International Journal of Pharmaceutical Research and Applications
Volume 9, Issue 6 Nov - Dec 2024, pp: 1101-1111 www.ijprajournal.com ISSN: 2456-4494

Determination of Mesalamine in Human Plasma by using Ultra Performance Liquid Chromatography – Mass Spectrometry/ Mass Spectrometry

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Date of Submission: 15-12-2024 Date of Acceptance: 25-12-2024

ABSTRACT

AIM: To develop and validate Mesalamine in human plasma for bio availability and bio equivalence studies by using LC-MS/MS.

MATERIALS AND METHODS: The Mesalamine D3 as internal solution (IS). Separation was achieved by liquid-liquid extraction on Phenomenex Luna 5u, C18 (150 mm x 4.6 mm). The mixture of Acetonitrile: 0.1% Formic Acid (60:40), v/v is used as a mobile phase 1.2 ml/min with splitter 50% flow will go inside used as a flow rate.

RESULTS: Ulcerative colitis is an idiopathic inflammatory colon disease that causes widespread friability and superficial erosions on the colonic wall, as well as bleeding. Mesalamine, a 5-aminosalicylic acid molecule (5-aminosalicylate, or 5-ASA), is the most often prescribed treatment for mild to severe ulcerative colitis. Around 80% of N-Ac-5-ASA is bound to plasma proteins, while 40% of mesalamine is protein bound. Mesalamine is an aminosalicylate with an atomic load of 153.1g/mol.

CONCLUSION: The calibration curve is linear and plotted. The developed method is accurate, precise, sensitive method for determination of mesalamine from human plasma solution. The reported method is suitable for bioequivalence and pharmacokinetic studies.

KEYWORDS: Ulcerative colitis, Mesalamine, Human Plasma, UPLC-MS/MS, Validation.

I. INTRODUCTION

Ulcerative colitis is an idiopathic inflammatory colon disease that causes widespread friability and superficial erosions on the colonic wall, as well as bleeding.[1] Irritable bowel illness is the most prevalent kind of inflammatory bowel disease in the globe.[2] It is characterized by inflammation that is limited to the mucosa and sub mucosa of the colon.[3] The illness usually begins in the rectum and progresses proximally in a continuous pattern. [4] Ulcerative colitis is a long-term immune-mediated inflammatory disorder of the large intestine that is most commonly linked with rectum inflammation but can also affect other parts of the colon.[5] Rectal involvement is absent in less than 5% of adult patients with UC at diagnosis, although it can be observed in up to one-third of children with colitis.[6] Symptoms of an inflamed rectum, including as bleeding, urgency are common in the early stages of new UC (a sense of pressure).[7]The illness can strike at any age and at any moment, however there is a clear age range for onset that peaks between the ages of 15 and 30.[8] The most common pattern of disease activity is relapsing and remitting, in which active illness symptoms alternate with periods of clinical quiescence, or remission.[9] Despite diagnosis and medical treatment, some people with UC have chronic disease activity, and a tiny proportion of patients develop the rapid-onset progressive form of colitis known as fulminant illness.[10] Mesalamine, a 5-aminosalicylic acid molecule (5-aminosalicylate, or 5-ASA), is the most often prescribed treatment for mild to severe ulcerative colitis.[11] However, the precise mechanism of action of mesalazine is yet unknown.[12] It is thought to inhibit the cyclooxygenase and lipoxygenase pathways, therefore lowering the production of pro-inflammatory prostaglandins and leukotrienes.[13] The peroxisome proliferator activated receptor-g, which has been identified as a target of 5-ASA activity, has also been linked to colonic inflammation. Furthermore, mesalazine may have antioxidant characteristics that help to prevent tissue damage and suppress T cell activation and

International Journal of Pharmaceutical Research and Applications
Volume 9, Issue 6 Nov - Dec 2024, pp: 1101-1111 www.ijprajournal.com ISSN: 2456-4494

proliferation.[14] UPLC-MS/MS is a chemical technology that combines liquid chromatography's physical separation capabilities with mass spectrometry's mass analysis capabilities.[15] (UP)LC-MS/MS is a versatile technology with good sensitivity and selectivity that is employed in a variety of applications.[16] The primary goal of this research is to develop and validate a simple extraction method (LLE) that is highly sensitive, robust, and repeatable in bioanalytical applications.

