Simultaneous quantification of mesalamine and its metabolite N-Acetyl mesalamine in human plasma by LC-MS/MS and its application to a bioequivalence study

Kanchanamala.Kanala[a,b*], Nagiat T. Hwisa[c], Babu Rao.Chandu[c] , Fathi H. Assaleh[c]
Mukkanti.K[d], Prakash.Katakam[c], Bala Sekhara Reddy. Challa[e]

[a] Jawaharlal Nehru Technological University Anantapur Andhrapradesh, India
[b] Ratnam Institute of Pharmacy, Pidatapolur, Muthukur, Nellore, Andhrapradesh, India
[c]Faculty of Pharmacy, University of Zawia, Al-Zawia, Libya
[d]Jawaharlal Nehru Technological University Hyderabad, Andhrapradesh, India
[e] Vagdevi College of Pharmacy, Gurazala, Guntur, Andhrapradesh, India.

**** Corresponding author:***

Kanchana mala.K, Ratnam Institute of Pharmacy, Pidatapolur, Muthukur, Nellore, Andhrapradesh, 524346, India

ABSTRACT

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used for simultaneous quantification of mesalamine and its metabolite N-acetyl mesalamine in human plasma with N-Acetyl mesalamine D3 as an internal standard (IS). Chromatographic separation was performed on a Thermo, HyPURITY C18 (150 x 4.6 mm, 5 μm) column with an isocratic mobile phase composed of 10 mM ammonium acetate and methanol in the ratio of 85:15 (%v/v), at the flowrate of 0.6 mL/min. The drug, metabolite and internal standard were extracted by liquid-liquid extraction. The method was validated over a linear concentration range of 2-1500 ng/mL for mesalamine and 10-2000 ng/ml for N-acetyl mesalamine, which demonstrated intra and inter-day precision ranging from 1.60 to 8.63% and 2.14 to 8.67% for mesalamine and 0.99 to 5.67% and 1.72 to 4.89% for N-acetyl mesalamine respectively. Similarly, the intra- and inter-day accuracy varied from 102.70 to 105.48% and 100.64 to 103.87% for mesalamine, 99.64 to 106.22% and 100.71 to 104.27% for N-acetyl mesalamine respectively. Both analytes were found to be stable throughout freeze–thawing cycles, bench top and postoperative stability studies. The method was successfully applied to support a bioequivalance study of healthy subjects.

Keywords

Mesalamine, N-Acetyl mesalamine, LC/MS/MS, bioequivalence, pharmacokinetics.

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. 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. The major metabolite of mesalamine (5-aminosalicylic acid) is N-acetyl-5- aminosalicylic acid or N-acetyl mesalamine. Its formation is brought about by N-acetyltransferase activity in the liver and intestinal mucosa (1,2). The recommended dosage for the induction of remission in adult patients with active, mild to moderate ulcerative colitis is two to four 1.2g tablets to be taken once daily with meal for a total daily dose of 2.4g or 4.8g (3,4). Treatment duration in controlled clinical trials was up to 8 weeks. The total absorption of mesalamine from Lialda® 2.4g or 4.8g given once daily for 14 days to healthy volunteers was found to be approximately 22% of the administered dose. Mesalamine is approximately 43% bound to plasma proteins at the concentration of 2.5 µg/mL. Mesalamine is mainly eliminated of via the renal route following metabolism to N-acetyl-5-aminosalicylic acid (acetylation). However, there is also limited parent drug excreted in urine. Of the approximately 22% of the dose absorbed, less than 8% of the dose compared with greater than 13% for N-acetyl-5- aminosalicylic acid was excreted unchanged in the urine (5-7).

The apparent terminal half-lives for mesalamine and its major metabolite were, on average of 8 h. About 80% of N-Ac-5-ASA is bound to plasma proteins, whereas 40% of mesalamine is protein bound. The mean elimination half-life was 5 h for 5-ASA, and 6 h for N-acetyl-5-ASA following the initial dosing. At steady state, the mean elimination half-life was 7 h for both 5-ASA and N-acetyl-5-ASA. Despite its effectiveness in Crohn's disease and mild to moderate ulcerative colitis the use of Mesacol®, the original product, is limited as it is very expensive (8-9). Availability of a more cost effective generic drug product will increase patient accessibility, but it requires bioequivalence data to prove the generic drug product is therapeutically equivalent and can be used interchangeably with the brand name product (10-11). Literature survey reveals several methods for quantification of mesalamine and N-Acetyl mesalamine by using LC-MS (12-16), HPLC (17-20), micellar electrokinetic capillary chromatography (32), differential pulse voltammetry (33), voltammetric studies (34) were reported. Amongthem , LC-MS (12-16) methods are most accurate. These methods were developed in biological matrices (12-14, Table 1), Gu et al. (12) reported sulphasalazine, its main metabolite sulphapyridine and 5-aminosalicylic acid in human plasma by LC-MS/MS and established pharmacokinetic study. The methods developed by Pastorini etal.(13) and Nobilis, et al. (14) require larger volumes of plasma sample for extraction**.** These methods (12-14) have some drawbacks in terms of sensitivity, extraction procedure, repeatability and matrix effect issues.

Table 1. The available LC-MS methods in the literature

	Proposed method	**Ref.12**	**Ref. 13**	**Ref .14**
Type of extraction method	LLE	PPT with 0.3mL of methanol	PPT with 1.0mL of methanol	PPT BY HCLO4 followed by LLE Derivatization
Plasma usage	100µL	100µL	490uL plasma	1 mL
IS	N-Acetyl mesalamine D3	dimenhydrinate	4-ASA and N-Ac-4-ASA	N-acetyl-4-ASA and N-propionyl-4-ASA
LOQ	2.0 ng/mL	10.0 ng/mL	50.0 ng/mL	43.3 ng/mL
Linearity	2.0-1500.0 ng/mL for Mesalamine and 10.0-2000.0 ng/ml for N-acetyl Mesalamine.	10–0,000ng/mL(??) (r>0.99) for sulphasalazine and 10–1000ng/mL for sulphapyridine and 5-minosalicylic acid	50-4000 ng/mL	43.3-4966.7 ng/mL
Instrument	LC–MS/MS API-4000	API-3000 LC–MS/MS	HPLC	HPLC, MS
Column	Thermo,	XBP Phenyl column	Synergi	LiChroCART®250mm×4

