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Determination of Mesalamine Related Impurities from Drug Product by Reversed Phase Validated UPLC Method

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Abstract: In the present study gradient reversed-phase UPLC method was developed for simultaneous determination and separation of impurities and degradation products from drug product. The chromatographic separation was performed on acquity UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 µm) using gradient elution. Other UPLC parameters which were optimised are flow rate, 0.7 mL/min; detection wavelength, 220 nm; column oven temperature, 40 °C; and injection volume 7 µL. Stability indicating capability was established by forced degradation experiments and separation of known degradation products. The method was validated as per International Conference on Harmonization (ICH) guideline. For all impurities and mesalamine, LOQ (limit of quantification) value was found precise with RSD (related standard daviation) of less than 2.0%. 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 successfully applied for quantification of impurities and degradation products in mesalamine delayed-release formulation. The same method can also be used for determination of related substances from mesalamine drug substance.

Keywords: Mesalamine, 5-Aminosalicylic acid, Stability indicating, UPLC, Validation.

Introduction

Mesalamine (5-aminosalicylic acid, 5-ASA), the therapeutically active moiety of sulfasalazine[1-3] 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[4].

Orally administrated mesalamine is rapidly and almost completely absorbed from the small intestine[5-7]. Formulations able to deliver the intact drug to the lower intestine are nowadays successfully used[8,9]. 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 spectrums of mesalamine and its six impurities are shown

in Figure 1. Mesalamine drug profile[10] and degradation mechanism in aqueous solution was reported[11]. Several articles for mesalamine metabolism[12,13] and its determination by HPLC[14-16] and HPLC-ESI-MS/MS[17] has been reported. Estimation of mesalamine and its metabolites in plasma and urine by HPLC[18-20] and by fluorescence detector[21] are also reported. Mesalamine HPLC determination in rectal tissue biopsies[22] and endoscopic intestinal biopsy in human has been reported[23]. Identification of unknown impurity in mesalamine was also reported[24,25].

Mesalamine protects against colorectal cancer in inflammatory bowel disease[26]. HPLC determination of mesalamine and related impurities using ion-pairing reagent[27] and by simple liquid chromatography[28] has been reported. Mesalamine formulation determination by spectrophotometric[29], by HPLC and ultraviolate[30] and by differential pulse voltammetry was reported[31]. Determination of mesalamine and its related impurity by micellar electrokinetic capillary chromatography has been reported[32]. Determination of mesalamine related impurities by micellar electrokinetic chromatography with an ion-pair reagent was reported[33]. Mesalamine determination in pharmaceutical dosage forms by HPLC, DPPH and nitrosation was also reported[34].

Literatures survey revealed that the mesalamine drug substance is official in US Pharmacopeia[37] as well as in British Pharmacopoeia[38]. Mesalamine extended release capsules[35] and mesalamine delayed-release tablets[36], formulation is also official in US Pharmacopoeia. In pharmacopeia 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. Further, mesalamine delayed-release tablet USP monograph having unknown single max limit 0.5% with the area percent method. Now a day 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[41]. 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[42]. Subsequent guidance from the U.S. Food and Drug Administration (USFDA)[41] 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 analytical method (with the low limit determination capability) is the first step to measure the quality of drug product.

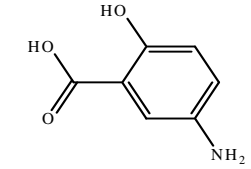
Comprehensive literatures for measure the quality of mesalamine delayed-release product by HPLC have long run time, limited solution stability, less efficiency (N), higher limit for unknown single maximum impurity, lack of related retention time for all impurities and less resolution, as such there is lack of a suitable procedure for the quantification and estimation for them. Therefore, the aim of the present work was 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.

Moreover, analytical column with sub 2-micron particle size technology was explored for better chromatography. Working concentration and RRT for all related substances was mentioned in Table 1.

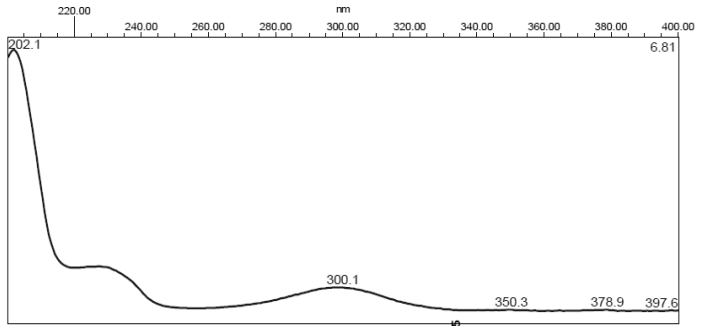
Table 1. Name of the impurities, working concentration (in % and µg/mL) and related retention time (RRT) with respect to mesalamine (5-ASA) are as follow.

