets. Thus, it is thought to undertake the development and validation of reversed phase UPLC method with lower LOQ for the quantification of aniline in delayed release mesalamine formulation.

The objectives of the present work are as follow:

- Development of rapid, stability indicating RP-UPLC method for determination of Aniline in mesalamine delayed release dosage form.
- Forced degradation study.
- Lower value of LOQ (limit of quantification) for Aniline determination.
- Perform analytical method validation for the proposed method as per ICH guideline.
5.3 DETERMINATION OF GENOTOXIC IMPURITIES

Ensuring the safety of pharmaceutical products is a primary responsibility of the chemists, engineers and formulators involved in their manufacture - regardless of whether the products are intended for commercial purposes or for clinical investigation. When focusing on safety, particular attention is paid to the quality and purity of the raw materials used in the formulation, especially of the active pharmaceutical ingredient(s) (API(s)). A drug substance will typically contain a range of low-level impurity compounds, for example arising as residues of starting materials, reagents, intermediates, or as side-products generated by the synthetic processes or degradation reactions; these need to be understood and controlled within tight limits. The drug substance itself is unlikely to be entirely safe, but a certain level of risk to the patient can be tolerated when weighed against the anticipated health benefits. This balance between risk and benefit has to be finely judged by pharmaceutical manufacturers and regulatory authorities on a case-by-case basis. Impurities, however, are expected to bring no benefits – only risk. Manufacturers must therefore eliminate them (or at least mitigate the risk associated with them) to the greatest extent possible.

The International Conference on Harmonization (ICH) began to publish definitive guidelines on impurities in drug substances and drug products in the late 1990s [21, 22]. These guidelines have been adopted by the regulatory bodies of, among others, the USA, the European Union, and Japan, and have enjoyed wide support within the pharmaceutical industry over the intervening decade. Under this guidance, the normal qualification threshold for impurities is 0.15% [1500 parts per million (ppm)] or 1 mg/day, whichever is lower, for drug substances whose intake is up to 2 g/day. Impurities which exceed this threshold must have their toxicity specifically investigated. Below the qualification threshold, no investigation is required, although impurities at levels above 1000 ppm (or 1 mg/day) are expected, at the least, to be identified [23]. While these thresholds are considered adequate for the general run of process-related impurities, the guideline also recognises that impurities which are “unusually toxic” are of increased concern and deserving of a qualification threshold significantly lower than the default values. Subsequent guidance from the European
Medicines Agency (EMEA) [24] and the U.S. Food and Drug Administration (USFDA) [25] confirmed that the ICH thresholds may not be acceptable for genotoxic or carcinogenic impurities. This, however, has proved to be more controversial within the industry - initially because of the lack of clear regulatory guidance, and subsequently because of the increased stringency imposed when more detailed guidance finally did emerge. Several commentators [26-28] have critically reviewed the history of the evolving guidance on genotoxic impurities. Genotoxic compounds are those which cause damage to DNA, for example by alkylation or intercalation, which could lead to mutation of the genetic code. The terms “genotoxic” and “mutagenic” are usually employed synonymously by chemists, although there is a subtle distinction [29]. The property, however named, can be easily demonstrated by subjecting the chemical to standard in Vitro tests [30], the best known being the Ames mutagenicity test. Whether a given genotoxic compound is also carcinogenic (the real worry from the safety viewpoint) is more difficult to determine; it relies on longer-term in vivo studies using animal models, and there is always a question of the extent to which the results of such studies can be extrapolated to humans. The conservative approach is to assess known genotoxic compounds as potential carcinogens unless there is experimental evidence to the contrary.