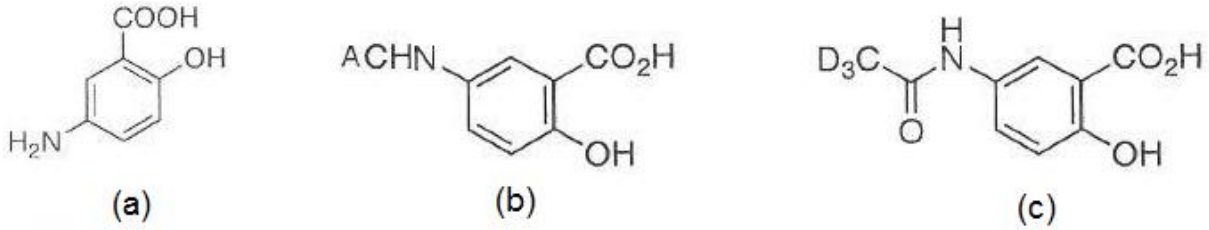
	HyPURITY C18 (150 x 4.6 mm, 5 μm)	(100mm×2.1mm, 5μm)	Hydro-RP (4μm, 150mm×2.0mm i.d.) protected by a guard col-umn (4μm, 10mm×2.0mm i.d.), both supplied by Phenomenex	mm column packed with Purospher RP-18e, 5μm and precolumn LiChroCART®4-4 with the same stationary phase
Mobile phase	10mM ammonium acetate and methanol in the ratio of 85:15 (%v/v)	0.2% formic acid, 2mM ammonium acetate in water (mobile phase A) and 0.2% formic acid, 2mM ammonium acetate in methanol (mobile phase B) by using gradient elution	17.5mmol/L acetic acid (pH 3.3):acetonitrile 85:15 (v/v)	acetonitrile–0.01M Na_2HPO4 buffer pH 3 in the 15:85 ratio (v/v)
Retention time	12 min	9 min	10 min	23 min
PK	400mg Tablet to 34 healthy volunteer	250mg SASP Tablet to 10 healthy volunteer	1200mg Tablet to 24 healthy volunteers	500mg Tablet to 14 healthy volunteers

The main purpose of the present study is to develop and validate simple extraction method (LLE), high sensitive (5 times higher than that proposed by Gu GZ et.al.(12)), rugged and reproducible bioanalytical method. At the same time suitable deuterated internal standard was used to compare analytes. Finally, the method was used to compute the pharmacokinetic parameters of two brands of mesalamine 400mg enteric coated tablets and then to evaluate the bioequivalence between the two Mesacol®(SUN Pharma Ltd., India) was selected as the reference produce, while APL Research Centre brand (Pv.T., Ltd, India) as the test formulation.

2. MATERIALS AND METHODS

2.1. Chemicals and Standards

Mesalamine, N-Acetyl mesalamine, and N-Acetyl mesalamine D3 (Fig.1) were obtained from Aurobindo Pharma Ltd. (Hyderabad, India). Ammonium acetate, formic acid, propionic anhydride (all in reagent grade) were obtained from Merck Specialities (Mumbai, India). Methanol, Acetonitrile and Methyl-t-butyl ether (HPLC grade) were purchased from J.T.Baker (Mumbai, India).



(a) (b) (c)

Figure.1. Chemical structures of Mesalamine (a), N-Acetyl Mesalamine (b), and N-Acetyl Mesalamine-D3 (c)

Test product: 400mg (APLRC)

Batch No: 006
Manufacturer: APL Research Centre(APLRC), Hyderabad, India.
Reference product: Mesacol® 400 mg tablets
Batch No: 7D0912
Manufacturer: SUN Pharma Ltd.,Baroda, India.
The label claim of each study product was 400 mg. The clinical study was conducted at Clinical and Pharmacological Research Unit, AXIS Life Sciences (Hyderabad,India.), sponsored by APL Research Centre, (a division of Aurobindo pharma Pv.t. Ltd., India).

2.2. Instrumentation and Conditions

The LC system was manufactured by (Agilent Technologies, model series 1200, Waldbronn, Germany). Mass spectrometric detection was performed on an API 4200 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada). Data were processed on Analyst 1.5.1 software package (SCIEX). Turbo ion spray (IS) negative mode with Unit Resolution, MRM was used for the detection. For mesalamine and N-acetyl mesalamine the $[M-H]^-$ were monitored at m/z 152.0 and m/z 194.2 as the precursor ion, and a fragment at m/z 108.0 and m/z 149.9 as the product ion, respectively. For internal standard N-Acetyl mesalamine D3 the $[M-H]^-$ was monitored at m/z 169.9 as the precursor ion and a fragment at m/z: 153.0, as the product ion. Mass parameters were optimized as source temperature 650 °C, nebulizer gas 20 psi, heater gas 30 psi , curtain gas 30 psi, CAD gas 4 psi, IS voltage - 2000 volts, source flow rate 600 µL/min without split, entrance potential 10 V, Collision cell exit potential (CXP) 12 V, Declustering potential (DP) 50 V, collision energy (CE) 35 V for mesalamine, N-acetyl mesalamine, and N-Acetyl mesalamine D3, respectively.

2.3. Chromatographic Condition

Chromatography was performed on a Thermo, Hypurity-C18 (150 x 4.6 mm, 5 µm) column. The mobile phase used as 10mM ammonium acetate : methanol, 85:15 v/v at the flow rate of 0.6 mL/min. Injection volume was 10µL, column temperature was 40°C with an isocratic elution mode.

2.4 Calibration and Quality Control Standards

Calibration curve of mesalamine/N-Acetyl mesalamine was prepared within the concentration range of 2.00-1500.00 ng/mL (2, 4, 10, 75, 150, 300, 600, 900, 1200 and 1500 ng/mL) for mesalamine and 10-2000 ng/mL (10, 20, 50, 100, 200, 400, 800, 1200,1600 and 2000 ng/mL) for N-Acetyl mesalamine (r^2 > 0.998). The calibration curve consisted of one replicate of 10 non-zero standards. The concentrations of quality control (QC) samples were 2, 6, 450 and 1050 ng/mL for mesalamine and 10, 30, 600 and 1400 ng/mL for N-Acetyl mesalamine as low (LQC), middle (MQC) and high (HQC) concentrations, respectively.

2.5. Sample Preparation

Liquid-liquid extraction was used for extraction of drug and IS. For this purpose, an 100 µL internal standard solution (150 ng/mL of N-Acetyl mesalamine-D3) was added to 100 µL of plasma sample (respective concentration) into riavial. To this, 25 µL of derivatisation solution(10%propionic anhydride in methanol) was added and vortexed briefly. After that, 100 µL of 0.5% formic acid was added into each tube and vortexed briefly again. Then, 3 mL of methyl t-butyl ether was added and vortexed for 10 minutes. Samples were then centrifuged for 5 minutes at 4000 rpm at 20°C. Supernatant from each sample was transferred into ria vial and evaporated to dryness. This was followed by reconstitution with 800 µL of reconstitution solution (10 mM ammonium acetate : methanol, 85:15 v/v). and vortex briefly. From this, 5 µL of sample was injected into the LC–MS/MS system through the autosampler.

2.6. Method Validation

2.6.1. Selectivity and Specificity

The selectivity of the method was determined by six different human blank plasma samples, which were pretreated and analyzed to test the potential interferences of endogenous compounds co-eluting with analyte and IS. Chromatographic peaks of analyte and IS were identified based on their retention times and MRM responses. The peak area of mesalamine and N-Acetyl mesalamine at the respective retention time in blank samples should not be more than 20% of the mean peak area of LLOQ of mesalamine and N-Acetyl mesalamine. Similarly, the peak area of N-Acetyl mesalamine -D3 at the respective retention time in blank samples should not be more than 5% of the mean peak area of LLOQ of N-Acetyl mesalamine-D3.

2.6.2. Matrix Effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by matrix factor, which was calculated as Peak response ratio in presence of extracted matrix (post extracted) to peak response ratio in aqueous standardsSix lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the Mid QC level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV in %) should be < 15%.