Compound	Working concentration		RRT
	In µg/mL	In %	
Imp-A	3.2	0.2	0.135
Imp-B	3.2	0.2	0.334
Imp-C	3.2	0.2	0.914
5-ASA	1600	100	1.000
Imp-D	3.2	0.2	1.320
Imp-E	3.2	0.2	1.666
Imp-F	3.2	0.2	1.904

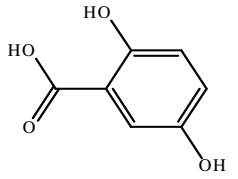
Mesalamine, Mesalazine, 5-Aminosalysilic acid, 5-ASA



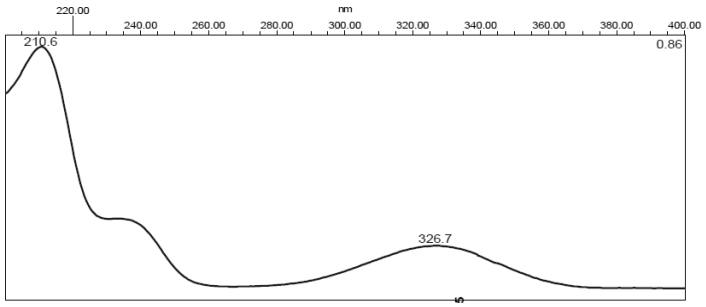
5-amino-2-hydroxybenzoic acid



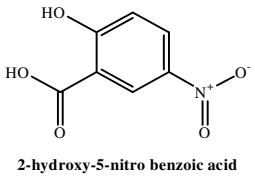
2,5-Dihydroxybenzoic acid; [Impurity-A]



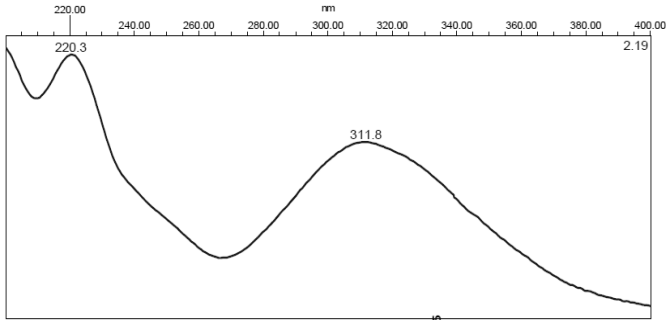
2,5-dihydroxy benzoic acid



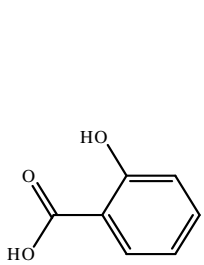
2-Hydroxy-5-nitrobenzoic acid; 5-nitrosalicylic acid; [Impurity-B]



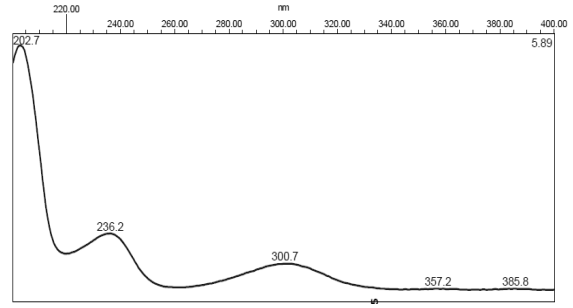
2-hydroxy-5-nitro benzoic acid



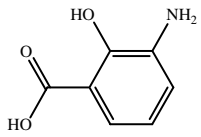
2-Hydroxybenzoic acid; Salicylic acid; [Impurity-C]



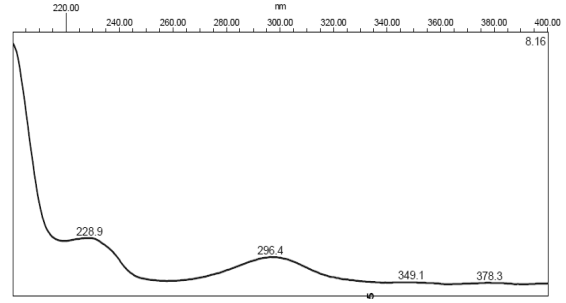
2-hydroxy benzoic acid



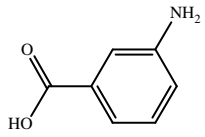
3-amino-2-hydroxybenzoic acid; 3-aminosalicylic acid; [Impurity-D]



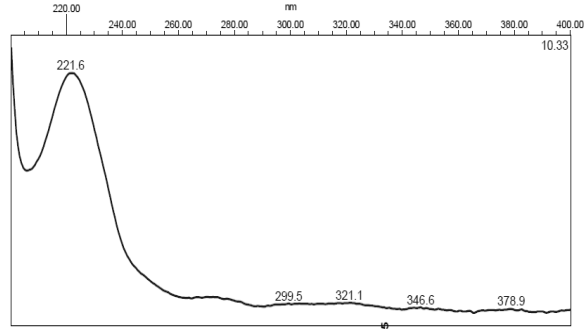
3-Amino salicylic acid



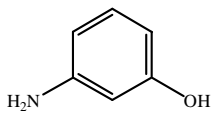
3-Aminobenzoic acid; [Impurity-E]



3-Amino benzoic acid



3-Aminophenol; [Impurity-F]



3-Amino phenol

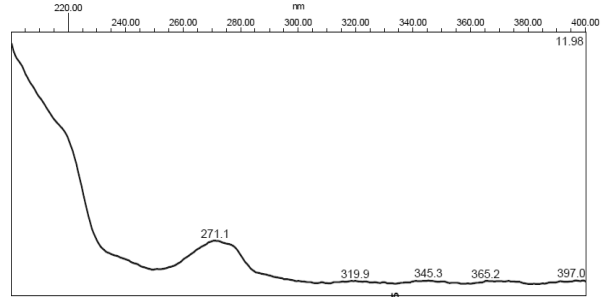


Figure 1. Chemical structures and UV spectrums of mesalamine and its six impurities

