



Evaluation of Pharmaceutical Quality of Mesalamine Delayed Release Tablets Using a New High Sensitivity Reversed-Phase UPLC Method for its Genotoxic/Aniline Impurity

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Received 16 May 2010; Revised 25 August 2010; Accepted 20 October 2010

Abstract: A reversed phase ultra performance liquid chromatography (UPLC) method was developed and validated for the quantification of aniline in mesalamine delayed-release tablets. The optimization of the experimental condition was carried out considering some important requirements like, detection limit, short run time and reproducibility. In the present study, isocratic reversed-phase UPLC method was developed for determination and separation of aniline from the drug product. The drug and impurity are well separated by using a reversed phase (Reprosil Gold C18-XBD) column and mobile phase comprising of buffer pH 6.0 and acetonitrile in the ratio of 90:10 v/v. Other UPLC parameters which were optimised are flow rate, 0.5 mL/min; detection wavelength, 200 nm; column oven temperature, 50 °C and injection volume 7 µL. Stability indicating capability was also established by forced degradation experiments. The method was validated as per ICH guideline. LOQ (limit of quantification) concentration (18 ng/mL) was found precise with RSD of less than 2%. In essence, the present study provides an improved low detection limit and lower run time for evaluation of pharmaceutical quality of mesalamine delayed-release formulation. Moreover, the developed method was also successfully applied for quantification of aniline in mesalamine delayed-release formulation. The same method can also be used for determination of aniline from drug substances.

Keywords: Mesalamine, 5-Aminosalicylic acid, Aniline, Genotoxicity, UPLC.

Introduction

Mesalamine (5-aminosalicylic acid, 5-ASA), a therapeutically active moiety of Sulfasalazine¹⁻³ 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^{4,6}. Mesalamine formulations which are able to deliver the intact drug to the lower intestine are nowadays successfully used⁷⁻⁸. 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. The chemical structure and UV spectrum of mesalamine and aniline are shown in Figure 1.

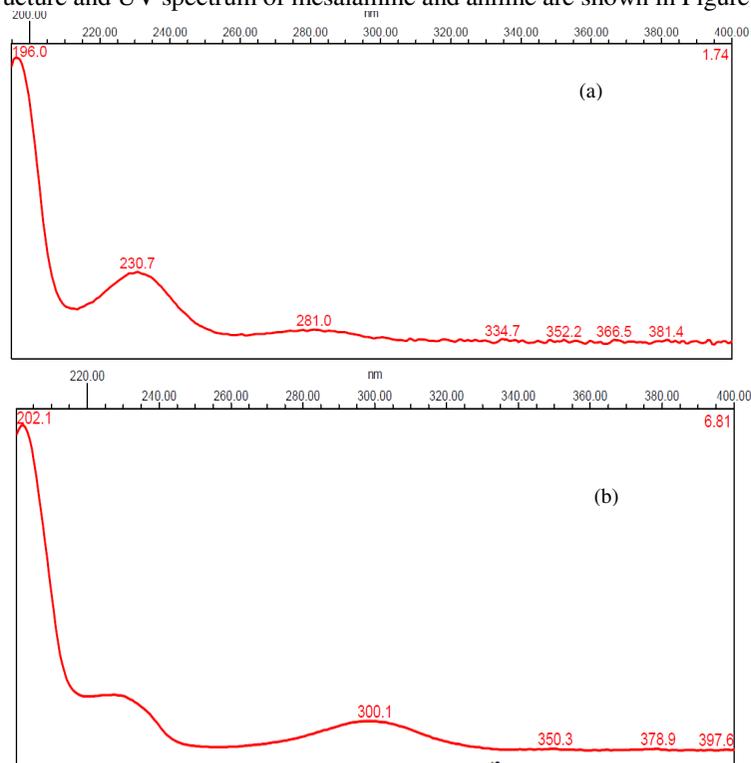


Figure 1. UV spectra of (a) aniline and (b) mesalamine

Moreover, recently the US-FDA has also published draft guidance for 'the genotoxic and carcinogenic impurities in drug substances and drug products'⁹. 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 was reported¹⁰ by Ernst and Bernd. Potent genotoxicity of aminophenylnorharman, formed from aniline in the liver of gpt delta transgenic mouse was reported¹¹ by Ken-ichi Masumura *et al.* Carcinogenicity of aminophenylnorharman, formed from aniline in F344 rats had been reported¹² by Toshihiko Kawamori *et al.* A few analytical methods has also been reported for quantification of aniline such as spectrometric¹³, azo-dye formation¹⁴, fluorescence^{15,16}, alternating-current oscillopolarographic titration¹⁷, NIR spectroscopy¹⁸, GC¹⁹, HPLC^{20,21} and LCMS^{22,23}.