2.6.3. Linearity , Precission and Accuracy

The calibration curve was constructed using values ranging from 2.0 to 1500.0 ng/mL for mesalamine and 10.0 to 2000.0 ng/mL for N-Acetyl mesalamine in human plasma respectively. Calibration curve was obtained by linear model with weighted $1/x^2$ regression analysis. The peak area ratio of mesalamine / N-Acetyl mesalamine-D3 was plotted against the mesalamine concentration in ng/mL for mesalamine. The peak area ratio of N-Acetyl mesalamine / N-Acetyl mesalamine-D3 was plotted against the N-Acetyl mesalamine concentration in ng/mL for N-Acetyl mesalamine. Calibration curve standard samples and quality control samples were prepared in replicates (n=6) for analysis. Precision and Accuracy for the back calculated concentrations of the calibration points, should be within ≤15 and ± 15% of their nominal values. However, for LLOQ, the Precision and Accuracy should be within ≤ 20 and ± 20%.

2.6.4. Recovery

The extraction recovery of mesalamine / N-Acetyl mesalamine and N-Acetyl mesalamine -D3 from human plasma was determined by analyzing quality control samples. Recovery at three concentrations (6, 450 and 1050 ng/mL for mesalamine and 30,600 and 1400 ng/mL for N-Acetyl mesalamine) was determined by comparing peak areas obtained from the plasma sample and those from the standard solution spiked with the blank plasma residue. A recovery of more than 50% was considered adequate to obtain required sensitivity.

2.6.5. LOQ and LOD

The response (peak area) was determined in blank plasma samples (six replicates from different plasma) and spiked LOQ sample prepared from the same plasma was determined. The peak area of blank samples should not be more than 20% of the mean peak area of LOQ of mesalamine / N-Acetyl mesalamine and not more than 5% of N-Acetyl mesalamineD3. The precision and mean accuracy of the back calculated LOQ replicate concentrations must be ≤ 20 and ± 20 %, respectively. The limit of detection (LOD) is a parameter providing the lowest concentration in a sample that can be detected from background noise but can not be quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analyte with blank samples.

2.6.6.Stability

Low quality control and high quality control samples (n=6) were retrieved from deep freezer after three freeze-thaw cycles according to the clinical protocols. Samples were stored at −30 ^{o}C in three cycles of 24, 36 and 48 h**.** In addition, the long-term stability of mesalamine / N-Acetyl mesalamine in quality control samples was also evaluated by analysis after 62 days of storage at −30 ^{o}C. Autosampler stability was studied following 48 h storage period in the autosampler tray with control concentrations. Bench top stability was studied for period of 25 h with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within ≤ 15 and ± 15 % of their nominal concentrations, respectively. Stability of the mesalamine / N-Acetyl mesalamine, N-Acetyl mesalamine-D3 in stock solution was evaluated for 9 days with comparision of freshly prepared stock solutions. Similarly stability of N-Acetyl mesalamine-D3 working solution was also proved for 9 days with comparision of freshly prepared working solutions

2.7. Bio equivalence study2.7.1. Study Subjects

The study was carried out in accordance with the current revision of the Declaration of Helsinki concerning medical research in humans. Study protocol was approved by IEC (Institutional Ethics committee) as per DCGI (Drug control general of india). Thirty four healthy male subjects were included in the study. All volunteers gave a written informed consent prior to participation, after they had been thoroughly informed and they understood the nature and details of the study. All clinical laboratory tests were

performed by the ISO 15189 certified laboratories. The daily results of the clinical laboratory tests including the quality control data were verified by its own independent quality assurance personnel before reporting. Subject inclusion criteria included Indian male, aged between 18-45 years, no consumption of drugs or food supplements for 4 weeks prior to the study, and no participation in any bioavailability or bioequivalence study at least 30 days prior to the present study.

2.7.2. Study Design

The study was conducted as an open label, randomized two-period, two-sequence, single-dose crossover bioequivalence study under fasting condition, and a wash-out period of 14 days. All subjects arrived at the clinical research laboratory, at least 12 h prior to the start of the study. They were housed in an air-conditioned facility and were given a standard dinner, which was finished at least 10 h before dosing in each period of the study. On the day of drug dosing in period 1, volunteers were randomly assigned to one of two treatment sequences TR (sequence 1) or RT (sequence 2), as indicated in a pre-printed randomization scheme, Subjects in sequence 1 received treatment T at the first dosing period and then crossed over to receive treatment R at the second dosing period (after the 7-day washout period). Subjects in sequence 2 received treatments in the order of R and T at the two dosing periods. The subjects administered the assigned mesalamine formulation with 240 mL of plain drinking water. This was followed by an oral cavity check to ensure completion of the dose administration. Subjects were required to refrain from lying down during the first 4 h after intaking the tablet. No meal was permitted until 4 h after dosing. Drinking water was restricted from 1 h before dosing till 2 h after excess water intake (> 100 mL/h) was not permitted. Lunch, snacks, and dinner were served as per the scheduled time. All subjects abstained from any xanthine-containing food or beverages for at least 72 h and alcoholic products for at least 7 days prior to formulation administration and throughout the sampling schedule during each period. No concomitant medication was permitted during the study period.

2.7.3. Blood Sampling

Blood samples were collected as the pre-dose 5 minutes prior to dosing followed by further samples at 2, 3, 4, 5, 6, 7, 8,9,10,11,12.5,14,16, 18, 20,22, 24, 26, 28, 30, 32, 36, 40, 44, 48, 60, 72, 84 and 96 h after dosing 2.5 mL blood was collected each time in vaccutainers containing K_2EDTA. A total of 60 time points (30 for Reference, 30 for Test) were collected by using centrifugation 3200 rpm, 10°C, 10 min and stored below -30 °C until sample analysis. Test and reference mesalamine tablets were administered to same human volunteers under fasting conditions separately with proper washing periods as per protocol (comparative, randomized, 2-way crossover) approved by IEC. During the sample collection, all subjects were under medical supervision. Vital signs were examined at scheduled time as described in the protocol.

2.7.4. Pharmacokinetics and Statistical Analysis

Pharmacokinetic parameters from the human plasma samples were calculated by a non-compartmental statistic model using Win Nonlin5.0. software (Pharsight, USA). Blood samples were taken for a period of 3 to 5 times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration time curve (AUC) ratio higher than 80% as per USFDA guidelines. Plasma mesalamine, N-Acetyl mesalamine concentration-time profiles were visually inspected, and C_{max} and T_{max} values were determined. The AUC_{0-t} was obtained by the trapezoidal method. $AUC_{0-\infty}$ was calculated up to the last measureable concentration and extrapolations were obtained using the last measureable concentration and the terminal elimination rate constant (K_e) was estimated from the slope of the terminal exponential phase of the plasma of the mesalamine, N-Acetyl mesalamine concentration-time curve (by linear regression method (36,37). The terminal elimination half-life ($t_{1/2}$), was then calculated as $0.693/K_e$ (38) . Regarding $AUC_{0-t,}$ $AUC_{0-\infty}$ and C_{max} bioequivalence were assessed by means of analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratio's test/reference (logarithmically transformed data). The bioequivalence was considered when the ratio of averages of log transformed data was within 80-125% for $AUC_{0-t,}$ $AUC_{0-\infty}$ and C_{max} (36-37).