5.4 THE REGULATORY VIEWPOINT

In December 2002 the Safety Working Party (SWP) of the European Committee for Proprietary Medicinal Products (CPMP) published a “Position paper on the limits of genotoxic impurities”, signaling an intention to fill the gap in ICH guidelines. Genotoxic impurities are topic selected for a joint meeting of the Drug Information Association (DIA) and the EMEA in October 2003, where scientific and regulatory updates are presented and discussed in the light of case studies. The outcome of these discussions, along with other industry and regulatory comment, has been subsequently published in a special edition of the International Journal of Pharmaceutical Medicine [31]. The EMEA has published a guideline on the subject in June 2006 that finally came into effect in January 2007 [24].
The EMEA guideline recommends that any potentially genotoxic impurities (PGIs) in the drug substance should be identified, either from existing genotoxicity data or through the presence of “structural alerts”. PGIs should then be dichotomized into those for which there is “sufficient (experimental) evidence for a threshold-related mechanism” and those “without sufficient (experimental) evidence for a threshold-related mechanism.” The former category would include *inter alia* compounds that induce aneuploidy by interfering with the mitotic spindle, compounds that interfere with the activity of topoisomerase, and/or compounds that inhibit DNA synthesis. The limits for impurities with clear evidence for a threshold mechanism can be addressed using methods similar to those recommended by ICH for setting limits on Class 2 solvents [32]. This approach calculates a “permitted daily exposure,” which is derived using the “no observed effect level” or, alternatively, the “lowest observed effect level” from the most relevant animal study and incorporating a variety of uncertainty factors. Where there is no, or insufficient, evidence that the genotoxic impurity acts via a threshold-related mechanism, establishing a safe limit is much more problematic. The EMEA approach has been to adopt a concept originally applied by the USFDA to contaminants leaching from food packaging materials and known as the “threshold of regulatory concern” [33]. This principle states that regulators ought not to be concerned with extremely low levels of contamination where the risk of harm is negligible. Subsequent research has aimed to quantify such a regulatory threshold, which is re-designated as the “threshold of toxicological concern” (TTC) [34].

On the basis of an analysis of the carcinogenic potency in rodents of over 700 carcinogens, [35, 36] it is estimated that exposures less than 0.15 μg/day of these substances are unlikely to increase a lifetime cancer risk by more than 1 in one million; it is therefore reasonable to regard 0.15 μg/day as a “virtually safe dose” for all but the most potent carcinogens. The actual limit recommended by the EMEA is 1.5 μg/day, representing a 1 in 105 excess lifetime cancer risk, the extra latitude being justified by the presumed benefit to the patient of taking the medicine. It is recognized that certain classes of compounds, specifically aflatoxin-like, N-nitroso, and azoxy compounds, will require even tighter control. This type of compound is unlikely to feature in a typical drug substance
synthesis. However, the general TTC limit, being several orders of magnitude lower than the normal qualification threshold suggested by ICH guidelines, still presents manufacturers with a host of technical and analytical challenges. The EMEA guideline summarises their recommendations in the form of a decision tree [Figure 5.1]. Their preferred option is, if possible, to eliminate the opportunities for the genotoxic impurity(ies) to appear at all. The second preference is for manufacturers to reduce the level(s) to as low as is reasonably practicable (ALARP principle). Application of TTC concepts is - according to this decision tree - only third favourite [17].

[Figure 5.1  EMEA decision tree for assessment of acceptability of genotoxic impurities]
One issue which the EMEA guideline has originally failed to address is appropriate limits for investigational drugs. At early stages of development the process understanding which is required to control trace-level impurities would likely be limited. On the other hand, clinical subjects are typically exposed to investigational drugs only for limited periods - certainly not the “lifetime” which is assumed in the TTC derivation. The industry group Pharmaceutical Research and Manufacturers of America (PhRMA) have therefore proposed a “staged TTC” approach for the intake of genotoxic impurities over various periods of exposure during clinical trials [37]. Extrapolating from the established acceptable intake over a lifetime, and resetting the acceptable risk to 1 in 106 (since clinical trial subjects may be volunteers who derive no specific benefit from the drug), they have proposed limits of up to 120 μg/day for exposures lasting up to one month, decreasing to 10 μg/day for > 6-12 month exposures. (In all cases a maximum limit of 0.5% would be observed on quality grounds.) This staged approach is subsequently endorsed by the EMEA in a 2008 “Question and Answer” document clarifying their original guidance [38], albeit with reduced limits for each duration of exposure. Similar limits are proposed by the USFDA in their draft guideline published in December 2008 [25]. A summary of the current recommendations from both authorities, as well as PhRMA, is provided in Table 5.1.