Aniline determination in mesalamine drug substance by GC is official in US Pharmacopeia²⁴ and by HPLC is official in British Pharmacopeia²⁵. The comprehensive literatures 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 have lack of 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 tables. Thus, the aim of the present work was to develop and validate a reversed phase UPLC method with lower LOQ for the quantification of aniline in delayed release mesalamine formulation.

Experimental

Mesalamine delayed-release tablets, placebo and aniline impurity standard (purity 98.7%) were provided by Dr. Reddy's Laboratory Ltd., Hyderabad. Acetonitrile of HPLC grade was obtained from J.T.Baker (NJ., USA), HPLC grade 1-octane sulphonic acid sodium salt was obtained from RANKEM (RFCL Ltd., Delhi, India), di-potassium hydrogen orthophosphate purified, GR grade orthophosphoric acid, GR grade sodium hydroxide pellets and GR grade hydrochloric acid were obtained from Merck (Mumbai, India). 0.2 μm nylon 66 membrane filter and 0.2 μm nylon syringe filter was purchased from Pall life science ltd. (India). 0.2 μm PVDF syringe filter was purchased from Millipore (India). Ultra pure water (resistivity 18.2M Ω cm) generated by a Milli-Q system (Millipore).

Buffer preparation

1.74 g of di-potassium hydrogen orthophosphate (K_2HPO_4) and 2.2 g of 1-octane sulphonic acid sodium salt was dissolved in one litre of milli-Q water. The pH of this solution was adjusted to 6.0 with orthophosphoric acid and then filtered through 0.2 μm pall nylon membrane filter. The buffer preparation was found stable with respect to pH and visual clarity up to 48 h.

Chromatographic system

Analyses were performed on AcquityTM 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 were accomplished using Waters Empower-2TM chromatography data software. The chromatographic condition was optimized using a column Reprosil Gold 100, C18-XBD 50 mm x 2.0 mm, 1.8 μm . Mobile phase was a mixture of buffer (pH 6.0) and acetonitrile at a ratio of 900:100 (v/v) respectively. It was filtered through 0.2 μm nylon filter and degassed before use. The separation of aniline from mesalamine was achieved by isocratic elution. The injection volume was 7 μL and flow rate was 0.5 mL/min. The detection was carried out at 200 nm and column oven temperature was set at 50 °C. The stress degraded samples and the solution stability samples were analyzed using a PDA detector covering the range of 195-400 nm. 1N hydrochloric acid and 1N sodium hydroxide solution in the ratio of 50:50 (v/v) was mixed and used as a diluent.

Impurity standard solution preparation

Impurity standard solution was prepared by dissolving standard substances in methanol to obtained solution containing 7.5 $\mu\text{g/mL}$ of aniline in methanol. Five millilitres of 7.5 $\mu\text{g/mL}$ of aniline solution was transferred to 250 mL volumetric flask, diluted to volume with the diluent and mixed well.

Sample solution preparation

Twenty tablets were crushed to fine powder. An accurately weighed portion of the powder equivalent to 750 mg of mesalamine was taken in 25 mL volumetric flask. About 12.5 mL of 1N hydrochloric acid was added to this flask and sonicated in an ultrasonic bath for 20 min.

This solution was then diluted up to the mark with 1N sodium hydroxide solution and mixed well. It was then filtered through 0.2 μ PVDF syringe filter and the filtrate was collected after discarding first few millilitres.