Experimental

Mesalamine delayed-release tablets, placebo of mesalamine tablets, mesalamine [5-ASA] USP reference standard, impurity-A to F, were provided by Dr. Reddy's laboratories Ltd., Hyderabad. HPLC grade acetonitrile and methanol were obtained from J.T.Baker (NJ., USA). HPLC grade 1-octane sulphonic acid sodium salt was obtained from RANKEM (RFCL Ltd., Delhi). Di-potassium hydrogen orthophosphate purified, potassium dihydrogen orthophosphate purified, GR grade orhtophosphoric acid 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 used was a product of Pall life science limited (India). 0.2 µm PVDF syringe filter used was manufactured by Millipore (India). High purity water was generated by using Milli-Q Plus water purification system (Millipore, Milford, MA, USA). All experiments were performed using 'A' class volumetric glassware and GLP compliance analytical laboratory.

Equipment

Cintex digital water bath was used for specificity study. Photo stability studies were carried out in a photo-stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

Buffer preparation

***pH 2.2 buffer preparation*:** 1.36 g of potassium dihydrogen orthophosphate and 5.5 g of 1-octane sulphonic acid sodium salt was dissolved in 890 mL of Milli-Q water. The pH of this solution was 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 g of dipotassium hydrogen orthophosphate and 5.5 g of 1-octane sulphonic acid sodium salt was dissolved in 890 mL of Milli-Q water. The pH of this solution was adjusted to 6.0 with orthophosphoric acid and then filtered through 0.2 µm nylon 66 membrane filter. Bench top stability at room temperature for the both buffer preparation was found stable with respect to pH and visual clarity up to 48 h.

Diluent preparation

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

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

Chromatographic system

Analyses were performed on 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 were accomplished using Waters Empower-2™ chromatography data software. The chromatographic condition was optimised using Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm) column. The buffer pH 2.2 was used as a mobile phase-A (M.P.-A). Mobile phase-B (M.P.-B) was a mixture of buffer pH 6.0, methanol and acetonitrile in the ratio of 890:80:30 (v/v/v) respectively. Mobile phase-A and mobile phase-B was filtered through 0.2 µm nylon membrane filter and degassed under vacuum prior to use. The separation of all impurities was achieved by gradient elution using M.P.-A and M.P.-B. The finally selected and optimized conditions were as follows: injection volume 7 µL, gradient elution (Table 2), 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 were analyzed using a PDA detector covering the range of 200-400 nm.

Table 2. Gradients program for elution of 5-ASA and all impurities

Time, min	Flow rate, mL/min	% M.P.-A	% M.P.-B	Gradient curve
Initial	0.7	90	10	Isocratic
3	0.7	90	10	Isocratic
13	0.7	10	90	Linear
13.1	0.7	90	10	Isocratic
15	0.7	90	10	Equilibration

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 was taken in 100 mL volumetric flask. About 6 mL of diluent-1 was added to this volumetric flask and sonicated in an ultrasonic bath for 10 min. This solution was then diluted up to the mark with diluent-2 and was mixed well. Three millilitres of this solution was transferred into 10 mL volumetric flask and diluted up to the mark with diluent-2 and was mixed well.

Standard solution preparation

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

Sample solution preparation

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

Placebo solution preparation

Tablets placebo powder was taken into 250 mL volumetric flask. About 15 mL of diluent-1 was added to this volumetric flask and sonicated in an ultrasonic bath for 10 minutes. This solution was then diluted up to the mark with diluent-2, mixed well. It was then filtered through 0.2 µm PVDF syringe filter and the filtrate was collected after discarding first few millilitres.

Method validation

The proposed method was validated as per ICH guidelines[39].

System suitability

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

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities[39,40].Forced degradation studies were performed to demonstrate selectivity and stability indicating capability of the proposed method. The sample solution was exposed to

acidic condition (2 N HCl, 60 °C, 6 h), alkaline condition (1 N NaOH, 60 °C, 1 h), strong oxidizing (6% H_2O_2, bench top for 2 h), Hydrolysis (water, 60 °C, 2 h). The powdered sample of tablets was exposed to UV light (short and longer wavelengths, 7 days), dry heat (105 °C, 12 h), humidity (25 °C, 90% RH, 7 days) degradation conditions. Also, placebo of the tablets was exposed to above all stress conditions to identify the source of degradation peak. The entire exposed samples were analyzed by the proposed method with PDA detector.

Precision

The precision of the related substances method verified by repeatability and by intermediate precision. Precision was 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 was performed with different column, different instrument, and different day by another analyst. Precision was 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 was also calculated for all substances.

Accuracy

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

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

The LOD and LOQ of mesalamine and all six impurities were determined (in placebo solution) by using signal to noise approach as defined in International Conference on Harmonization (ICH) guideline[39,40]. Increasingly dilute solution of drug and each 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 250% of working concentration by using minimum seven calibration levels for the mesalamine compound and all impurity standards. The method of linear regression was used for data evaluation. Peak area of 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 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.