### Table 5.1 Proposed allowable daily intake (μg) for genotoxic impurities of unknown carcinogenic potential during clinical development

<table>
<thead>
<tr>
<th></th>
<th>Single dose</th>
<th>NMT 14 days</th>
<th>14 days to 1 month</th>
<th>1-3 months</th>
<th>3-6 months</th>
<th>6-12 months</th>
<th>&gt;12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhRMA recommendation</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>40</td>
<td>20</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>EMEA limits</td>
<td>120</td>
<td>60</td>
<td>60</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>Draft USFDA limits</td>
<td>120</td>
<td>120</td>
<td>60</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

An example of this “staged TTC” approach has been presented by Syntagon in a recent web newsletter [39]. They evaluated the synthetic route to an investigational API [Figure 5.2] intended for a phase I study with a duration of 20 days and a daily dose of approximately 100 mg. They have identified two reagents, benzene and thiourea, as known genotoxins, and the iodo-intermediate 3 as
potentially genotoxic. For the initial clinical study they set specifications of NMT 0.06% w/w (equivalent to 60 μg/day) for all three.

![Chemical structure](image)

**Figure 5.2** Four last chemical steps in the manufacture of an API [39]

It could be argued that the available compound-specific data for benzene and thiourea should have been consulted in preference to applying a staged TTC. In the case of benzene, this would have justified a higher limit of 800 μg/day (0.80% w/w) for the short study, but they defaulted to the lower level as it is easily controlled for using standard analytical techniques (HPLC-UV and GC-FID). It is possible to argue that the “staged” levels can be equally valid for approved drugs which are intended to be administered only for short periods [40]. This suggestion, however, has been specifically dismissed by the USFDA on the grounds that a drug may be used multiple times by the same individual, or may be used outside of its approved indication. However, applicants may provide the agency with a detailed rationale to support higher limits on a case-by-case basis - for example if the patient is likely to be exposed to higher levels of the potential genotoxic impurity (PGI) from other sources or if the drug is intended to treat a life-threatening condition. The EMEA’s 2008 Question and Answer document made a number of other welcome clarifications.

- The guideline need not be applied retrospectively to existing authorised products so long as the manufacturing procedure remains essentially unchanged, unless newly acquired knowledge flags a potential problem.
• It is not necessary to apply ALARP principles to genotoxic impurities which are controlled below the TTC, unless they have structures of very high concern. (This, as pointed out by Snodin et al. [28], actually contradicts the original guideline.)

• Absence of a “structural alert” based on a well performed assessment allows the impurity concerned to be classified as nongenotoxic, without the requirement for specific testing.

• A negative Ames test (properly conducted) would be sufficient to overrule any structural alerts and allow an impurity to be classified as nongenotoxic.

• It is acceptable to assume that an identified PGI is in fact genotoxic without specifically testing it.

• Unidentified impurities which occur below the ICH identification threshold (0.10% or 1 mg/day intake) need not be considered further.

• Identified impurities, even those below the identification threshold, should always be screened for structural alerts.

• When more than one genotoxic impurity is present, the TTC value of 1.5 μg/day can be applied to each, provided the impurities are not structurally related. Where structurally related PGIs are present, the 1.5 μg/day limit should be applied to the whole group.

