IJFANS INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES
ISSN PRINT 2319 1775 Online 2320 7876
Research Paper © 2012 IJFANS. All Rights Reserved Journal UGC CARE Listed (Group-I) Volume 10, Issue 01 2021

Development of a Robust Analytical Method Based on Design Space Methodology

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Abstract

Introduction:

Mesalamine (5-aminosalicylic acid) is an anti-inflammatory agent widely used in the treatment of ulcerative colitis and Crohn's disease. The need for a simple, robust, and cost-effective analytical method for the routine quantification of Mesalamine in bulk and pharmaceutical formulations prompted this study.

Materials and Methods:

A rapid, selective, and precise RP-HPLC method was developed using an Xterra ODS C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of phosphate buffer (pH 6.5) and methanol (65:35 v/v), under isocratic conditions at a flow rate of 1 mL/min. Detection was carried out at 300 nm with a 20 µL injection volume. The method was validated as per ICH Q2 (R1) guidelines for parameters such as linearity, accuracy, precision, specificity, robustness, and system suitability.

Results:

Mesalamine exhibited a retention time of 2.47 min with well-resolved, symmetric peaks. The calibration curve was linear over the range of 10–60 µg/mL ($r^2 = 0.9986$). Accuracy studies showed mean recoveries between 98.74 % and 99.15 %. Precision results indicated %RSD < 2 %, demonstrating repeatability and intermediate precision. The method was robust with respect to minor variations in chromatographic conditions, and the assay of marketed tablets showed 100.07 % of label claim.

Conclusion:

The proposed RP-HPLC method is simple, accurate, precise, and robust, suitable for routine quality-control analysis of Mesalamine in bulk and tablet dosage forms. Its short run time and cost-effectiveness make it ideal for pharmaceutical laboratories engaged in regular testing and stability assessment.

Keywords: Mesalamine, RP-HPLC, phosphate buffer, method validation, ICH Q2 (R1).

Introduction

Mesalazine (INN, BAN), also known as Mesalamine (USAN) or 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug used to treat inflammation of the digestive tract (Crohn's disease) and mild to moderate ulcerative colitis[1]. Mesalazine is a bowel-specific aminosalicylate drug that is metabolized in the gut and has its predominant actions there, thereby having fewer systemic side effects. As a derivative of salicylic acid, 5-ASA is also an antioxidant that traps free radicals, which are potentially damaging by-products of metabolism[2].

Chemically Mesalamine is 5-Amino-2-hydroxybenzoic acid. It is in solid state and It is having solubility in slightly soluble in distilled water,very slightly soluble in methanol, practically insoluble in ethyl acetate and soluble in 0.1N Dil alkali hydroxide n in 1N HCL solution[3]. The empirical formula is $C_7H_7NO_3$ and the molecular weight is Average: 153.1354 Monoisotopic: 153.042593095. The structural formula is represented in Figure Although the mechanism of action of mesalazine is not fully understood, it appears to be topical rather than systemic. Mucosal production of arachidonic acid metabolites, both through the cyclooxygenase pathways, i.e., prostanoids, and through the lipoxygenase pathways, i.e., leukotrienes and hydroxyeicosatetraenoic acids, is increased in patients with chronic inflammatory bowel disease, and it is possible that mesalazine diminishes inflammation by blocking cyclooxygenase and inhibiting prostaglandin production in the colon[4].

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

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

Approximately 28% of the mesalamine in Asacol tablets is absorbed after oral ingestion, leaving the remainder available for topical action and excretion in the feces. It is excreted mainly by the kidney as N-acetyl-5-aminosalicylic acid[6].

The mean elimination half-life was 5 hours for 5-ASA and six hours for N-acetyl-5-ASA following the initial dose. At steady state, the mean elimination half-life was seven hours for both 5-ASA and N-acetyl-5-ASA.melting point - 283 °C.

Material and Method

Mesalamine purchased from Mesalamine (Drug samples collected from Ipca Laboratory Limited Mumbai) All the chemicals were of analytical reagent grade of Merck (Germany) unless otherwise specified. HPLC grade acetonitrile, Phosphate Buffer(6.5) and water, AR grade hydrochloric acid, sodium hydroxide and 30% hydrogen peroxide was supplied by Shivaji Scientific, Pune.