II. MATERIALS AND METHODS

The raw materials mesalamine and mesalamine D3 are procured from MTR lab, Chennai. Melting and solubility tests were used to verify the samples. The UPLC technique has breathed fresh life into liquid chromatography. It may be used to increase resolution, speed, and sensitivity on particles with a diameter of less than 2mm. Human plasma was obtained from volunteers. In HPLC grade, methanol and water were utilised. This strategy aids in the reduction of both cost and time. Table 1 and Figure 1 provide the structure and profile information.

CHROMATOGRAPHIC CONDITIONS

UPLC Conditions: Column used Phenomenex Luna 5u, C18 (150 mm x 4.6 mm)
Mobile Phase Acetonitrile: 0.1% Formic Acid (60:40)
Injection volume: 10 uL
Flow rate: 1.2 ml/min with splitter 50% flow will go inside
Column oven temperature: 45° C Auto sampler temperature 10°C
Retention Time: Mesalamine : 2.00+0.5min, Mesalamine D3: 2.00+0.5min
Total run time: 3.5 min.
Mode of ionization: ES +
LCMS/MS conditions and source conditions are given in Table 2 and Table 3.

Preparation of Reagents and Solutions

Preparation of Buffer-1: (0.1% Formic Acid, v/v)

1ml formic acid was added in 1000 ML reagent bottle containing 500ml of water and make up the volume to 1000ml by using water. Mix well, filter and sonicate. Provide a batch number and complete the Solution Preparation Form'. Use this solution for four days from the date of preparation.

Preparation of Derivatizing Agent (T % Propionic Anhydride in Acetone-M)

Measure and transfer 1 ml of Propionic anhydride into a 100 ml Volumetric flask and make up the volume up with Acetone-M. Mix well and sonicate. Transfer the contents to a reagent bottle. Provide a batch number to the solution and use. Prepare the solution on daily basis.

Preparation of Strong Wash Solution [Acetonitrile: Isopropyl alcohol: Ammonia (90:10:0.2%, v/v/v)]

Measure and transfer 900 mL of Acetonitrile into a 1000 mL reagent botle and add 100 mL. of isopropyl alcohol. Add 2 mL of Ammonia and mix thoroughly. Filter and sonicate the contents of the reagent bottle. Provide a batch number and completc the Solution Preparation Form. Use this solution for four days from the date of preparation.

Preparation of Weak Wash Solution [Acetonitrile: Water Isopropyl alcohol: Ammonia (50:30:20;0.2%,v/v/v)]

Measure and transfer 500 mL of Acetonitrile into a 1000 mL reagent bottle and add 300 mL of water and 200 mL of Isopropyl Alcohol. Add 2mL of ammonia, Mix well, filter and sonicate the contents of the reagent bottle. Provide a batch number and complete the Solution Preparation Form". Use this solution for four days from the date of preparation.

Preparation of Diluent [Acetone-M:Water (50:50; v/v)]

Measure and transfer 500 mlL Acetone-M into a 1000 mL reagent bottle and add 500 mL of Water using a measuring cylinder. Mix well and sonicate. Provide a batch number and complete the "Solution Preparation Form. Use this solution for four days from the date of preparation.

Preparation of 0.2% Formic Acid Solution

Measure 500mL of water in 1000mL measuring cylinder and make up the volume 1000 ml, add 2ml of fomic acid. Mix well and sonicate. Provide a batch number and complete the "Solution Preparation Form'. Use this solution for four days from the date of preparation.

Preparation of Diluent-I [Acetone-M:0.2 % Formic Acid (50:50; v/v)]

Measure and transfer 500 mL Acetone-M into a 1000 mL reagent botle and add 500ml of 0.2% formic acid solution. Mix well and sonicate

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the contents of the reagent bottle. Provide a batch number and complete the Solution Preparation Form. Use this solution for four days from the date of preparation.

Preparation of Mobile Phase [Acetonitrile: Buffer-1 (60:40:v/v]

Measure and transfer 600 mL Acetonitrile into a 1000 mL reagent bottle and add 400 ml of Buffer-1 using a measuring cylinder. Mix well and sonicate. Provide a batch number and complete the 'Solution Preparation Form'. Use this solution for four days from the date of preparation.