5.5 MESALAMINE

Mesalamine (5-aminosalicylic acid, 5-ASA), a therapeutically active moiety of Sulfasalazine [41-43] is routinely employed in the treatment of inflammatory bowel disease like ulcerative colitis and Crohn’s disease. Orally administrated mesalamine is rapidly and almost completely absorbed from the small intestine [44-46]. Mesalamine formulations which are able to deliver the intact drug to the lower intestine are nowadays successfully used [47, 48]. The purity evaluation of mesalamine in drug product by determination of related substances is a critical step in examination of the safety and quality of the drug product. Aniline is the critical genotoxic impurity to be considered for mesalamine drug substance and its drug product. Chemical structure and UV spectra of mesalamine
and aniline are shown in [Figure 5.3]. Molecular formula of Mesalamine is C₇H₇NO₃ with molecular weight 153.135 g/mole. Its melting point is 283°C and reported predicted pKa is 15.48.

Aniline; Phenylamine; Aminobenzene; Benzenamine

![Aniline chemical structure](image)

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

![Mesalamine chemical structure](image)

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.

5.6 EXPERIMENTAL

5.6.1 Materials and reagents

Mesalamine delayed-release tablets, placebo and related aniline impurity standard (purity 98.7 %) are provided by Dr. Reddy’s Laboratory Ltd., Hyderabad. Acetonitrile of HPLC grade is obtained from J.T.Baker (NJ, USA), HPLC grade 1-octane sulphonic acid sodium salt is obtained from RANKEM (RFCL ltd., Delhi, India), di-potassium hydrogen orthophosphate purified, GR grade orthophosphoric acid, GR grade sodium hydroxide pallets and GR grade hydrochloric acid are
obtained from Merck (Mumbai, India). 0.2 µm nylon 66 membrane filter and 0.2 µm nylon syringe filter is manufactured by Pall life science ltd. (India). 0.2 µm PVDF syringe filter is manufactured by Millipore (India). Ultra pure water generated by a Milli-Q system (Millipore, Milford, MA, USA).

5.6.2 Equipments

Acquity UPLC™ system (Waters, Milford, USA), consists 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 study is carried out in a photo-stability chamber (SUNTEST XLS+, ATLAS, Germany).

5.6.3 Preparation of mobile phase and its gradient program

**Buffer preparation**

1.74 g of di-potassium hydrogen orthophosphate (K₂HPO₄) and 2.2 g of 1-octane sulphonic acid sodium salt is dissolved in one liter of milli-Q water. The pH of this solution is adjusted to 6.0 with orthophosphoric acid and then filtered through 0.2 µm pall nylon membrane filter. The buffer preparation is found stable with respect to pH and visual clarity up to 48h.

**Mobile phase**: Mixture of buffer (pH 6.0) and acetonitrile at a ratio of 900:100 (v/v) respectively. It is filtered through 0.2 µ nylon filter and degassed before use.

5.6.4 Diluent preparation

1N hydrochloric acid and 1N sodium hydroxide solution in the ratio of 50:50 (v/v) is mixed and used as a diluent.

5.6.5 Impurity standard solution preparation (approx 0.156 µg/mL solution)

Impurity standard solution is prepared by dissolving standard substances in methanol to obtained solution containing 7.5 µg/mL of aniline in methanol. Five millilitres of 7.5 µg/mL of aniline
solution is transferred to 250 mL volumetric flask, diluted to volume with the diluent and mixed well.

[Note: Working concentration of aniline is 0.0005 % with respect to Mesalamine]

5.6.6 Sample solution preparation (30,000 µg/mL solution)

Twenty tablets are crushed to fine powder. An accurately weighed portion of the powder equivalent to 750 mg of mesalamine is taken in 25 mL volumetric flask. About 12.5 mL of 1N hydrochloric acid is added to this flask and sonicated in an ultrasonic bath for 20min. This solution is then diluted up to the mark with 1N sodium hydroxide solution and mixed well. It is then filtered through 0.2 µ PVDF syringe filter and the filtrate is collected after discarding first few millilitres.