A JASCO-HPLC system equipped with manual injector of 20 µl loop, UV-Visible detector and Hypersil C18 column (250mm×4.6 mm ID × 5µm) was used. An Shimadzu (BL-220H) analytical balance and an ultra sonicator were used. The methods were conducted using a gradient reverse phase technique. The mobile phase comprised of Buffer: ACN (65:35, v/v) at flow rate of 1 ml/min. The mobile phase filtered through nylon 0.45 µm membrane filters and degassed before use. The determination was performed at 300 nm using UV probe (Version-2.35) software. The injection volume was 20 µl and the total run time was 10 min[8]

The mobile phase was prepared freshly and degassed by sonicating for 5 min before use. The UV spectrum of Mesalamine was taken using an Shimadzu UV 1800 UV-Visible spectrophotometer. Stock and working standard solutions Accurately weigh and transfer 10 mg

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of Mesalamine working standard into a 10 mL volumetric flask, add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.1 mL of the above stock solution into a 10 mL volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45 µm filter. The calibration curve was plotted with the five concentrations of the 10-60 µg/mL working standard solutions. Chromatogram was recorded thrice for each dilution. Calibration solutions were prepared daily and analyzed immediately after preparation[9].

Assay of Mesalamine tablets

Sample Preparation:

Twenty Mesalamine tablets were accurately weighed, and the average tablet weight was calculated. A sample equivalent to 10 mg of Mesalamine was precisely weighed and transferred into a 100 mL volumetric flask. Approximately 70 mL of diluent was added, and the solution was sonicated until the drug dissolved completely. The volume was made up to the mark with diluent, mixed thoroughly, and filtered through a 0.45 µm membrane filter. From this stock solution, 0.1 mL was pipetted into a 10 mL volumetric flask and diluted to the mark with the same diluent. The resulting solution was mixed well and filtered again through a 0.45 µm filter. An aliquot of this solution was injected into the HPLC system, and the peak area of Mesalamine was recorded for quantification[[10].

Method Validation:

The objective of method validation was to demonstrate that the developed method is suitable for its intended analytical purpose in accordance with ICH Q14 guidelines. The method was validated for parameters such as linearity, precision, accuracy, specificity, stability, and system suitability.

Linearity:

Standard calibration plots were prepared using five concentrations ranging from 6–14 µg/mL, each in triplicate. The peak area of Mesalamine was plotted against its corresponding concentration to generate a calibration curve. Linearity was assessed using linear regression analysis, calculated by the least squares method[10].

Precision:

Precision was evaluated through repeatability and intermediate precision. Repeatability was determined by six replicate injections of a freshly prepared Mesalamine test solution (10 µg/mL; 100% of the test concentration) using the same equipment on the same day[11]. Intermediate precision was assessed by repeating the experiment on two consecutive days with freshly prepared solutions. Precision was expressed as the percentage relative standard deviation (%RSD) of the peak areas[12].

Accuracy:

Accuracy was determined by recovery studies at three concentration levels using pure Mesalamine solutions, with three replicates for each level. The results were expressed as the percentage recovery and %RSD for each determination.

Stability:

Short-term stability of the sample solution was assessed at ambient temperature (20 ± 1°C) for three days. To confirm the stability of both standard and tablet sample solutions at the 100% level, the solutions—protected from light—were re-injected after 24 and 48 hours and compared with freshly prepared solutions[13].

Selection of Detection Wavelength:

The UV spectrum of Mesalamine in a 65:35 (v/v) mixture of phosphate buffer and methanol was recorded between 200–400 nm. The drug exhibited a maximum absorbance (λmax) at 300 nm, which was selected as the detection wavelength.

Optimization of Chromatographic Conditions:

The selection of a suitable stationary phase was based on the physicochemical properties of Mesalamine, including its molecular weight and solubility. As Mesalamine is a non-polar compound, a reverse-phase chromatographic approach was employed. Between C8 and C18 columns, the C18 column was chosen for optimal performance. The mobile phase consisted of phosphate buffer and methanol, and the effect of their ratio on retention time was systematically studied. The optimized mobile phase ratio of 65:35 (v/v) provided a symmetric peak, short run time, and consistent peak asymmetry, confirming it as the optimal composition for Mesalamine analysis[14].

Result

Analysis of tablet formulation

Sample Preparation:

Not less than ten Mesalamine tablets were randomly selected and weighed to calculate the average tablet weight. The tablets were finely powdered, and an accurately weighed quantity of powder equivalent to 25 mg of Mesalamine was transferred to a 100 mL volumetric flask. The volume was made up to the mark with diluent and mixed thoroughly. The solution was filtered through a 0.45 µm nylon membrane filter. From this filtrate, 2 mL was transferred to a 10 mL volumetric flask and diluted to the mark with the mobile phase to obtain a final concentration of 100 µg/mL. The prepared solution was injected into the HPLC system, chromatograms were recorded, and the peak areas were used to determine Mesalamine content.