Method validation

System suitability

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

Specificity

Forced degradation studies were performed to demonstrate selectivity and stability indicating capability of the proposed method. The powdered sample of tablets was exposed at the following conditions; acidic (1N HCl, 60 °C, 6 h.), alkaline (1N NaOH, 60 °C, 6 h.), strong oxidizing (6% H₂O₂, 60 °C, 6 h.), hydrolysis (water, 60 °C, 6 h.), thermal (105 °C, 12 h.), humidity (25 °C, 90 % RH, 7 days), sunlight (for 7 days), UV light (short and longer wavelengths). Also, placebo of tablets was exposed to above all stress conditions to identify source of degradation peaks. The entire exposed samples were analyzed by the proposed method with photo diode array detector.

Precision

Precision was 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 was performed with different column, different instrument and different day by another analyst. Precision was also performed at LOQ and 100% of standard concentration level. The mean of percentage impurity (n=6) and the relative standard deviation was calculated for aniline impurity.

Accuracy

To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique. Six different levels (LOQ, 50%, 70%, 100%, 130% and 150%) of impurity standard were 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 were determined. The mean of percentage recoveries (n=18) and the relative standard deviation was calculated.

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

The LOD and LOQ of aniline impurity were determined by using signal to noise ratio method as defined in International Conference on Harmonization (ICH) guideline²⁶. Increasingly dilute solution of aniline impurity was injected into the chromatograph and signal to noise (S/N) ratio was calculated at each concentration.

Linearity

Linearity was 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 was used for data evaluation. Peak area of standard compound was plotted against respective concentrations. Linearity was described by regression equation, correlation coefficient and Y-intercept bias.

Robustness

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was 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 were measured for above all experiments.

Stability of standard and sample preparation

Stability of standard and sample solution was established by storage of sample solution (duplicate preparation) and standard solution at ambient temperature for 20 h. Sample solution stability was demonstrated by spiking impurity standard in pre-analyzed tablet sample. Standard and sample solutions were re-analyzed in between duration and after 20 h. Percentage difference were calculated against fresh injected sample solution and system suitability requirements were measured for the standard preparation.

Filter compatibility

Filter compatibility was 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 were carried out by sample filtration technique. The working concentration level impurity standard was added to pre-analyzed tablet sample in duplicate. Spiked impurity samples were filter through both syringe filter and percentage difference was calculated against centrifuged sample.

Results and Discussion

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 behaviour of aniline compound with change in percentage of organic solvent (acetonitrile) and with change in pH of buffer was studied on Reprosil Gold100 C18-XBD column (50 mm x 2.0 mm, 1.8 μ). 1-octane sulphonic acid sodium salt ion pair reagent was 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 was found more appropriate for robust peak shape and RT (retention time) performance of interested aniline substance. The final isocratic run was chosen with regards to the peak resolution and analysis time (total run time 5 min for one injection) as well. The flow rate of 0.5 mL/min was optimized with regard to the back pressure and analysis time as well. Detection wavelength 200 nm was selected for aniline due to higher detector response at this nm. Diluent was optimised based on solubility of aniline and mesalamine.

Analytical parameters and validation

After satisfactory development of method it was subjected to method validation as per ICH guideline²⁶. The method was 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.

System suitability

The percentage RSD of area count of six replicate injections was below 2.0%. The results of system suitability are presented in Table 1. 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 1. 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 0.9 to 1.1 and theoretical plates are not less than 5000. Overlay chromatogram of replicate standard injections are presented in Figure 2. Results of system suitability parameters from different studies are presented in Table 1.