3.RESULTS AND DISCUSSION

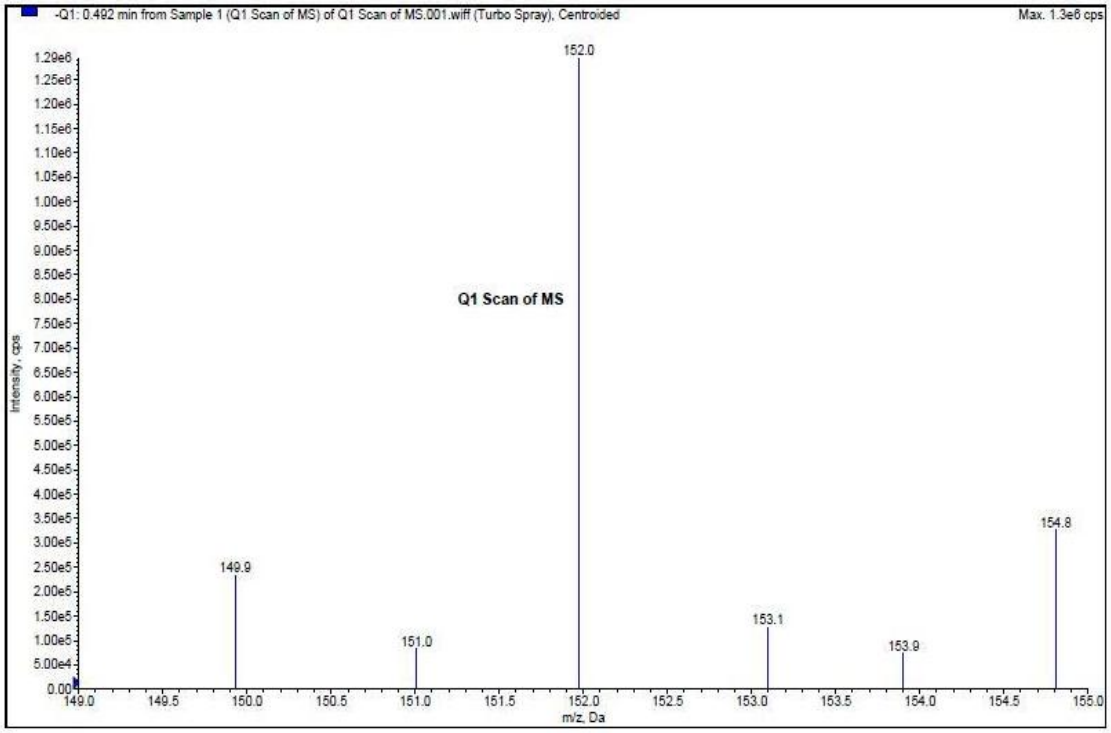
3.1.Method development

LC-MS/MS has been recognized as one of the most powerful analytical techniques in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility (12). The goal of this work was to develop and validate a simple, sensitive and rapid assay method for the quantitative determination of mesalamine, N-

acetyl mesalamine from plasma samples. A simple liquid-liquid extraction technique was utilized in the extraction of mesalamine, N-acetyl mesalamine and N-Acetyl mesalamine-D3 from the plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, were optimized through several trials to achieve best resolution and increase the signal of mesalamine, N-acetyl mesalamine and N-Acetyl mesalamine-D3. The MS optimization was performed by direct infusion of solutions of both mesalamine, N-acetyl mesalamine and N-Acetyl mesalamine D3 into the ESI source of the mass spectrometer.

Other parameters, such as the nebulizer and the heater gases were optimized to obtain a better spray shape, resulting in better ionization. mesalamine, N-acetyl mesalamine and N-Acetyl mesalamine-D3 were detected with proton adducts at m/z 152.0→108.0, 194.2→149.9, and 169.9→153.0 in multiple reaction monitoring (MRM) negative mode respectively (Fig. 2a-2b, Fig. 2c-2d, and Fig. 3a-3b) . After the MRM channels were tuned, the mobile phase was changed from more organic phase to an aqueous phase to obtain a fast and selective LC method. A good separation and elution were achieved using the proposed LC study condition.

(a)



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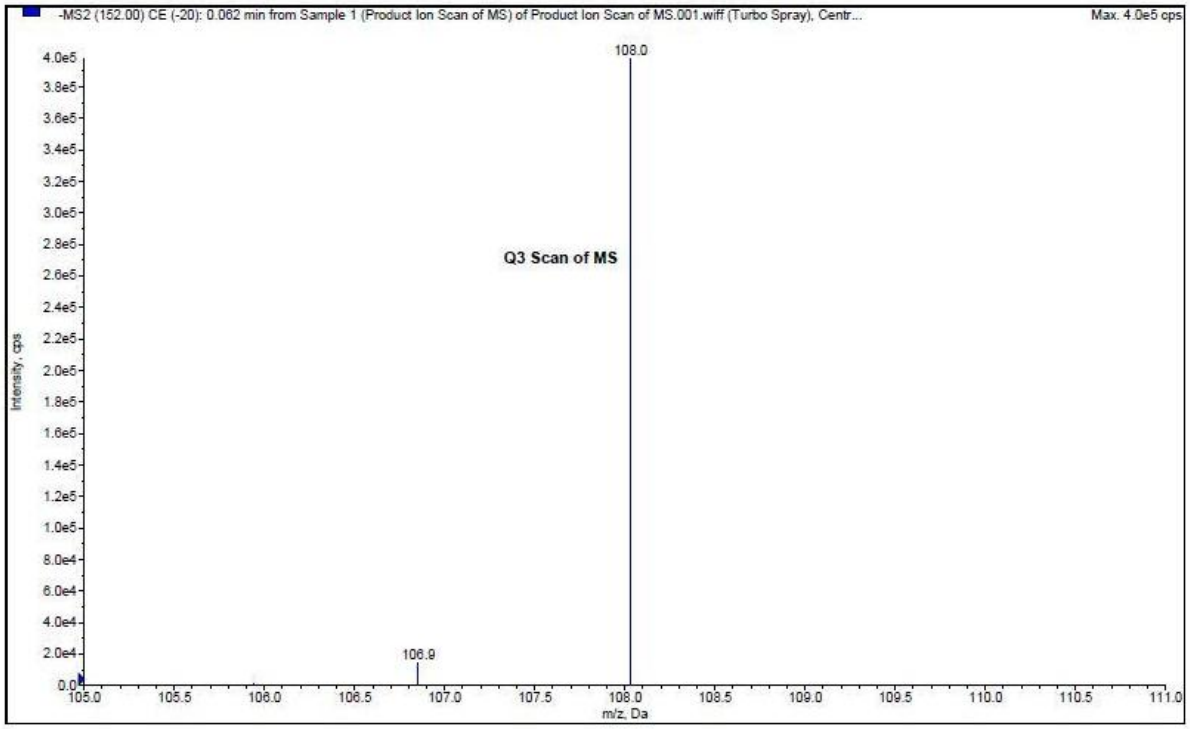
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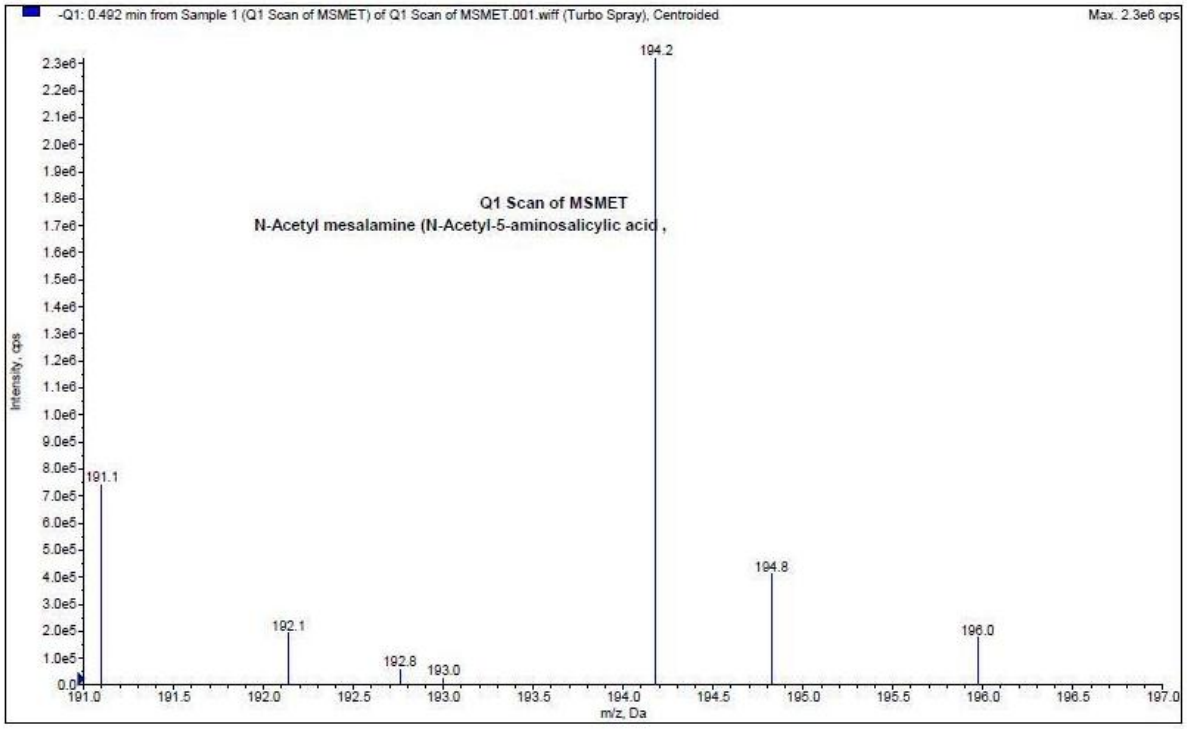
(c)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Max. 2.3e6 cps
Q1 Scan of MSMET
N-Acetyl mesalamine (N-Acetyl-5-aminosalicylic acid ,
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192.8
193.0