Preparation of Analytical Solutions

Mesalamine Stock Solution for CC (1mg /mL)

Weigh accurately about 2 mg of Mesalamine working Standard and transfer into a 2 mL volumetric flask. Add I mL of Diluent-I to dissolve completely and make up the volume with the same. Sonicate for few minutes. Calculate the final concentration of Mesalamine in Hg/ml as follows:

$$\frac{\text{Weight of Mesalamine (mg)}}{2\text{ mL}} \times \frac{\text{Potency (as is basis)}}{100} \times \frac{M_1}{M_2} \times 1000$$

Where, M1 is the molecular weight of Mesalamine (salt free) and M2 is the molecular weight of Mesalamine (salt).

Internal Standard Stock Solution Mesalamine D3 (1 mg /mL)

Weigh accurately about 2 mg equivalent of Mesalamine D3 and transfer into a 2 mL volumetric flask. AddI mL of Diluent-I to dissolve and make up the volume with same. Sonicate for few minutes. Calculate the final concentration of Mesalamine D3 in Hg/mL as follows:

$$\frac{\text{Weight of Mesalamine D3 (mg)}}{2\text{ mL}} \times \frac{\text{Potency (as is basis)}}{100} \times \frac{M_1}{M_2} \times 1000$$

Where, M is the molecular weight of Mesalamine D3 (salt free) and M2 is the molecular weight of Mesalamine D3 (salt).

Internal standard solution

Prepare a solution of internal standard mixed dilution in the concentration of 5.000 Hg mL of Mesalamine D3 in diluent as described in the Table 4.

Mesalamine Stock Dilutions for CC

Use Mesalamine stock solution for CC. Just prior to spiking. prepare stock dilutions of Mesalamine in the concentration range using diluent as described in the Table 5.

Spiked Calibration Curve Standards

Use Mesalamine stock dilutions for CC

Transfer 0.200 mL of corresponding concentrations of Mesalamine standard stock dilution into 10 mL volumetric flask and make up the volume with K2EDTA screened plasma to achieve the spiked calibration curve standards described in the Table 6.

Mesalamine Stock Solution for QC (1 mg /ml)

Weigh accurately about 2 mg of Mesalamine WS and transfer into a 2 mL volumetric flask. Add I mL of Diluent-I to dissolve and make up the volume with same. Sonicate for few minutes. Calculate the final concentration of Mesalamine in ug/mL as follows: As described in Table 7.

$$\frac{\text{Weight of Mesalamine (mg)}}{2\text{ mL}} \times \frac{\text{Potency (as is basis)}}{100} \times \frac{M_1}{M_2} \times 1000$$

Spiked Quality Control Samples

Use stock dilutions of Mesalamine for QC.

Transfer 0.200 mL of corresponding concentrations of quality control each of Mesalamine stock dilutions into 10 mL volumetric flask and make up the volume with K2EDTA pooled plasma to achieve the following concentrations as described in the Table 8.

III. RESULTS

A selective and sensitivity LC-MS/MS method to quantify mesalamine in human plasma over the concentration range 3780.638 to 7.984 ng/ml was developed and validated. The aim of our work was to develop a validated method for the determination of mesalamine in plasma by using LC MS/MS. Mesalamine D3 as internal solution (IS). Separation was achieved by liquid-liquid extraction on Phenomenex Luna 5u, C18 (150 mm x 4.6 mm).

Biological matrix

The blank K_2EDTA human plasma lots were used for screening loot detail and the characteristics. The

International Journal of Pharmaceutical Research and Applications
Volume 9, Issue 6 Nov - Dec 2024, pp: 1101-1111 www.ijprajournal.com ISSN: 2456-4494

plasma sample are procured from Micro Therapeutics Research Labs Pvt Limited,chennai.

Calibiration curve standard and quality control samples:
Calibiration curve standard and quality control all samples were prepared as per ICH guidelines and stored in -70° ± 15°C and -20° ± 5° C.

Validation and characteristics of method
Specificity and selectivity

Six lots of blank plasma, one lot of heparin plasma, one lot of lipemic plasma, and one lot of hemolyzed plasma were analysed for specificity and selectivity, confirming that the acceptance requirements were fulfilled and the sample passed the test. Selectivity and Specificity. The matrix lots analysed had Signal-to-Noise ratios ranging from 156.175 to 290.810, indicating acceptable S/N intensity. Refer Table 9.

Carry over test

The percentage peak area seen in a processed blank plasma injected in duplicate immediately after a processed ULOQ calibration standard utilised from a PA batch sample is computed as carry over. For Mesalamine and as an internal standard, no significant carryover was seen. As described in Table 10.