Table 1. System suitability results

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

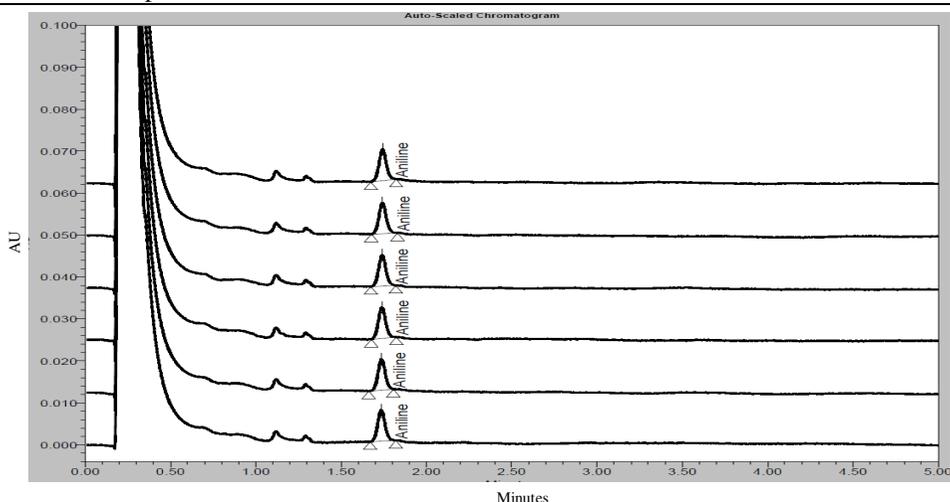


Figure 2. Overlay chromatograms of replicate standard injections.

Specificity

Typical overlaid chromatograms are presented in Figure 3, which show separation of aniline compound from blank and placebo. No interference was observed at the RT of aniline due to blank and placebo. In all the degradation conditions, no interference was observed at the RT of aniline. It