(d)

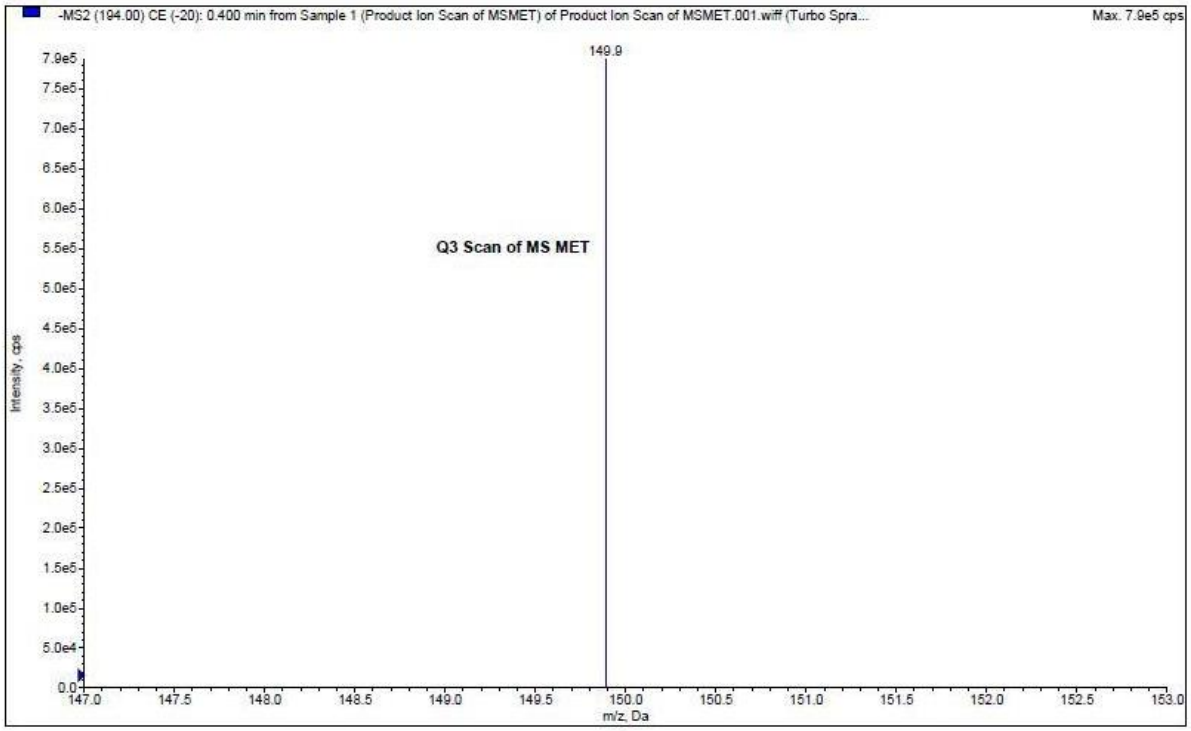
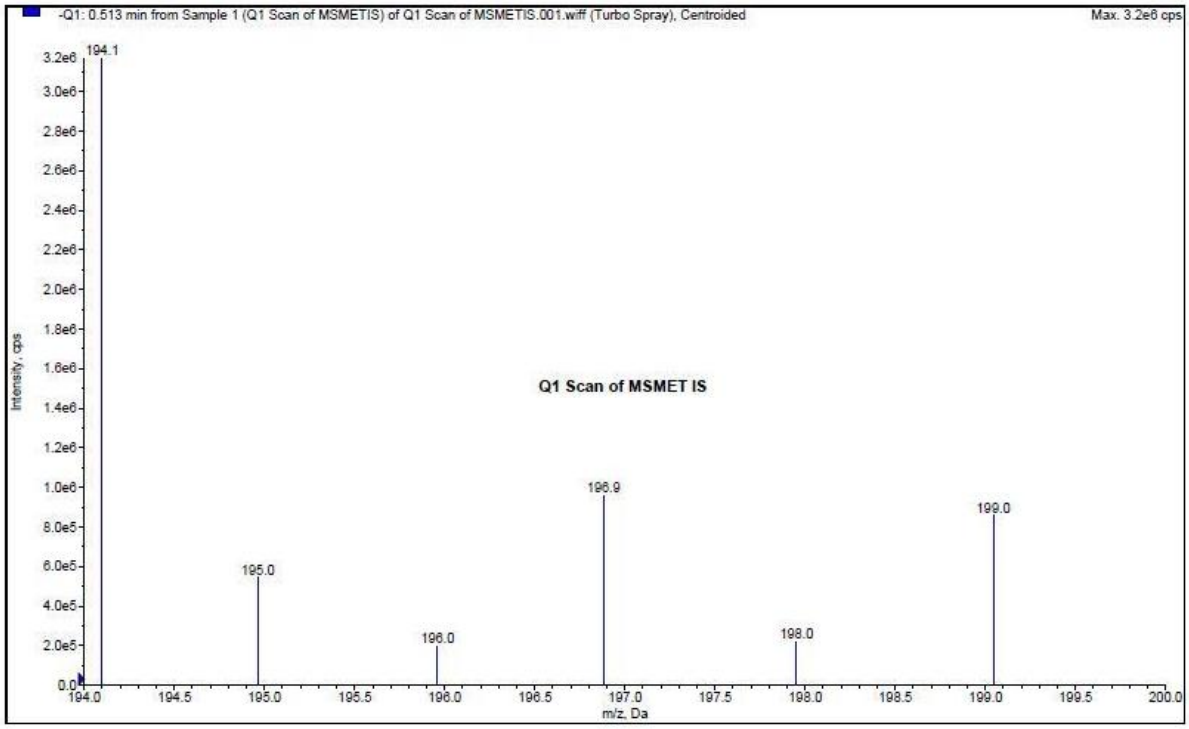