5.6.7 Placebo solution preparation

Twenty placebo tablets are crushed to fine powder. An accurately weighed portion of the powder equivalent to 750 mg of mesalamine is taken in 25 mL volumetric flask. About 12.5 mL of 1N hydrochloric acid is added to this flask and sonicated in an ultrasonic bath for 20min. This solution is then diluted up to the mark with 1N sodium hydroxide solution and mixed well. It is then filtered through 0.2 µ PVDF syringe filter and the filtrate is collected after discarding first few millilitres.

5.6.8 Chromatographic conditions

The chromatographic condition is optimized using a column Reprosil Gold 100, C18-XBD 50 mm x 2.0 mm, 1.8 µm. The separation of aniline from mesalamine is achieved by Isocratic elution. The injection volume is 7 µl and flow rate is 0.5 mL/min. The detection is carried out at 200 nm and column oven temperature is set at 50°C. The stress degraded samples and the solution stability samples are analyzed using a PDA detector covering the range of 195-400 nm.

5.6.9 Method Validation

The RP-UPLC method described herein has been validated for Assay determination of Aniline.
5.6.9.1 System suitability

System suitability parameters are measured so as to verify the system performance. Standard preparation is considered for the system suitability. All important characteristics including capacity factor, peak tailing and theoretical plate number are measured. The similarity factor for the peak of aniline in the duplicate standard preparation is measured. Related standard deviation for the peak areas of aniline for five replicate (standard solution) injections is also measured. All these system suitability parameters covered the system, method and column performance.

5.6.9.2 Specificity

Forced degradation studies are performed to demonstrate selectivity and stability indicating capability of the proposed method. The powdered sample of tablets is exposed to acidic [1N HCl (5 mL), 60°C, 6h], alkaline [1N NaOH (5 mL), 60°C, 6h], strong oxidizing [6% w/v H₂O₂ (5 mL), 60°C, 6h], hydrolysis [water (5 mL), 60°C, 12h] and photolytic [1.2 million Lux hours] degradation conditions. Also, placebo of tablets is exposed to above all stress conditions to identify source of degradation peaks. The entire exposed samples are analyzed by the proposed method with photodiode array detector.

5.6.9.3 Precision

Precision is investigated using sample preparation procedure for six real samples (with spiked impurity in standard 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 and 100% of standard concentration level. The mean of percentage impurity (n=6) and the relative standard deviation is calculated for aniline impurity.

5.6.9.4 Accuracy

To confirm the accuracy of the proposed method, recovery experiments are carried out by standard addition technique. Six different levels (LOQ, 50%, 70%, 100%, 130% and 150%) of impurity
standard are added to pre-analyzed tablet samples in triplicate. The percentage recoveries of aniline at each level (LOQ to 150% of standard concentration) and each replicate are determined. The mean of percentage recoveries (n=18) and the relative standard deviation is calculated.

5.6.9.5  **Limit of detection (LOD) and limit of quantification (LOQ)**

The LOD and LOQ of aniline impurity are determined by using signal to noise ratio method as defined in International Conference on Harmonization (ICH) guideline [46]. Increasingly dilute solution of aniline impurity is injected into the chromatograph and signal to noise (S/N) ratio is calculated at each concentration.

5.6.9.6  **Linearity**

Linearity is demonstrated from LOQ to 150% of standard concentration using minimum seven calibration levels (LOQ, 25%, 50%, 75%, 100%, 125% and 150%) for aniline impurity standard. The method of linear regression is used for data evaluation. Peak area of standard compound is plotted against respective concentrations. Linearity is described by regression equation, correlation coefficient and Y-intercept bias.

5.6.9.7  **Robustness**

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions is studied by testing influence of small changes in flow rate (± 0.05 mL/min), column oven temperature (45°C to 55°C) and pH of buffer (pH 5.9 to pH 6.1). System suitability parameters are measured for above all experiments.

5.6.9.8  **Stability of standard and sample preparation**

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 impurity standard in pre-analyzed tablet sample. Standard and sample solutions are re-analyzed in between duration and after 24h. Percentage difference is calculated
against fresh injected sample solution and system suitability requirements are measured for the standard preparation.