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 24 h. Sample solution stability was demonstrated by spiking impurity standards in pre-analyzed tablet sample. Standard and sample solutions were re-analyzed after 12 h and after 24 h. For sample solution, percentage difference in impurities was calculated against fresh injected sample solution. Percentage RSD was calculated for standard preparation.

Filter compatibility

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

Results and Discussion

Method development

The important criteria for development of successful RP-UPLC method for determination of mesalamine related substances in delayed-release tablets were: 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 was studied on Waters Acquity BEH C18 column (50 mm x 2.1 mm, 1.7 µm). 1-Octane sulphonic acid ion pair reagent was 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 M.P.-A was found more appropriate for robust resolution, peak shape and RRT performance of all the interested substances. The final gradient run was chosen with regards to the peak resolution and analysis time as well. The gradient program is given in Table 2. The flow rate of 0.7 mL/min was optimized with regard to the back pressure and analysis time as well. Diluents concentration was optimized to improve the solution stability and peak shape. Detection wavelength 220 nm was selected for mesalamine and its related substances due to higher detector response at this wavelength. Thus, determination of impurities was possible in single run.

Analytical parameters and validation

After satisfactory development of method it was subjected to method validation as per ICH guideline[39]. The method was 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).

System suitability

The percentage RSD of mesalamine area count of six replicate injections was 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 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 3. Overlay chromatograms of replicate standard injection are presented in Figure 2.

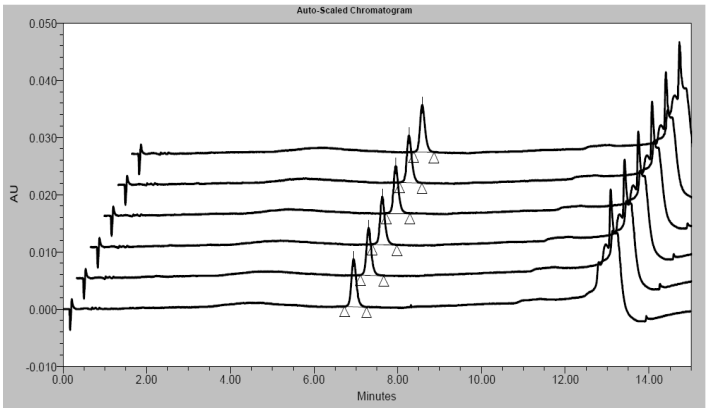


Figure 2. Overlay chromatograms of replicate standard injections

Table 3. System suitability results (precision, intermediate precision and robustness)

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

Specificity

Typical overlaid chromatograms are presented in Figure 3 and 4, 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. Chromatograms of base and peroxide degraded (with its 3D plot and purity plot) tablet samples are presented in Figure 5 and 6 respectively. Mesalamine was found to be stable under acid, heat, humidity, and light degradation condition. Further, spectra of unknown degradation products in tablet sample were similar to that of unknown degradation products of individual standards eluting at respective retention time. Also spectra of known impurities in degraded tablet sample were similar to its respective impurity standard substance, indicating that there was no co-elution of unknown degradation peak at retention times of respective known impurities. Peak due to mesalamine was investigated for spectral purity in the chromatogram of all exposed samples and found spectrally pure. The max plot of chromatograms degradation samples was 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 4.

Table 4. Forced degradation data and purity results of mesalamine (5-ASA)

Stress condition	5-ASA Purity flag	% Degradation
Refluxed with 2N HCl solution at 60 °C for 6 h.	No	0.33
Refluxed with 1N NaOH solution at 60 °C for 1 h.	No	5.05
6 % H_2O_2 solution bench top for 2 h.	No	4.30
Refluxed with water at 60 °C for 2 h.	No	0.22
Exposed to UV light.	No	0.35
Dry heated at 105 °C for about 12 h.	No	0.52
Exposed to humidity (25 °C, 90 %RH) about 7 days.	No	0.18

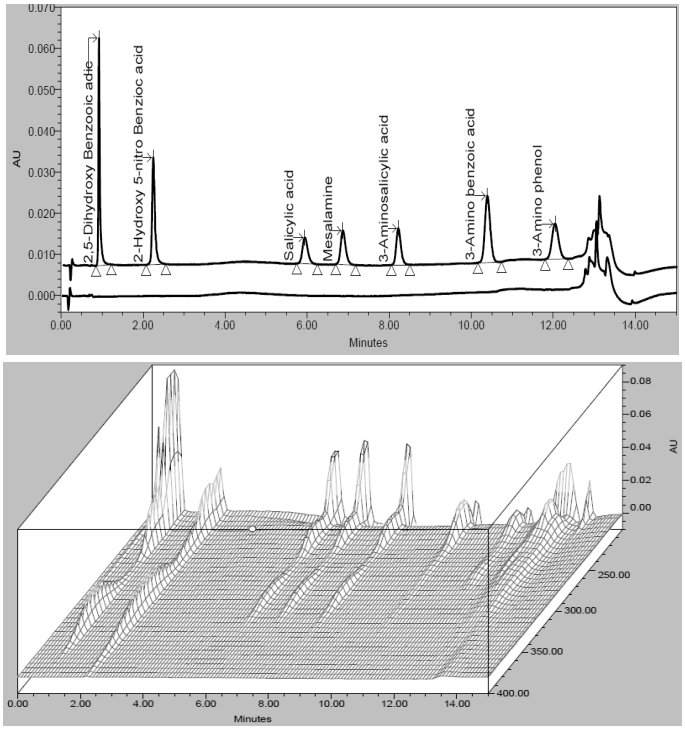


Figure 3. Overlay chromatograms of placebo (bottom) and spiked impurities (top) with its 3D plot