Linearity

Preparing 8 point standard calibration curve in human plasma is used to determine linearity concentration. Using mesalamine D3 as an internal standard and a CC curve, the concentration of mesalamine varied from 7.984 to 3780.638 ng/ml. using mesalamine D3 as IS and for CC curve refer in Figure 2

Precision and Accuracy

The precision and accuracy of the assay were determined by analysing six samples of each of the LLOQC, LOC, MQC, and HQC samples (Table 11). For chromatogram A refer in Figure 3. H refer in Figure 4.

Recovery studies of mesalamine

The recovery of mesalamine was determined by comparing the detector response of analyte extracted low, medium, and high quality quality control samples from extracted CC and QC samples from PA batch with detector response from un-extracted aqueous samples at three different levels. 's average recovery rate was 85.30 percent. At 3 different QC levels, the percent CV of mesalamine was 6.52 percent. For mesalamine D3, the average IS recovery was 87.05 percent.

IV. DISCUSSION

Six lots of blank plasma, one lot of heparin plasma, one lot of lipemic plasma, and one lot of hemolyzed plasma were analysed for specificity and selectivity, confirming that the acceptance requirements were fulfilled and the sample passed the test. A selective and sensitivity LC-MS/MS method to quantify mesalamine in human plasma over the concentration range 3780.638 to 7.984 ng/ml was developed and validated. The aim of our work was to develop a validated method for the determination of mesalamine in plasma by using LC MS/MS. Mesalamine D3 as internal solution (IS).

V. CONCLUSION

The bio analytical method development was developed as per ICH guidelines, a selective and sensitivity LC-MS/MS method to quantify mesalamine in human plasma over the concentration range 7.984 to 3780.638 ng/ml. It has been successfully validated in the experimental parameters and resulted values are shown in **Table 11**. The aim of our work is to develop a validated method for the determination of mesalamine in plasma by using LC MS/MS and separation was achieved by liquid-liquid extraction.

ACKNOWLEDGMENT

The authors would express their thanks to the Department of pharmaceutical chemistry and analysis, Vels institute of science and technology and advanced studies (VISTAS), Pallavaram, Chennai-600 117 Tamil Nadu. India. For providing infrastructure facility and support.

FUNDING SOURCES
No funding is provided.

CONFLICT OF INTEREST
The authors declare that No conflict of interest among us

ABBREVIATIONS:
AR : Analytical Reagent
Amu: Atomic mass units
BA: Bio-Analytical
CC: Calibration Curve
GR: General Reagent
GL : Group leader

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g : Gram
HQC : Higher Quality Control
UPLC: Ultra Performance Liquid Chromatography
INTQC: Intermediate Quality Control
KV : Kilo Volts
K_2EDTA: Di Potassium Ethylene Diamine Tetra Acetic Acid
LC-MS/MS : Liquid Chromatography-Mass Spectrometry/Mass spectrometry
LOQQC: Limit of Quantification Quality Control
LQC : Lower Quality Control
mg: Milligram
ml: Milliliter
mM: Millimolar
MQC : Middle-Quality control
MTR: Micro Therapeutic Research Labs Pvt. Ltd.
ng/ml : nanogram/milliliter
No : Number
PD: Positive Displacement
QA: Quality Assurance
QC : Quality Control
RIA : Radio Immuno Assay
RA: Research Associate
RS : Research Scientist.
rpm : Revolution per minute.
SOP : Standard Operating Procedure
TL : Team Leader
ULOQ: Upper Limit of Quantification
v/v : Volume by volume
TFA : Trifluro Acetic acid

VI. SUMMARY

A selective and sensitivity LC-MS/MS method to quantify mesalamine in human plasma over the concentration range 3780.638 to 7.984 ng/ml was developed and validated. The aim of our work was to develop a validated method for the determination of mesalamine in plasma by using LC MS/MS. Mesalamine D3 as internal solution (IS). Separation was achieved by liquid-liquid extraction on Phenomenex Luna 5u, C18 (150 mm x 4.6 mm). Calibration curve standard and quality control all samples were prepared as per ICH guidelines and stored in -70° ± 15°C and -20° ± 5° C. The percentage peak area seen in a processed blank plasma injected in duplicate immediately after a processed ULOQ calibration standard utilised from a PA batch sample is computed as carry over

Table Figure and Legends

Table 1: Drug profile.