Figure 2. Mass spectra: (a) mesalamine parent ion, (b) mesalamine product ion, (c) N-Acetyl mesalamine parent ion, and (d) N-Acetyl mesalamine product ion

(a)



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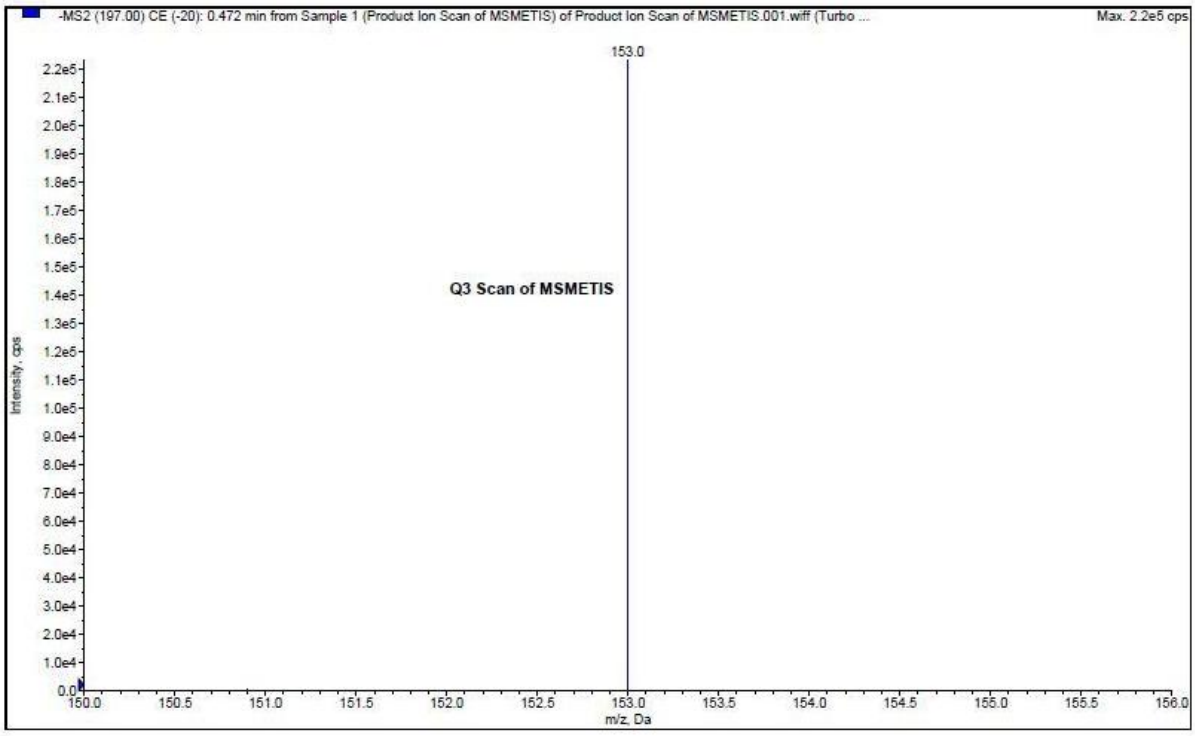

Figure 3. Mass spectra: (a) N-Acetyl mesalamine-D3 parent ion, and (b) N-Acetyl mesalamine-D3 product ion

3.2.Method Validation

3.2.1.Selectivity and Specificity

The analysis of mesalamine, N-acetyl mesalamine and N-Acetyl mesalamine-D3 using MRM function was highly selective with no interfering compounds. Fig. 4 shows the chromatograms of one blank human plasma. Chromatograms obtained from plasma spiked with mesalamine (2.0 ng/mL), N-Acetyl mesalamine (10.0 ng/mL) and N-Acetyl mesalamine D3 (150 ng/mL) are shown inFigures 4 and 5.

3.2.2.Matrix Effect

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with aqueous standards of same concentration in alternate injections. The overall precision of the matrix factor is 2.83 for mesalamine, and 2.80 for N-Acetyl mesalamine, respectively. There was no ion- suppression and ion- enhancement effect observed due to IS and analyte at respective retention time.