Method Validation:

The developed method was validated as per ICH Q2 (R1) guidelines for parameters such as specificity, linearity, precision, and accuracy.

Specificity:

Specificity was evaluated by comparing chromatograms of standard and sample solutions of Mesalamine to ensure no interference from excipients or other components in the sample matrix.

Linearity (Calibration Curve):

A series of standard solutions were prepared from the stock solution (100 µg/mL) to obtain

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concentrations ranging from 10–60 µg/mL. Each concentration was analyzed in triplicate. The calibration curve was constructed by plotting peak area versus concentration, and the linearity was confirmed by correlation coefficient (r^2) values within the acceptable range.

Precision:

Precision was determined by intra-day and inter-day variation studies. For intra-day precision, three concentrations (30, 40, and 50 µg/mL) were analyzed in triplicate under identical chromatographic conditions. For inter-day precision, the same concentrations were analyzed on three consecutive days. Precision was expressed as %RSD, which was found to be within acceptable limits, confirming method reproducibility.

Accuracy (Recovery Studies):

Accuracy was evaluated by recovery experiments at three concentration levels—50%, 100%, and 150%. A pre-analyzed sample solution (20 µg/mL) was spiked with known amounts of standard Mesalamine solution (40 and 60 µg/mL). Each level was analyzed in triplicate under optimized chromatographic conditions. The percentage recovery and %RSD were calculated, demonstrating the method's accuracy**.**

Sensitivity

The sensitivity of the measurement of Mesalamine was estimated as a limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ for the sample were calculated by slope and intercept by using following formula;

$$LOD = 3.3 \times S.D. / Slope$$

$$LOQ = 10 \times S.D. / Slope$$

Where,

S.D = Standard deviation of the response

Assay:

Standard Préparation:

Transfer 10 ml of standard stock solution in to 100 ml volumetric flask and make up to volume with diluent.

Sample Preparation:

Transfer sample quantitatively equivalent to 40 mg of Mesalamine in to 100 mL volumetric flask add 100 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through filter paper. Dilute 10 ml of filtra to 100 ml with mobile phase.

Procedure:

Inject 20 µL of blank solution, standard solution, and sample solution record the chromatogram. And calculate the percentage of assay the results are shown in table

Assay Result

ISSN PRINT 2319 1775 Online 2320 7876
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Mesalamine = 100.07%

System suitability

The system suitability parameter like capacity factor, asymmetry factor, tailing factor, HETP and number of theoretical plates were also calculated. It was observed that all the values are within the limits. The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and let us to the conclusion that it could be used for the rapid and reliable determination of Mesalamine in tablet formulation. The results are furnished in Table 3.

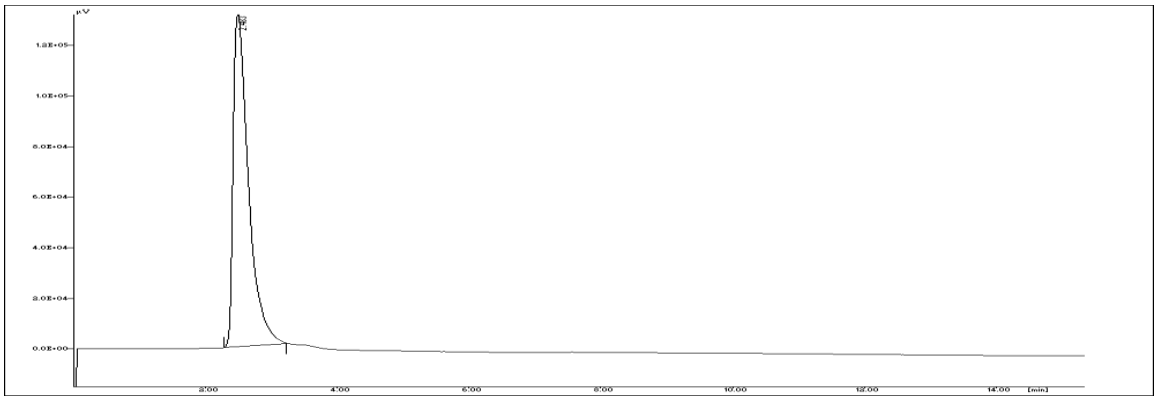

Fig 1 : Typical chromatogram of Mesalamine

Conc (µg/ml)	**Area**
10	480938
20	815491
30	1228631
40	1679153
50	2088232
60	2494042