indicates that method is specific for the aniline substance in mesalamine drug product. Therefore, the method is specific and suitable for routine work. The standard chromatogram with its 3D plot and purity plot is presented in Figure 4.

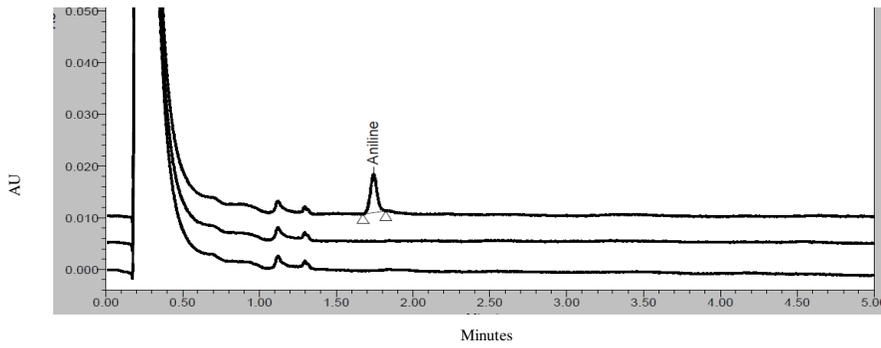


Figure 3. Overlay chromatogram of blank (bottom), placebo (middle) and standard preparation (top)

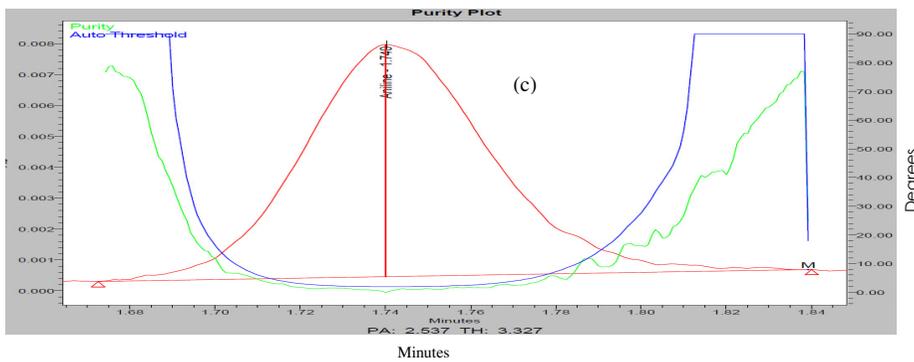
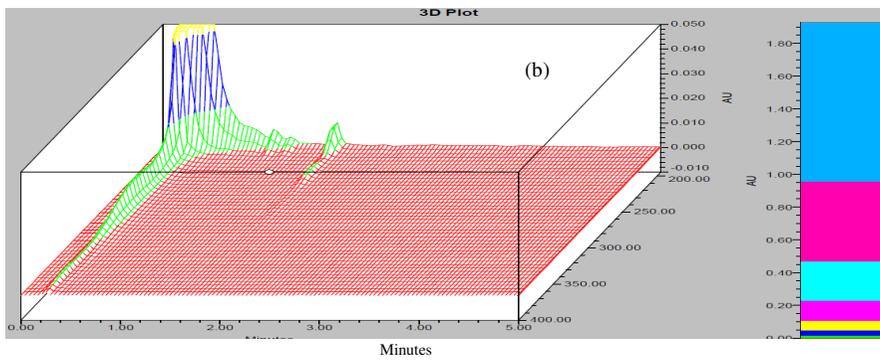
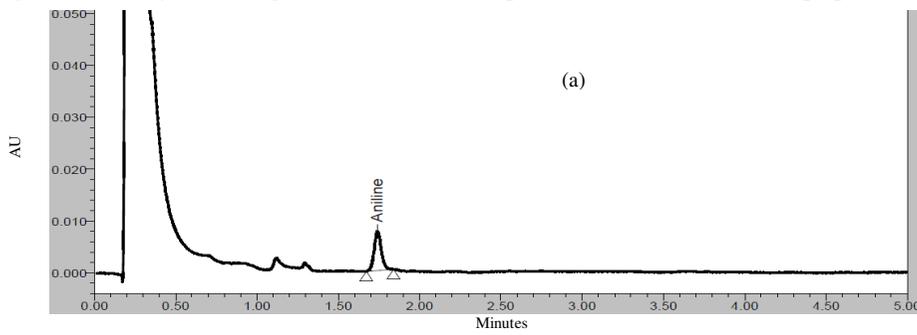