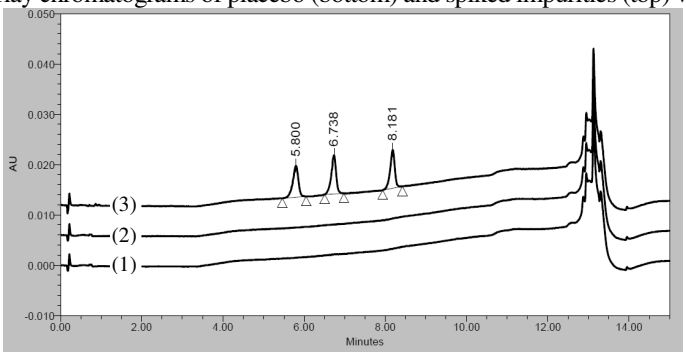


Figure 4. Overlay chromatograms of (1) blank; (2) placebo and (3) system suitability standard

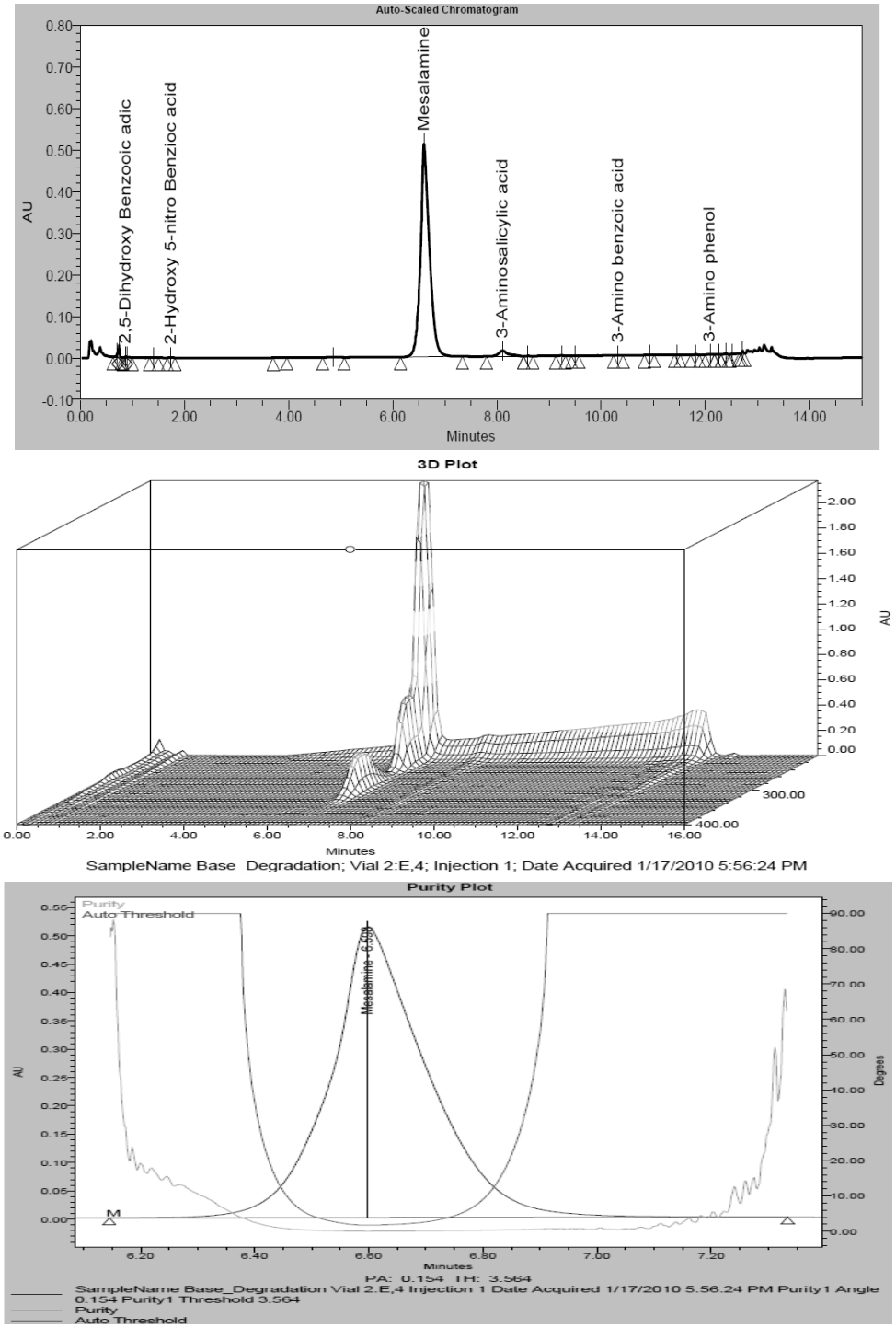


Figure 5. Chromatogram of alkali degraded tablet sample with its 3D plot and purity plot.