PROFILE	MESALAMINE	MESALAMINE D3
CHEMICAL NAME	5-Amino-2-hydroxybenzoic acid	5-Amino-2-hydroxybenzoic acid D3
CHEMICAL FORMULA	$C_7H_7NO_3$	$C_7H_4D_3NO_3$
MOLECULAR WEIGHT	153.135 g/mol	156.15 g/mol
SOLUBILITY	Water, Alcohol, HCl, Methanol, DMSO	DMSO, Methanol
PHYSICAL PROPERTIES	Appearance: Crystalline Colour : white to pinkish crystalline solid	Appearance: Crystalline Colour : white to pinkish crystalline solid
HALF LIFE	7 hrs	7 hrs

Table 2 : LCMS/MS conditions.

Molecule	Parent (m/Z)	Daughter (m/Z)	Dwell (sec)	Cone (Volts)	Collision energy (ev)
Mesalamine	210.1500	136.0600	0.200	35.00	17.00
Mesalamine D3	213.1000	138.1500	0.200	35.00	17.00

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Table 3: Source Condition.

Parameters - value

Capillary (kV)	3.50
Desolvation Gas (L/Hr)	1000
Cone Gas Flow (L/Hr)	150
SourceT emperature (°C)	150
Desolvation Temperature (°C)	500

Table 4: Preparation of Internal Standard solution for Mesalamine D3.

Stock Concentration (µg/mL)	Stock Aliquot (mL)	Diluent (mL)	Final Volume (mL)	Final Concentration (µg/mL)
1000.0000	0.500	99.500	100.000	5.000

Table 5: Preparation of Mesalamine stock dilutions.

Stock ID	Stock Concentration (µg/mL)	Stock Aliquot (mL)	Diluents Added (mL)	Final Volume (mL)	Final Concentration (µg/mL)	Stock QCID
MESA-CC	1018.4805	0.491	1.509	2.000	250.0370	AQ-H
AQ-H	250.0370	1.515	0.485	2.000	189.4030	AQ-G
AQ-G	189.4030	1.000	1.000	2.000	94.7015	AQ-F
AQ-F	94.7015	0.500	1.500	2.000	23.6754	AQ-E
AQ-E	23.6754	0.500	1.500	2.000	5.9189	AQ- D
AQ-D	5.9189	0.500	1.500	2.000	1.4797	AQ-C
AQ-C	1.4797	0.570	1.430	2.000	0.4217	AQ-B
AQ-B	0.4217	0.712	1.288	2.000	0.1501	AQ-A

Table 6: Preparation of Mesalamine spiked calibration curve standards.

Stock CCID	Stock Concentration (µg/mL)	Stock Aliquot (mL)	Plasma Added (mL)	Final Volume (mL)	Final Concentration (ng/mL)	Spiked CCID
AQ-H	250.0370	0.200	9.800	10.000	5000.7400	STD-H
AQ-G	189.4030	0.200	9.800	10.000	3788.0600	STD-G
AQ-F	94.7015	0.200	9.800	10.000	1894.0300	STD-F
AQ-E	23.6754	0.200	9.800	10.000	473.5080	STD-E
AQ-D	5.9189	0.200	9.800	10.000	118.3780	STD-D
AQ-C	1.4797	0.200	9.800	10.000	29.5940	STD-C
AQ-B	0.4217	0.200	9.800	10.000	8.4340	STD-B
AQ-A	0.1501	0.200	9.800	10.000	3.0020	STD-A

Table 7: Spiked Quality Control Samples.

Stock QC ID	Stock Concentration (µg/Ml)	Stock Aliquot (Ml)	Plasma Added (Ml)	Final Volume (Ml)	Final Concentration (µg/Ml)	Stock QCID
AQ-HQC	188.9281	0.200	9.800	10.000	3778.5620	HQC
AQ-MQC	94.4641	0.200	9.800	10.000	1889.2820	MQC
AQ-INTQC	23.6160	0.200	9.800	10.000	472.3200	INTQC
AQ-LQC	0.4204	0.200	9.800	10.000	8.4080	LQC
AQ-LOQQC	0.1503	0.200	9.800	10.000	3.0060	LOQQC

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Table 8: specificity and selectivity.