5.6.9.10 Filter compatibility

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

5.7 RESULTS AND DISCUSSION

5.7.1 Method Development and Optimization

The important criteria for development of successful RP-UPLC method for determination of aniline from the mesalamine drug product are: the method should be able to determine aniline in single run and it should be accurate, reproducible, robust, stability indicating, free of interference from blank/placebo/degradation product/other impurities and straightforward enough for routine use in quality control laboratory testing.

To develop the stability indicating method, first the retention behavior of aniline compound with change in percentage of organic solvent (acetonitrile) and with change in pH of buffer is studied on Reprosil Gold 100 C18-XBD column (50 mm x 2.0 mm, 1.8 µ). 1-octane sulphonic acid sodium salt ion pair reagent is used in buffer preparation to improve the peak shape and avoid the other substances co-elution at the same retention time in RP chromatography. The buffer pH 6.0 is found more appropriate for robust peak shape and RT (retention time) performance of interested aniline substance. The final isocratic run is chosen with regards to the peak resolution and analysis time (total run time 5min for one injection) as well. The flow rate of 0.5 mL/min is optimized with regard to the back pressure and analysis time as well. Detection wavelength 200 nm is selected for
aniline due to higher detector response at this nm. Diluent is optimized based on solubility of aniline and mesalamine.

5.7.2 Analytical Parameters and Validation

After satisfactory development of method it is subjected to method validation as per ICH guideline [49]. The method is validated to demonstrate that it is suitable for its intended purpose by performing system suitability, accuracy, precision, linearity, robustness, ruggedness, solution stability, LOD, LOQ, filter compatibility and stability indicating capability.

5.7.2.1 System suitability

The percentage RSD of area count of six replicate injections is below 2.0%. The results of system suitability are presented in Table 5.2. Low values of % RSD of replicate injections indicate that the system is precise. Results of other system suitability parameters such as capacity factor, tailing factor, similarity factor (between two standard preparation), and theoretical plates are presented in Table 5.2. As seen from this data, the acceptable system suitability parameters are: relative standard deviation of replicate injections is not more than 1.5, similarity factor should be in between 0.95 to 1.05 and theoretical plates are not less than 5000. Overlaid chromatograms of replicate standard injections are presented in Figure 5.4. Results of system suitability parameters from different studies are presented in Table 5.2.
Table 5.2  System suitability results

<table>
<thead>
<tr>
<th>Condition</th>
<th>USP tailing factor</th>
<th>USP theoretical plates</th>
<th>Capacity factor</th>
<th>Similarity factor of standard</th>
<th>% RSD of standard area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>1.06</td>
<td>7643</td>
<td>9.2</td>
<td>1.02</td>
<td>1.5</td>
</tr>
<tr>
<td>Intermediate Precision</td>
<td>1.06</td>
<td>7825</td>
<td>9.2</td>
<td>1.03</td>
<td>1.2</td>
</tr>
<tr>
<td>At 0.45mL/min flow rate</td>
<td>1.10</td>
<td>7596</td>
<td>10.3</td>
<td>0.96</td>
<td>0.9</td>
</tr>
<tr>
<td>At 0.55mL/min flow rate</td>
<td>1.10</td>
<td>8386</td>
<td>8.3</td>
<td>0.97</td>
<td>1.7</td>
</tr>
<tr>
<td>At 45°C Column oven temp.</td>
<td>1.13</td>
<td>7411</td>
<td>9.7</td>
<td>1.01</td>
<td>0.8</td>
</tr>
<tr>
<td>At 55°C Column oven temp.</td>
<td>1.04</td>
<td>7704</td>
<td>8.8</td>
<td>0.98</td>
<td>0.9</td>
</tr>
<tr>
<td>With Buffer pH 5.9</td>
<td>1.03</td>
<td>7740</td>
<td>9.2</td>
<td>1.02</td>
<td>1.2</td>
</tr>
<tr>
<td>With Buffer pH 6.1</td>
<td>1.12</td>
<td>7799</td>
<td>9.2</td>
<td>1.01</td>
<td>1.3</td>
</tr>
</tbody>
</table>