Figure 4. (a) Standard chromatogram (b) 3D plot and (c) purity plot

Precision

The average % impurity (n=6) and % RSD results are shown in Table 2. Precision (at LOQ and at 100% level) results are shown in Table 2 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 and 6.

Table 2. Precision at LOQ, precision at 100% and intermediate precision results[@]

Impurity	Precision at LOQ		Precision at 100%		Intermediate precision	
	%impurity [#]	%RSD [*]	%impurity [#]	%RSD [*]	%impurity [#]	%RSD [*]
Aniline	0.00005591	1.98	0.0005253	0.75	0.0005136	0.97

[#]Average of six determinations, ^{*}Determined on six values, [@]Demonstrated by spiking known impurities into sample.

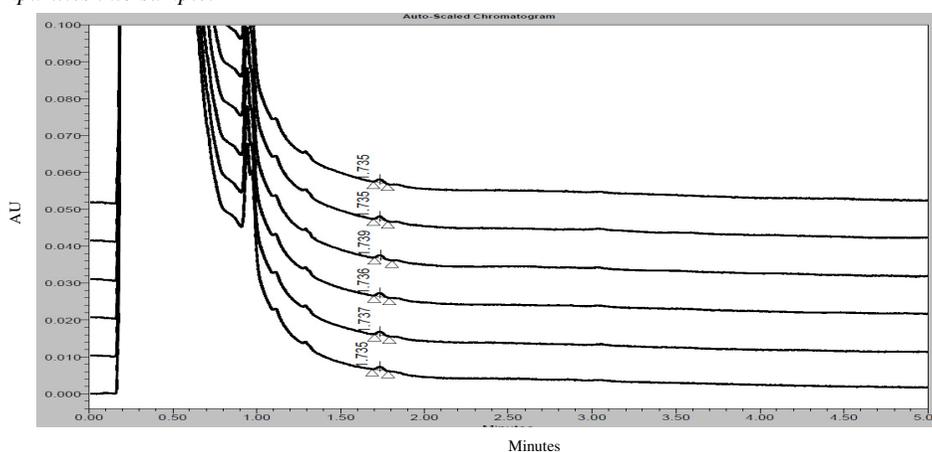


Figure 5. Overlay chromatograms of precision at LOQ level

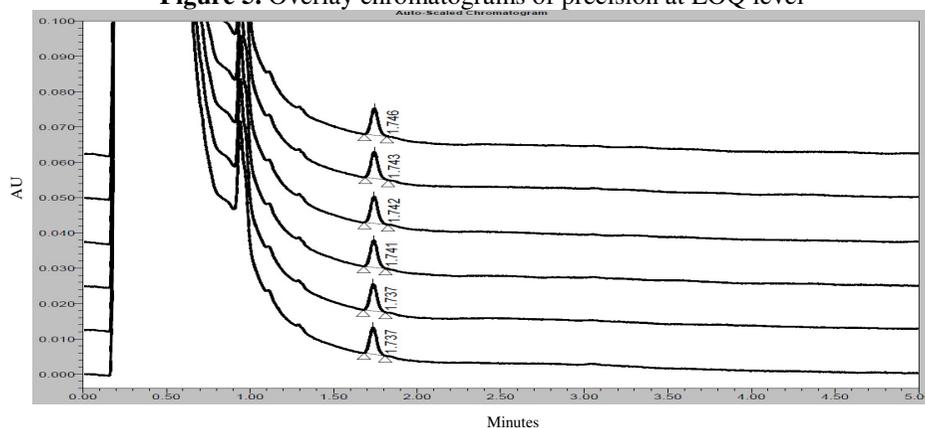


Figure 6. Overlay chromatograms of precision at 100% level

Accuracy by recovery

The accuracy by recovery was performed on aniline impurity, from LOQ to 150% of standard concentration. Recovery of aniline was performed in triplicate for each level.

The results of recoveries (LOQ, 50%, 70%, 100%, 130% and 150%) for aniline are shown in Table 3. The amount recovered was within $\pm 10\%$ 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 7.

Table 3. Accuracy results for aniline

Aniline	Recovery (With triplicate determination at each level)					
	at LOQ	at 50%	at 70%	at 100%	at 130%	at 150%
Mean %	106.75	102.24	98.20	99.42	97.14	97.04
% R.S.D*	0.96	1.14	1.57	0.50	0.76	0.11

* Determined on three values

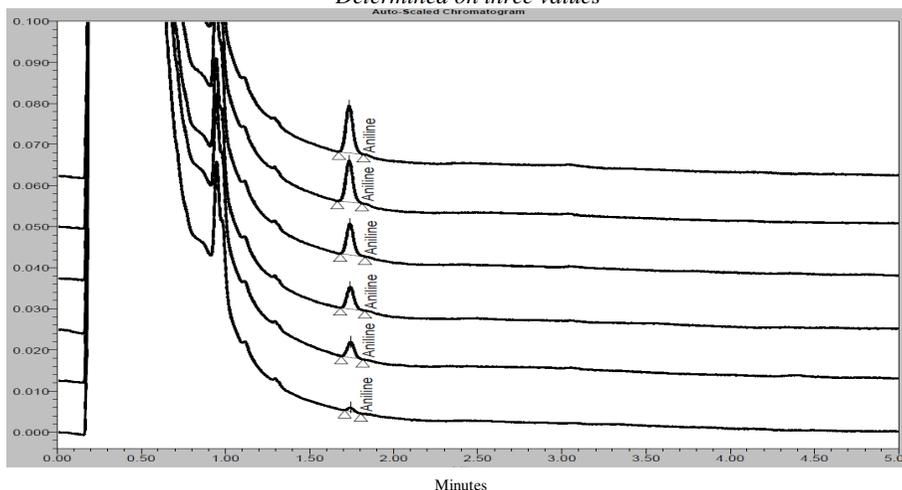


Figure 7. Overlay chromatogram of accuracy study at LOQ, 50, 70, 100, 130 and 150%.

LOD and LOQ

The concentration (in $\mu\text{g/mL}$) with signal to noise ratio of at least 3 was taken as LOD and concentration with signal to noise of at least 10 was taken as LOQ, which meets the criteria defined by ICH guidance²⁶. The LOD and LOQ result of aniline substance is presented in Table 4.