Plasma Lot ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio	S/N Ratio (≥5)
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS(<5%)	Analyte/IS	Analyte
MAT-17-0242-II	346	111	15443	224163	1.1656	0.0825	0.0689	156.175
MAT-17-0244-II	180	185	14627	215145	0.2393	0.0390	0.0680	403.977
MAT-17-0245-II	35	84	12864	185116	1.0028	0.1334	0.0695	344.602
MAT-17-0246-II	129	247	12971	182586	1.0716	0.0170	0.0710	208.679
MAT-17-0247-II	139	31	12524	176867	1.1099	0.0175	0.0708	208.263
MAT-17-0249-II	184	48	12282	171955	1.4981	0.0279	0.0714	488.269
MAT-16-0094-XIV(H)	674	198	12190	167908	5.5291	0.1179	0.0726	493.259
MAT-16-0224-VII(L)	45	112	12380	169006	0.3635	0.0663	0.0733	290.810
				Mean	**1.49749**	**0.06270**	**0.07069**	
						SD	**0.001810**	
% of Lots passing =	**100.00**	%				**%CV**	**2.56**	
						Result	**Pass**	

Table 9: Carry over test for mesalamine and mesalamine D3.

Sample ID	Analyte peak area	IS peak area
Extracted blank	**770**	**654**
Extracted LLOQ+IS	**12096**	**1045739**
Extracted ULOQ+IS	**5813386**	**596671**
Extracted Blank I	**839**	**79**
Extracted Blank II	**506**	**208**
% Carry Over Blank-I	**0.57**	**-0.05**
% Carry Over Blank-II	**-2.18**	**-0.04**
RESULT	**Pass**	**Pass**
	Pass	**Pass**

Table 10: Precision and Accuracy study for mesalamine.

QC ID	LOQQC	LQC	INTQC	MQC	HQC
Actual Concentration (ng/mL)	**1.2260**	**3.1620**	**70.2880**	**433.8740**	**867.7460**
Calculated	1.0928	2.8531	73.4700	446.1507	898.5121

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Concentrations (ng/mL)	1.0678	2.6295	72.2905	436.2671	902.0712
	0.8899	2.9235	73.1852	450.4104	897.8388
	0.9960	2.8015	72.7152	451.1252	913.9201
	1.0196	2.8568	74.3900	451.8975	896.5145
	0.9034	2.8757	73.0501	453.9389	900.5734
Mean	**0.99492**	**2.82335**	**73.18350**	**448.29830**	**901.57168**
SD	**0.083555**	**0.102791**	**0.717255**	**6.427829**	**6.365533**
%CV	**8.40**	**3.64**	**0.98**	**1.43**	**0.71**
%Nominal	**81.15**	**89.29**	**104.12**	**103.32**	**103.90**

Table 11: Summary of Experimental Parameters and Results of Validated LC-MS/MS Method for the Quantification of mesalamine in K2EDTA human plasma.

SI.No	Experimental Parameters	Acceptable Range/Criteria (in %)	Result Obtained (in %)
1	Specificity and Selectivity	>80	100
2	Recovery	≤110%	Analyte -82.20%,IS-85.25%
3	Carry Over Test	Analyte < 20 IS<5	Analyte –2.18, IS- -0.04
4	Precision and Accuracy	80 ± 115%	LOQQC 93.21 LQC 101.94 INTQC 101.50 MQC 107.76 HQC 103.67

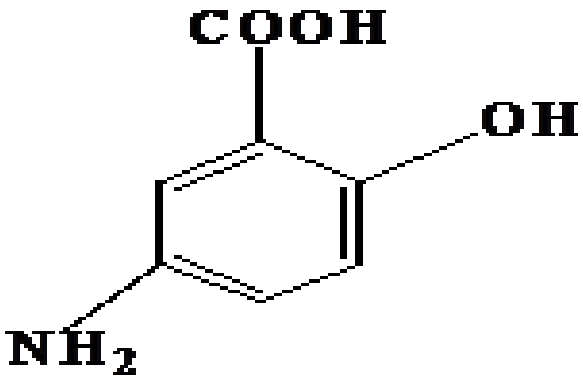

Figure 1: Chemical structure of Mesalamine.