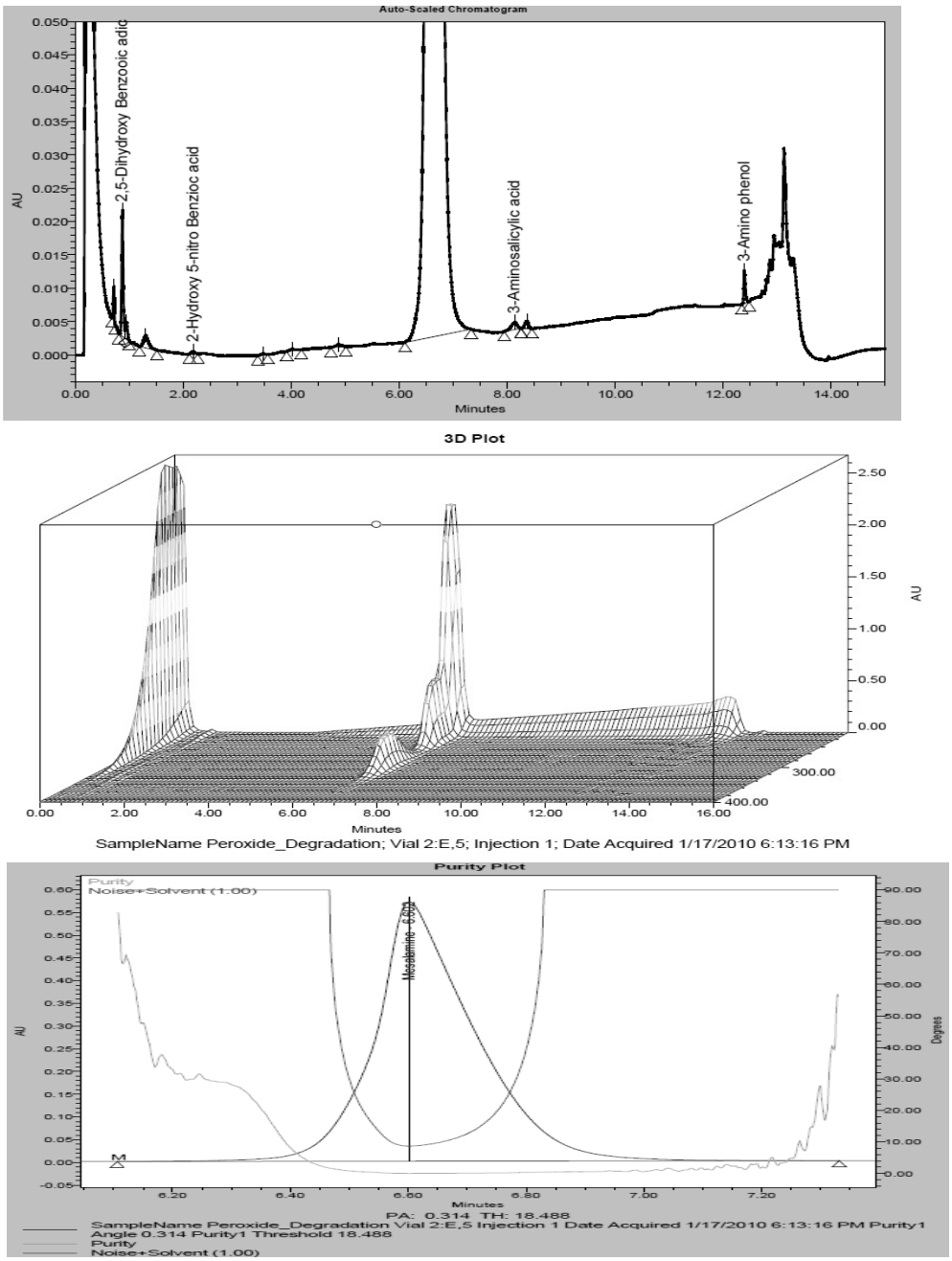


Figure 6. Chromatogram of peroxide degraded tablet sample with its 3D plot and purity plot.

Precision

Precision (at LOQ, 100% and 150%) results are shown in Table 5 along with intermediate precision data. Low values of RSD, indicates that the method is precise. Overlay chromatograms of precision at LOQ are presented in Figure 7.