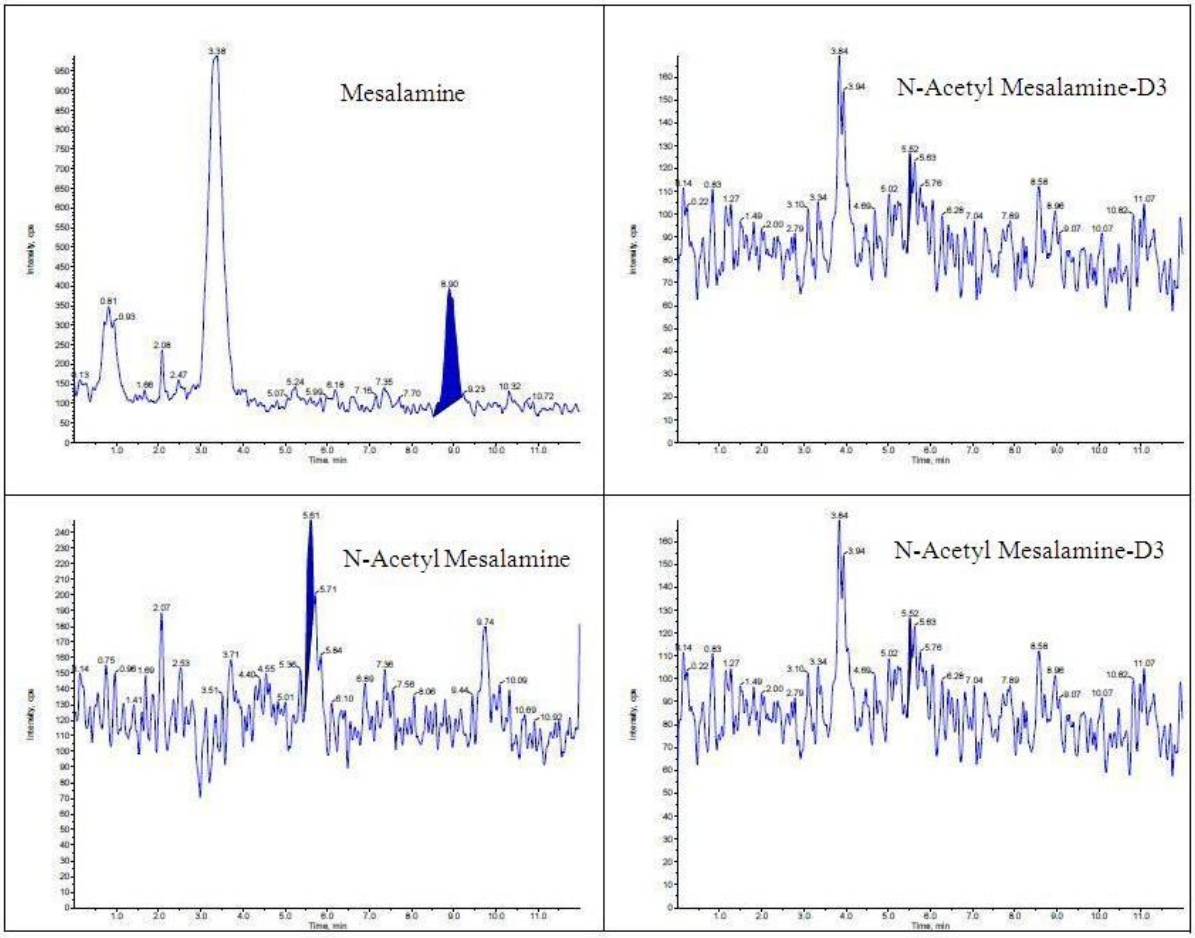

Figure 4. Chromatogram of blank human plasma

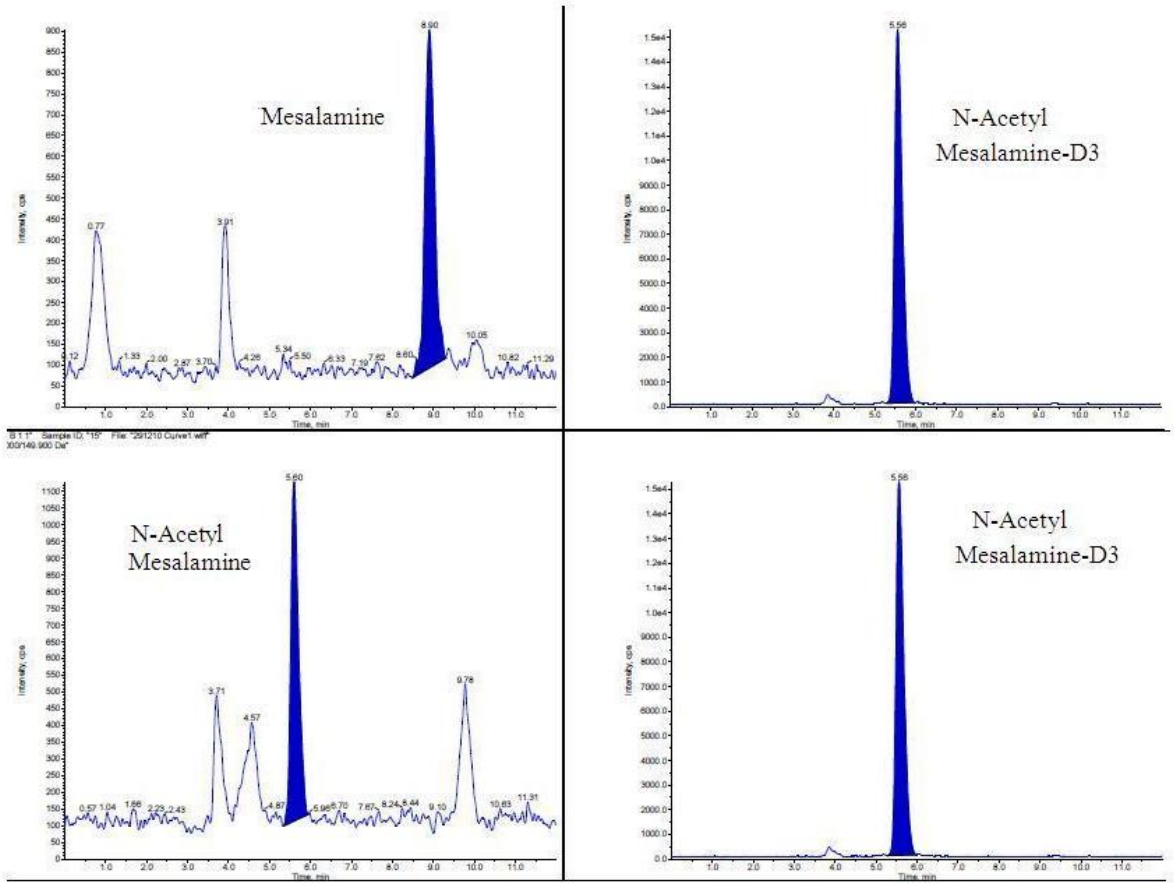

Figure 5. LOQ Chromatograms of mesalamine and N-Acetyl mesalamine

3.2.3.Linearity, Precision and Accuracy

Calibration curves were plotted as the peak area ratio (Mesalamine / N-Acetyl mesalamine-D3) versus (mesalamine) concentration for Mesalamine and the peak area ratio (N-Acetyl mesalamine / N-Acetyl mesalamine-D3) versus (N-acetyl mesalamine) concentration for N-acetyl mesalamine. Calibration was found to be linear over the concentration range of 2–15000 ng/mL for mesalamine and 10–2000 ng/mL for N-Acetyl mesalamine. The Precision (CV %) for mesalamine , N-Acetyl mesalamine was less than 8.67% and 5.67% respectively. The accuracy ranged from 100.64 to 105.48% for mesalamine and 98.55 to 106.22% for N-acetyl mesalamine. The determination coefficients (r^2) for mesalamine and N-acetyl mesalamine were greater than 0.9998 and 0.9987 respectively for all curves (Table 2).

Table 2. Details of Mesalamine and N-Acetyl Mesalamine calibration curves

Analyte	Spiked plasma concentration (ng/mL)	Concentration measured(mean±SD; ng/mL)	CV (%) (*n* = 5)	Accuracy (%)
Mesalamine	2	1.96±0.05	2.32	98.20
	4	4.14±0.22	5.27	103.40
	10	10.12±0.34	3.33	101.20
	75	74.12±1.08	1.45	98.83
	150	148.20±2.59	1.75	98.80
	300	305.00±4.58	1.50	101.67
	600	593.80±19.89	3.35	98.97
	900	874.20±20.14	2.30	97.13
	1200	1204.00±8.94	0.74	100.33
	1500	1524.00±15.17	1.00	101.60
N-Acetyl Mesalamine	10	9.85±0.1	0.99	98.48
	20	20.38±0.33	1.61	101.90
	50	52.58±1.11	2.11	105.16
	100	96.52±1.01	1.04	96.52
	200	194.6±3.97	2.04	97.30
	400	399.6±4.28	1.07	99.90
	800	799.8±3.11	0.39	99.98
	1200	1158±8.37	0.72	96.50
	1600	1636±26.08	1.59	102.25
	2000	2040±27.39	1.34	102.00

Precision and accuracy for this method was controlled by calculating the intra and inter-batch variations of QC samples in six replicates at four concentrations (2, 6, 450 and 1050 ng/mL) for mesalamine and(10, 30, 600 and 1400 ng/mL) N-Acetyl mesalamine respectively as shown in Table 3 .