Table 1: Linearity of Mesalamine

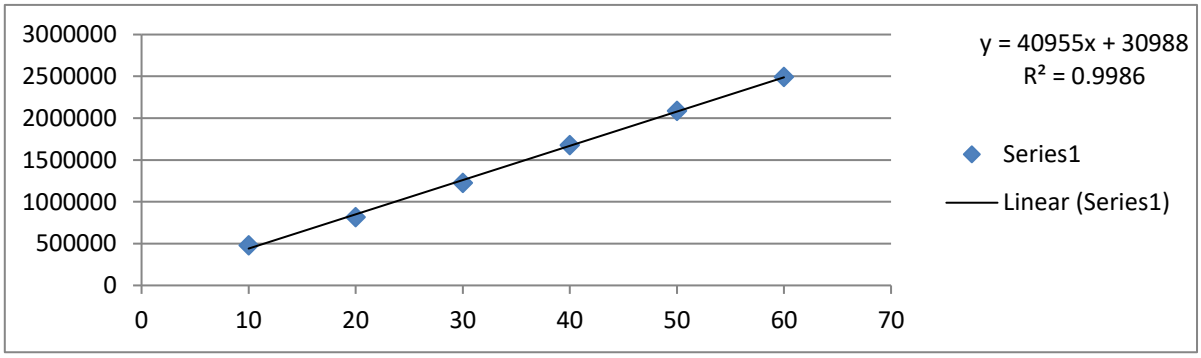

Fig 2. : Calibration curve of Mesalamine HPLC method

Sr. no.	**Parameters**	**Mesalamine**
1	Detection of wavelength (nm)	300
2	Linear range (µg/ml)	10-60
3	Correlation coefficient (r^2)	0.9986
4	Linear regression equation (y=mx+c)	y = 40955x + 30988

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5	LOD(µg/ml)	0.0302
6	LOQ(µg/ml)	0.0916

Table 2 : Linear regression data of Mesalamine

Precision:

Sr. No	**Area**
1	1763478
2	1786713
3	1765368
4	1769023
5	1775435
6	1759085
Avg	1769850
Std Dev	9927.9
% RSD	0.56

Table 3 : System Precision of Mesalamine

Accuracy:

Table 4: Accuracy of mesalamine

Accuracy 50%		**Accuracy 100%**		**Accuracy 150%**	
Sr.no.	Area	Sr.no.	Area	Sr.no.	Area
Injection -1	835385	Injection -1	1672098	Injection -1	2499132
Injection -2	835690	Injection -2	1673319	Injection -2	2501354
Injection -3	835902	Injection -3	1674178	Injection -3	2502210
Avg	835659	Avg	1673198	Avg	2500898.667
Amt Recoverd	49.49	Amt Recoverd	99.15	Amt Recoverd	148.11
% Recovery	98.98	% Recovery	99.15	% Recovery	98.74

Ruggedness:

Table 5 : Ruggedness of Mesalamine day1

Sr. No.	**Name**	**RT**	**Area**
1	Injection-1	3.235	1685389
2	Injection-2	3.237	1690156
3	Injection-3	3.239	1693424
4	Injection-4	3.24	1701543
5	Injection-5	3.242	1695635
6	Injection-6	3.241	1698905
Avg		0.003	1694175
Std Dev		0.081	5877.1
% RSD		3.235	0.35

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Table 6 : Ruggedness of Mesalamine day 2

Sr. No.	Name	RT	Area
1	Injection-1	3.231	1658905
2	Injection-2	3.233	1659683
3	Injection-3	3.235	1663065
4	Injection-4	3.237	1664324
5	Injection-5	3.23	1660964
6	Injection-6	3.239	1669689
Avg		3.234	1662772
Std Dev		0.003	3952.3
% RSD		0.108	0.238