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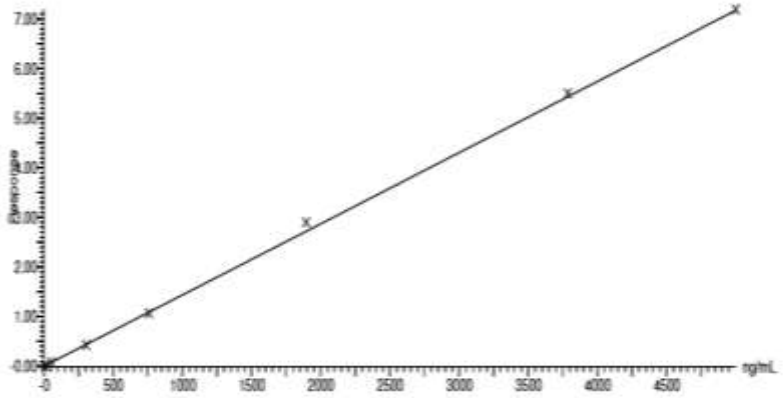

Figure 2: Calibration curve for mesalamine.

Compound name: MESALAMINE Correlation coefficient: r = 0.999124, r^2 = 0.998248 Calibration curve: 0.00143477 * x + 0.0012525 Response type: Internal Std, Area * (IS Conc. / IS Area) Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None.

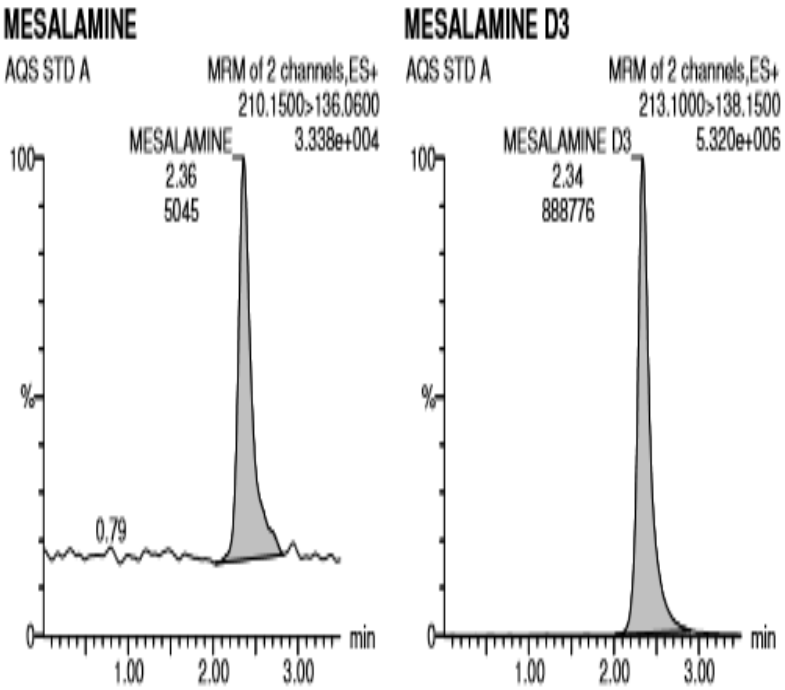

Figure 3: Representative chromatogram of standard A for mesalamine.

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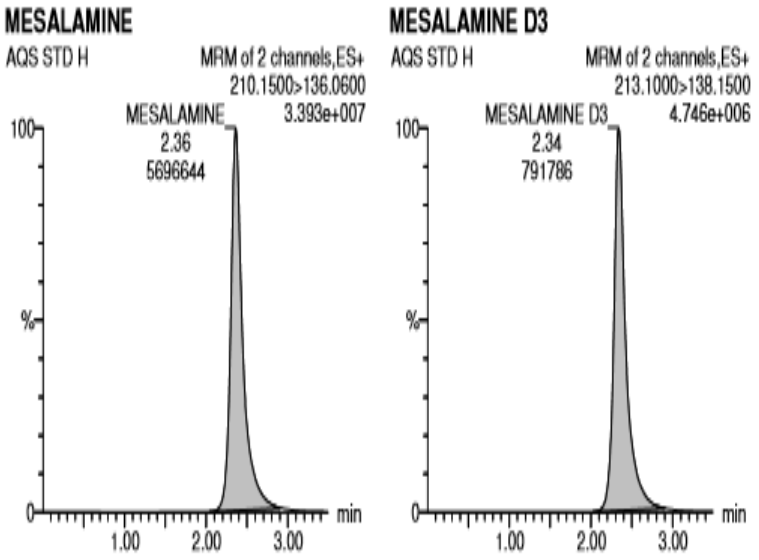

Figure 4: Representative chromatogram of standard H for mesalamine.

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