Table 5. Precision at three levels (LOQ, 100% and 150%) and intermediate precision results[§]

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

[#] *Average of six determinations;* * *Determined on six values*

[§] *Demonstrated by spiking known impurities into sample*

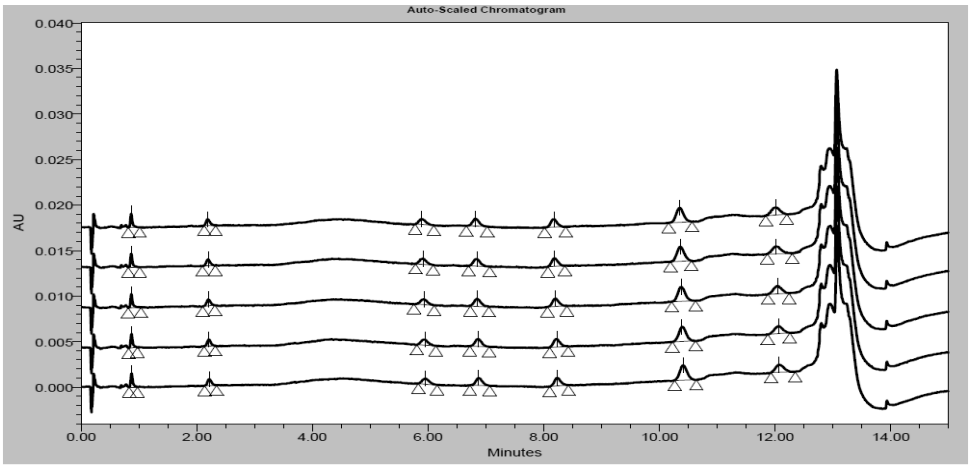


Figure 7. Overlay chromatograms of precision at LOQ level

Accuracy by recovery

The amount recovered was 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. Overlay chromatograms of accuracy are presented in Figure 8.

Table 6. Accuracy results

Substances	Mean % Recovery (With triplicate determination at each level)			
	at LOQ [#]	at 50% [#]	at 100% [#]	at 150% [#]
Imp-A	105.5	102.1	100.8	98.9
Imp-B	102.4	101.5	99.4	101.1
Imp-C	97.4	98.5	101.3	103.2
5-ASA	95.9	99.5	102.7	99.5
Imp-D	104.3	100.3	101.5	98.7
Imp-E	101.1	98.6	101.2	99.1
Imp-F	103.9	97.9	102.6	100.6

[#] *Average of three determinations*

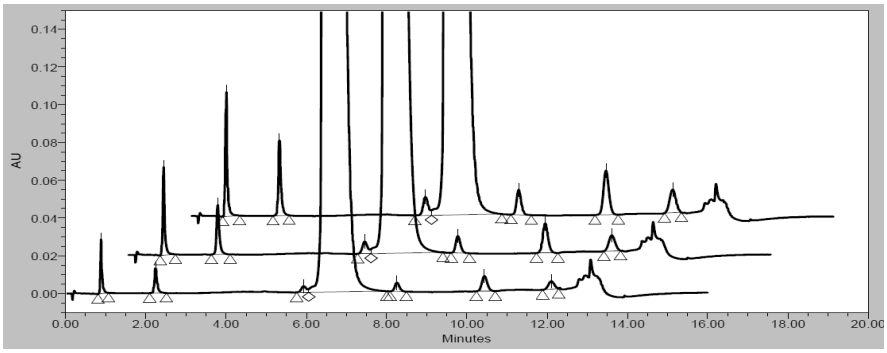


Figure 8. Overlay chromatograms of accuracy (at 50%, 100% and 150%)

LOD and LOQ

The concentration (in %) 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 guidelines[39,40]. The LOD and LOQ results of all substances are presented in Table 7. Precision result at LOQ was also presented in Table 5.

Table 7. Limit of detection and limit of quantification

Substances	In % (w.r.t. working conc.)		Signal to noise ratio	
	LOD	LOQ	LOD	LOQ
Imp-A	0.0018	0.006	2.9	10.4
Imp-B	0.0018	0.006	3.2	10.0
Imp-C	0.006	0.020	2.7	10.0
5-ASA	0.006	0.020	2.4	9.7
Imp-D	0.006	0.020	3.0	9.9
Imp-E	0.006	0.020	3.3	13.5
Imp-F	0.006	0.020	3.1	10.2

Linearity

The response was found linear for all substances from LOQ to 250% of working concentration. This test was 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 was greater than 0.999. Correlation coefficients, Y-intercept bias and linearity equations for mesalamine and impurities are presented in Table 8. Overlay chromatograms of different linearity levels are also presented in Figure 9.

Table 8. Linearity results for 5-ASA and impurities

Compound	Linearity range, µg/mL	Correlation coefficient (r^2)	Linearity (Equation)	Y- Intercept bias
Imp-A	0.096 to 8.00	0.9996	y = 44871(x) - 2380	-1.692
Imp-B	0.096 to 8.00	0.9998	y = 40980(x) - 1678.8	-1.301
Imp-C	0.32 to 8.00	0.9998	y = 18233(x) - 711.35	-1.223
5-ASA	0.32 to 8.00	0.9997	y = 22128(x) - 1366.6	-1.964
Imp-D	0.32 to 8.00	0.9997	y = 21633(x) + 129.75	0.186
Imp-E	0.32 to 8.00	0.9999	y = 46186(x) - 624.15	-0.421
Imp-F	0.32 to 8.00	0.9997	y = 30622(x) - 1182.2	-1.212