USP = United state pharmacopeia

5.7.2.2  Specificity

Typical overlaid chromatograms are presented in Figure 5.5, which show separation of aniline compound from blank and placebo. Overlaid specimen chromatograms of standard, drug product (sample) and spiked sample (aniline spiked in drug product) are presented in Figure 5.6. No interference is observed at the RT of aniline due to blank and placebo (without mesalamine drug). The standard chromatogram with its purity plot is presented in Figure 5.7. In all the degradation conditions, no interference is observed at the RT of aniline [Figure 5.8]. It’s indicating that method is specific for the aniline substance in mesalamine drug product. Therefore, the method is specific and suitable for routine work.

[Figure 5.5   Overlaid specimen chromatograms of specificity study]
Figure 5.6  Overlaid specimen chromatograms of standard, sample and spiked drug product preparation

Figure 5.7  Standard chromatogram with its purity plot
5.7.2.3 Precision

The average % impurity (n=6) and % RSD results are shown in Table 5.3. Precision (at LOQ and at 100% level) results are shown in Table 5.3 along with intermediate precision data. Low values of RSD, indicates that the method is precise. Overlay chromatograms of precision at LOQ and precision at 100% are presented in Figure 5.9 and 5.10.
5.7.2.4 Accuracy

The accuracy by recovery is performed on aniline impurity, from LOQ to 150% of standard concentration. Recovery of aniline is performed in triplicate for each level. The results of recoveries (LOQ, 50%, 70%, 100%, 130% and 150%) for aniline are shown in Table 5.4. 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 the tablets. Overlay chromatograms of accuracy are presented in Figure 5.11.

Table 5.4 Accuracy results for aniline

<table>
<thead>
<tr>
<th>Aniline</th>
<th>Recovery (triplicate determination at each level)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At LOQ</td>
</tr>
<tr>
<td>Mean %</td>
<td>106.75</td>
</tr>
<tr>
<td>% R.S.D.*</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* Determined on three values.
5.7.2.5 LOD and LOQ

The concentration (in µg/mL) 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 [49]. The LOD and LOQ result of aniline substance is presented in Table 5.5.

Table 5.5  Limit of detection and limit of quantification

<table>
<thead>
<tr>
<th>Aniline</th>
<th>Concentration (ng/mL)</th>
<th>% Impurity (w.r.t. 5-ASA)</th>
<th>Signal to Noise Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>5.5</td>
<td>0.000018</td>
<td>2.9</td>
</tr>
<tr>
<td>LOQ</td>
<td>18</td>
<td>0.00006</td>
<td>10.4</td>
</tr>
</tbody>
</table>

5.7.2.6 Linearity

The response is found linear for aniline from LOQ to 150% of standard concentration. This test is performed on seven different levels of aniline solution, which gave us a good confidence on analytical method with respect to linear range. For aniline substance the correlation coefficient is greater than 0.999. Correlation coefficients and linearity equations of impurity and Y- intercept bias
are presented in Table 5.6. Overlaid linearity chromatograms and linearity curves are presented in Figure 5.12 and 5.13 respectively.

**Table 5.6 Linearity results (determined on LOQ, 25%, 50%, 75%, 100%, 125% and 150%)**

<table>
<thead>
<tr>
<th>Substance</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>Aniline</td>
<td>0.018 to 0.225</td>
<td>0.9998</td>
<td>( y = 155496(x) - 429.33 )</td>
<td>-1.865</td>
</tr>
</tbody>
</table>

[Figure 5.12  Overlaid specimen chromatograms of linearity study]

\[ y = 155496x - 429.33 \]
\[ R^2 = 0.9998 \]

[Figure 5.13  Linearity curve of aniline impurity]
5.7.2.7 Robustness

No significant effect is observed on system suitability parameters such as tailing factor, capacity factor and theoretical plates of aniline compound, when small but deliberate changes are made to chromatographic conditions. The results are presented in Table 5.2 along with system suitability parameters. Thus, the method is found to be robust with respect to variability in variable conditions.