Table 4. Limit of detection and limit of quantification

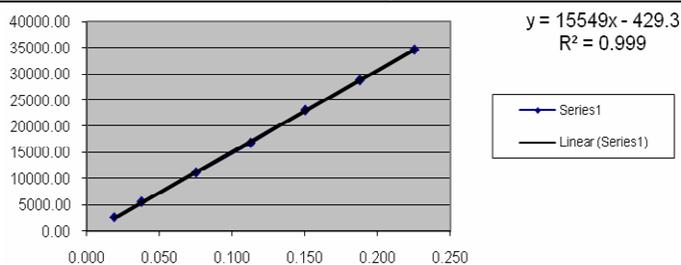
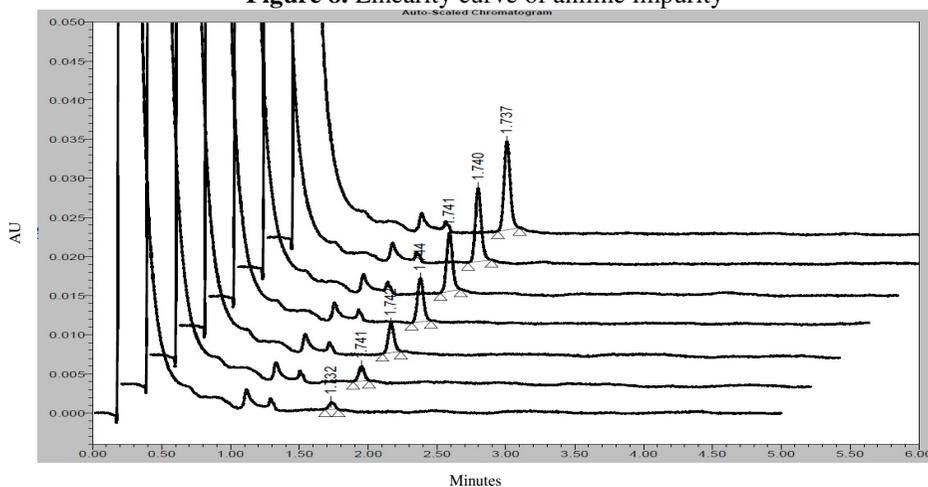
Aniline	Concentration (ng/mL)	% Impurity (w.r.t. 5-ASA)	Signal to Noise Ratio
LOD	5.5	0.000018	2.9
LOQ	18	0.00006	10.4

Linearity

The response was found linear for aniline from LOQ to 150% of standard concentration. This test was 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 was greater than 0.999. Correlation coefficients and linearity equations of impurity and Y- intercept bias are presented in Table 5. Linearity curve is presented in Figure 8 and overlay linearity chromatograms are presented in Figure 9.

Table 5. Linearity results (determined on LOQ, 25, 50, 75, 100, 125 and 150%)

Substance	Linearity range $\mu\text{g/mL}$	Correlation Coefficient (r^2)	Linearity (Equation)	Y- intercept bias
Aniline	0.018 to 0.225	0.9998	$y = 155496(x) - 429.33$	-1.865

**Figure 8.** Linearity curve of aniline impurity**Figure 9.** Overlay chromatograms of aniline linearity (LOQ to 150%)

Robustness

No significant effect was observed on system suitability parameters such as tailing factor, capacity factor and theoretical plates of respective component, when small but deliberate changes were made to chromatographic conditions. The results are presented in Table 1 along with system suitability parameters. No significant effect was observed on retention time (RT) of impurity, when deliberate changes were made to chromatographic condition. Thus, the method was found to be robust with respect to variability in variable conditions.

Stability of standard and sample solution

Sample solution did not show any appreciable change in impurity value when stored at ambient temperature up to 20 h. Percentage impurity result of 9 h and 20 h was compared with freshly prepared sample solution. Percentage difference in impurity was calculated with respect to freshly injected sample solution. Results are presented in Table 6. Standard solution did not show any unknown peak during this 20 h study and also fulfil the requirements of system suitability. Standard solution stability results are presented in Table 7.

Table 6. Sample solution stability results

Impurity	Initial Sample		Sample after 9 hours				Sample after 20 hours			
	Imp. in %		Imp. in %		Difference in %		Imp. in %		Difference in %	
	Sam-1	Sam-2	Sam-1	Sam-2	Sam-1	Sam-2	Sam-1	Sam-2	Sam-1	Sam-2
Aniline	0.000505	0.000502	0.000506	0.000500	0.000001	0.000002	0.000496	0.000498	0.000009	0.000004

Table 7. Standard solution stability results

Substance	Initial		After 9 h.		After 20 h.	
	Area	%RSD [#]	Area	%RSD [*]	Area	%RSD [@]
	Aniline	22449	0.78	22246	0.79	22356

[#]Determined on five replicate injections, ^{*}Determined on five initial and 9 h standard injections, (% RSD of six inj.) [@]Determined on five initial, 9 h and 20 h standard injections. (% RSD of seven inj.)