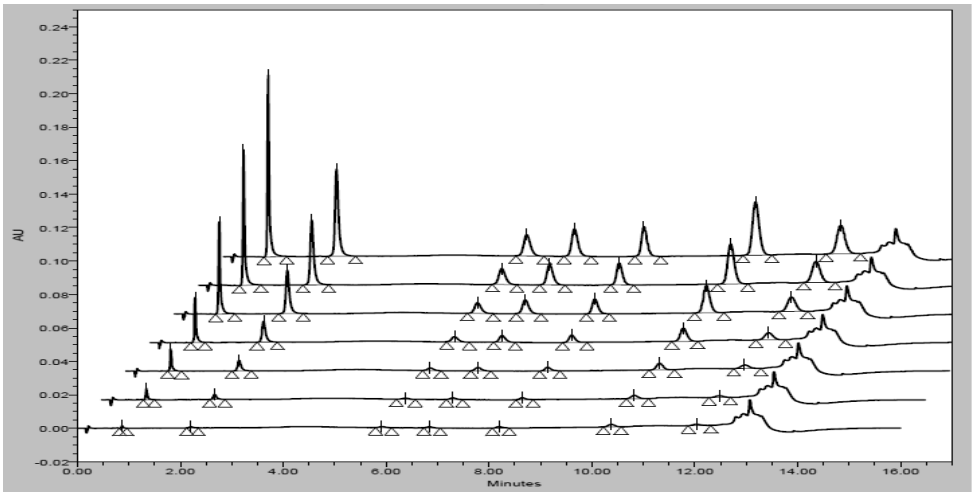


Figure 9. Overlay chromatograms of linearity study from LOQ to 250%

Robustness

No significant effect was observed on system suitability parameters such as resolution, theoretical plates, tailing factor, similarity factor and RSD of respective components, when small but deliberate changes were made to chromatographic conditions. The results are presented in Table 3 along with system suitability parameters of normal methodology. No significant effect was observed on related retention time (RRT) of all impurities, when deliberate changes were made to chromatographic conditions. The impurities RRT variation results are presented and compared with normal methodology result, which was presented in Table 9. Thus, the method was found to be robust with respect to variability in variable conditions

Table 9. Robustness results

Compound	Related retention time of Impurities						
	Initial	Flow rate per minutes		Column oven temperature		M.P.-A pH variation	
		0.65 mL	0.75 mL	38 °C	42 °C	pH 2.1	pH 2.3
Imp-A	0.134	0.139	0.139	0.139	0.139	0.139	0.139
Imp-B	0.334	0.348	0.348	0.348	0.348	0.349	0.348
Imp-C	0.914	0.915	0.915	0.915	0.914	0.914	0.914
5-ASA	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Imp-D	1.320	1.302	1.302	1.302	1.303	1.302	1.302
Imp-E	1.666	1.631	1.631	1.631	1.634	1.632	1.634
Imp-F	1.904	1.855	1.855	1.854	1.859	1.856	1.858

Stability of standard and sample solution

Solution stability was performed and determined with duplicate spike sample preparation. Percentage difference in all impurities was calculated with respect to freshly injected sample solution. Sample solution did not show any appreciable change in all impurities value when stored at ambient temperature up to 24 hours, which was presented in Table 10. Standard solution did not show any unknown peak during this 24 h study and also full fill the requirement of % RSD, which was also presented in Table 11.

Table 10. Solution stability results (test preparation)

Impurity	Initial sample		Sample after 12 hours				Sample after 24 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
Imp-A	0.207	0.208	0.205	0.204	0.002	0.004	0.204	0.206	0.003	0.002
Imp-B	0.210	0.210	0.206	0.207	0.004	0.003	0.207	0.206	0.003	0.004
Imp-C	0.200	0.200	0.203	0.204	0.003	0.004	0.209	0.208	0.009	0.008
Imp-D	0.207	0.205	0.210	0.210	0.003	0.005	0.205	0.208	0.002	0.003
Imp-E	0.212	0.212	0.208	0.209	0.004	0.003	0.210	0.209	0.002	0.003
Imp-F	0.204	0.206	0.205	0.205	0.001	0.001	0.203	0.205	0.001	0.001

Table 11. Solution stability results (standard preparation)

Compound	Initial		After 12 h		After 24 h	
	Area	% RSD[#]	Area	% RSD[§]	Area	% RSD[*]
Mesalamine	66552.8	0.60	66710.8	0.79	66766.4	0.75

[#] *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 24 h standard injections (RSD of seven Inj.)*

Filter compatibility

Filter compatibility with 0.2 µm syringe filter of PVDF and nylon filter was determined with duplicate sample preparation. Filtered sample solution was not shown any significant changes in impurities percentage with respect to centrifuge samples impurities percentage. Difference in all impurities percentage results was presented in Table 12. In displayed result difference in % of impurities was not observed more than 0.002 %, which indicates that both syringe filters are having good compatibility with sample solution.

Table 12. 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
Imp-A	0.205	0.204	0.205	0.205	0.000	0.001	0.206	0.205	0.001	0.001
Imp-B	0.206	0.207	0.207	0.206	0.001	0.001	0.206	0.205	0.000	0.002
Imp-C	0.203	0.204	0.203	0.204	0.000	0.000	0.203	0.204	0.000	0.000
Imp-D	0.210	0.210	0.209	0.212	0.001	0.002	0.210	0.210	0.000	0.000
Imp-E	0.208	0.209	0.210	0.209	0.002	0.000	0.210	0.209	0.002	0.000
Imp-F	0.205	0.205	0.203	0.204	0.002	0.001	0.205	0.205	0.000	0.000

Conclusion

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

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