5.7.2.8 Stability of standard and sample solution

Sample solution does not show any appreciable change in impurity value when stored at ambient temperature up to 24h. Percentage impurity result of 12h and 24h is compared with freshly prepared sample solution. Percentage difference in impurity is calculated with respect to freshly injected sample solution. Results are presented in Table 5.7. Standard solution does not show any unknown peak during this 24h study and also fulfill the requirements of system suitability. Standard solution stability results are presented in Table 5.8.

**Table 5.7 Sample solution stability results**

<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>Spl-1</td>
<td>0.000505</td>
<td>0.000506</td>
<td>0.000001</td>
</tr>
<tr>
<td>Spl-2</td>
<td>0.000502</td>
<td>0.000500</td>
<td>0.000002</td>
</tr>
<tr>
<td>Aniline</td>
<td>0.000496</td>
<td>0.000498</td>
<td>0.000009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.000004</td>
</tr>
</tbody>
</table>

**Table 5.8 Standard solution stability results**

<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>Aniline</td>
<td>22449</td>
<td>0.78</td>
<td>22446</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, 12h and 24h standard injections. (% RSD of seven Inj.)
5.7.2.9 Filter compatibility

Filter compatibility of PVDF and Nylon syringe filter (0.2 µ) is performed on duplicate sample preparation. Filtered sample solution does not show significant changes in aniline percentage with respect to centrifuged samples. Difference in impurity percentage result is presented in Table 5.9. In displayed result difference in % of impurity is not observed more than 0.000007%, which indicates that both syringe filter having a good compatibility with sample solution. Overlay chromatograms are presented in Figure 5.14.

Table 5.9 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>Spl-1</td>
<td>Spl-2</td>
<td>Spl-1</td>
<td>Spl-2</td>
</tr>
<tr>
<td>Aniline</td>
<td>0.000503</td>
<td>0.000493</td>
<td>0.000498</td>
</tr>
</tbody>
</table>

[Figure 5.14 Overlaid specimen chromatograms of filter compatibility study]
5.8 CALCULATION FORMULA

5.8.1 Impurity (% w/w)

Calculated the quantity, in mg, of Aniline in mesalamine dosage form, using the following formula:

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

Where,

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

5.8.2 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,

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

5.8.3 Accuracy (% Recovery)

It is calculated using the following equation:

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

A novel RP-UPLC method is successfully developed and validated for determination of aniline impurity from mesalamine drug product. The total run time is 5min, within which drug and their degradation products are separated from aniline. Method validation results have proved that the method is selective, precise, accurate, linear, rugged, robust and stability indicating with low LOD and LOQ. Sample solution stability and filter compatibility is also established. This method can successfully be applied for routine analysis and stability study of mesalamine delayed release drug product. Thus, the method provides high throughput solution for determination of aniline in the mesalamine delayed-release formulation with excellent selectivity, precision and accuracy. This method can also be applied for quantifying the trace levels of aniline from drug substances, drug products and different type of samples.

Note: Intellectual Property Management (IPM) clearance number for the present research work is: PUB-00041-10.
5.10 REFERENCES


Mesalazine, “*British Pharmacopeia*” BP-2010, Page 2894.


A recent FDA guideline (July 2009) on impurities in generic drugs would effectively reduce the qualification threshold to 1000 ppm or 1 mg/day for new impurities which were not present in the reference listed drugs.


FDA center for drug evaluation and research, “Genotoxic and carcinogenic impurities in drug substances and products” Recommended approaches, Guidance for industry (Draft); 03 December 2008.


Snodin DJ, “EU guideline on genotoxic impurities needs updating” RAJ Pharma, 2008; Part I September (593-598) and Part II October (663-670).