Table 7: Ruggedness of Mesalamine D_1 & D_2

Sr.No.	Name	RT	Area
1	Injection-1	3.235	1685389
2	Injection-2	3.237	1690156
3	Injection-3	3.239	1693424
4	Injection-4	3.24	1701543
5	Injection-5	3.242	1695635
6	Injection-6	3.241	1698905
7	Injection-7	3.231	1658905
8	Injection-8	3.233	1659683
9	Injection-9	3.235	1663065
10	Injection-10	3.237	1664324
11	Injection-11	3.23	1660964
12	Injection-12	3.239	1669689
	Avg	3.237	1678473.50
	Std Dev	0.00387	17081.028
	% RSD	0.12	1.02

Robustness:

Table 8 : Robustness of Mesalamine

Sr. no	Peak name	RT	Area	Height	%Area	USP Tailing	USP Plate count
1	Mesalamine (Flow rate)	3.871	1996189	229383	100.000	1.30	4652
2		3.214	1427701	202976	100.000	1.20	3605
3	Mesalamine (Coloumn oven temp)	3.242	1668274	217834	100.000	1.24	4213
4		3.241	1681028	220686	100.000	1.23	4239

Assay:

Analysis of tablet formulation

Lable Claim- Mesalamine 400mg tablet

Table 9 : Assay of mesalamine

1686733	40	10	100	100	99.5	682	100.000	Result
1690478	100	100	68	10	100	400		100.07%

System suitability

The system suitability parameter like capacity factor, asymmetry factor, tailing factor, HETP and number of theoretical plates were also calculated. It was observed that all the values are within the limits. The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and let us to the conclusion that it could be used for the rapid and reliable determination of Mesalamine in tablet formulation. The results are furnished in Table

Table 10 : System Suitability Parameters

S. No.	Parameters	Values
1	λmax (nm)	300
2	Beer's law limit (µg/mL)	6-14
3	Retention time (min)	2.483
4	Correlation coefficient	0.989
5	Tailing factor	2.44
6	Theoretical plates	1200
7	Limit of quantification (µg/mL)	0.03
8	Limit of detection (µg/mL)	0.10

Discussion

The present study focused on the development and validation of a simple, precise, and accurate HPLC method for the quantitative estimation of Mesalamine in tablet dosage form. The analytical conditions were optimized to achieve sharp, symmetric peaks with good resolution and acceptable retention time. The selection of the mobile phase was based on the solubility and polarity of Mesalamine, ensuring optimal separation efficiency and reproducibility. The use of a C18 reverse-phase column and a phosphate buffer–methanol mixture provided well-defined chromatograms with minimal tailing.

The method validation, performed in accordance with ICH Q2 (R1) guidelines, confirmed the reliability and robustness of the proposed method. Specificity studies demonstrated that there was no interference from excipients or degradation products, confirming the method's selectivity. Linearity was established over the concentration range of 10–60 µg/mL, with a correlation coefficient (r^2) value close to 1.000, indicating an excellent linear relationship between peak area and concentration. Precision studies, including intra-day and inter-day variations, showed %RSD values well within the acceptable limit (<2%), confirming the method's repeatability and intermediate precision.

Accuracy was assessed by recovery experiments conducted at three levels—50%, 100%, and 150%—with recovery values close to 100%, demonstrating that the method is free from interference and highly reliable for quantitative determination. The results of solution stability studies indicated that both standard and sample solutions were stable for at least 48 hours at ambient conditions.

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Overall, the developed HPLC method proved to be simple, economical, and robust, making it suitable for routine analysis of Mesalamine in bulk and tablet formulations. The validation results confirmed that the method meets all the required parameters for quantitative analysis, ensuring its applicability for quality control and pharmaceutical formulation studies.

Conclusion

A simple, accurate, and precise HPLC method was successfully developed and validated for the quantitative estimation of Mesalamine in tablet dosage form. The optimized chromatographic conditions provided sharp and symmetrical peaks with satisfactory retention time and resolution. Validation studies performed as per ICH Q2 (R1) guidelines confirmed that the method is specific, linear, precise, accurate, and robust. The recovery and precision data were within acceptable limits, indicating the reliability of the method for routine quality control analysis. Hence, the developed method can be effectively applied for the routine estimation and stability testing of Mesalamine in pharmaceutical formulations.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the research work presented in this report.

Consent for Publications

All authors have read and approved the final version of this manuscript for publication.

Availability of Data and Material

All data generated or analyzed during this study are included in the manuscript and are available within the document.

Authors' Contributions

Ms. Surekha N. Shegar and Dr. Bhalekar S. Mahadu designed and conducted the research work, performed data analysis, and prepared the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The study was carried out with institutional support from JSPM's Charak College of Pharmacy and Research Wagholi, Pune.

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