Filter compatibility

Filter compatibility of PVDF and nylon syringe filter (0.2 μ) was performed on duplicate sample preparation. Filtered sample solution was not shows any significant changes in aniline percentage with respect to centrifuged samples. Difference in impurity percentage result was presented in Table 8. In displayed result difference in % of impurity was 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 10.

Table 8. Filter compatibility results

Impurity	Centrifuged		PVDF syringe filter 0.2μ (Millipore)				Nylon syringe filter 0.2μ (Pall Life Sciences)			
	Imp. in %		Imp. in %		Difference in %		Imp. in %		Difference in %	
	Sam-1	Sam-2	Sam-1	Sam-2	Sam-1	Sam-2	Sam-1	Sam-2	Sam-1	Sam-2
Aniline	0.000503	0.000493	0.000498	0.000487	0.000005	0.000006	0.000496	0.000487	0.000007	0.000006

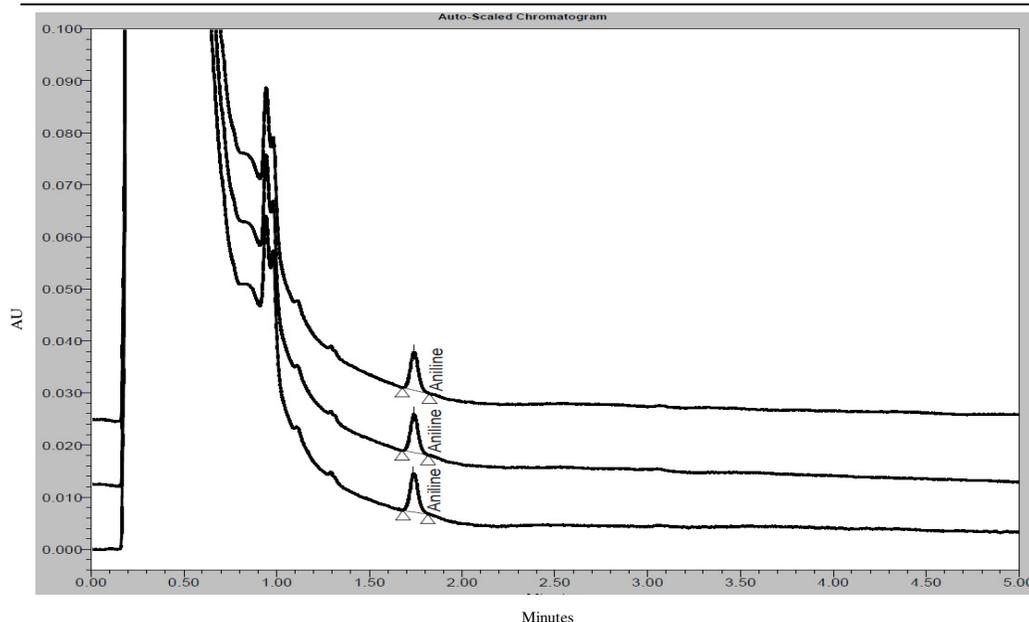


Figure 10. Overlay chromatograms of centrifuge, nylon and PVDF filtered sample

Conclusion

A novel RP-UPLC method was successfully developed and validated for determination of aniline impurity from mesalamine drug product. The total run time was 5min, within which drug and their degradation products were 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 was 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.

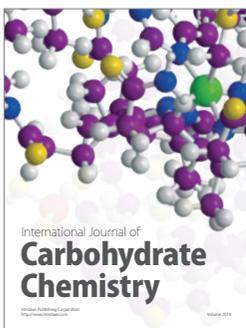
Acknowledgment

The authors would like to thank M/s Dr. Reddy's Laboratories Ltd. for supporting this work. Authors also would like to acknowledge Mr. Amit R. Kharkar for his immense support. All the development and validation work was performed at Analytical Research and Development (AR&D) Lab., Dr. Reddy's Laboratory Ltd., IPDO, Bachupally, Hyderabad, India.

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