Table 3. Precision and accuracy details for mesalamine and N-Acetyl mesalamine.

Analyte	Spiking plasma concentration (ng/mL)	Within-run			Between-run		
		Concentration measured (mean±SD) (n=6; ng/mL)	CV (%)	Accuracy (%)	Concentration measured (mean±SD) (n=6; ng/mL)	CV (%)	Accuracy (%)
Mesalamine	2	2.07±0.18	8.63	103.25	2.01±0.17	8.67	100.64
	6	6.29±0.43	6.79	104.83	6.23±0.33	5.31	103.87
	450	474.67±17.08	3.60	105.48	461.2±14.37	3.12	102.49
	1050	1078.33±17.22	1.60	102.70	1067.67±22.85	2.14	101.68
N.Acetyl Mesalamine	10	10.14±0.57	5.67	101.42	9.86±0.48	4.89	98.55
	30	31.87±0.52	1.64	106.22	31.28±0.71	2.26	104.27
	600	602.33±7.15	1.19	100.39	605.77±10.42	1.72	100.96
	1400	1395±13.78	0.99	99.64	1410±30.51	2.16	100.71

This method was demonstrated intra and inter-day precision 1.60 to 8.63 and 2.14 to 8.67% for mesalamine 0.99 to 5.67 and 1.72 to 4.89% for N-Acetyl mesalamine. This method demonstrated intra and inter-day accuracy 102.70 to 105.48 and 100.64 to 103.87% for mesalamine, 99.64 to 106.22 and 100.71 to 104.27% for N-acetyl mesalamine. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

3.2.4. Recovery

The recovery following the sample preparation using Liquid-Liquid extraction method with t-butyl methyl ether was calculated by comparing the peak areas of drug in plasma samples with the peak area ratios of solvent samples and was estimated at control levels of drug .The recovery of mesalamine (at concentrations 6 , 450 and 1050 ng/mL) and N-acetyl mesalamine (at concentrations 30 , 600 and 1400 ng/mL) were found to be 95.97, 91.79, 98.87%, and 88.27, 89.24,90.16 %, respectively . The overall mean recovery of mesalamine, N-Acetyl mesalamine, and N-Acetyl mesalamine-D3 were 95.54%, 89.22% and 86.71% respectively.

3.2.5. LOQ and LOD.

The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 2.00 ng/mL for mesalamine and 10.00 ng/mL for N-acetyl mesalamine. The LOD was determined using aqueous solutions. For mesalamine 10 μL of a 1.0 pg/mL solution was injected to give an on-column mass of 10.0 Femtogram (fg) and for N-acetyl mesalamine, 10 μL of a 2.0 pg/mL solution was injected to give an on-column mass of 20.0 fg respectively.

3.2.6.Stability

Quantification of the mesalamine, N-acetyl mesalamine in plasma subjected to 3 freeze–thaw (from -30^{o}C to room temperature) cycles showed the stability of the analyte and its metabolite. The accuracy ranged from 99.33 to 100.64% for mesalamine and 98.10 to 102.11% for N-Acetyl mesalamine of the theoretical values. No significant degradation of mesalamine and N-Acetyl mesalamine was observed even after 48 h storage period in the autosampler tray and accuracy of mesalamine and N-Acetyl mesalamine were between 98.50 and 96.86% and 100.95 and 105.72% of the theoretical values. The room temperature stability of mesalamine and N-Acetyl mesalamine in QC samples after 25 h was also evaluated and accuracy ranged from 98.25 to 98.61% for mesalamine and 99.17 to 102.67 % for N-Acetyl mesalamine of the theoretical values. In addition, the long-term stability of mesalamine, N-acetyl mesalamine in QC samples after 62 days of storage at −30 ^{o}C was also evaluated and accuracy ranged from 97.94 to 102.54% for mesalamine and 102.26 to 106.73% for N-Acetyl mesalamine of the theoretical values. These results confirmed the stability of mesalamine and N-Acetyl mesalamine in human plasma for at least 62 days at -30 ^{o}C (Table-4). Stability of the mesalamine and N-acetyl mesalamine in stock solution was proved for 9 days, and N-Acetyl mesalamine-D3 working solution was proved for 9 days with freshly prepared stock solutions and working solutions.

Table 4 Stability of mesalamine and N.Acetyl mesalamine in six plasma samples

Stability paameters	Plasma concentration (ng/mL)	Concentration Measured(mean± SD ng/mL)	CV (%)	Accuracy
		Mesalamine		
Room temperature stability	6	5.92±0.34	5.76	98.61
(25 h)	1050	1031.67±24.01	2.33	98.25
Autosampler stability (48 h)	6	5.91±0.22	3.68	98.50
	1050	1017.00±22.76	2.24	96.86
Freeze-thaw stability	6	5.96±0.3	4.99	99.33
(3cycles)	1050	1056.67±21.60	2.04	100.64
Long term stability (62 days)	6	5.88±0.23	3.84	97.94
	1050	1076.67±42.27	3.93	102.54
		N-Acetyl mesalamine		
Room temperature stability	30	30.8±0.52	1.68	102.67
(25 h)	1400	1388.33±21.37	1.54	99.17
Autosampler stability (48 h)	30	31.72±0.50	1.56	105.72
	1400	1413.33±29.44	2.08	100.95
Freeze-thaw stability	30	30.63±0.53	1.74	102.11
(3cycles)	1400	1373.33±27.33	1.99	98.10
Long term stability (62 days)	30	32.02±1.14	3.56	106.73
	1400	1431.67±47.92	3.35	102.26

3.3. Application to biological samples

The above validated method was used in the determination of mesalamine and N-Acetyl mesalamine in plasma samples for establishing the bioequivalence of a single 400 mg dose (one 400 mg tablet) in Thirty four healthy volunteers. Typical plasma concentration versus time profiles is shown in Figures 6 and 7. All the plasma concentrations of mesalamine and N-Acetyl mesalamine were within the standard curve region and retained above the 2.0 ng/mL as the LOQ of mesalamine and above 10.0 ng/mL as the LOQ of N-Acetyl mesalamine for the entire sampling period (Tables-5 and 6). The ANOVA results revealed that period, sequence and treatment had no statistically significant effects on C_{max}, $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$. Since the sequence or carry-over effect was not significant, the ANOVA test was valid. The statistically significant subject within sequence effect on C_{max}, $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$ were observed that are usually seen in large sample size study as in crossed over bioequivalence studies. Bioequivalence between the 400 mg enteric coated tablet formulations of mesalamine under fasting condition was demonstrated by the 90% CI of the geometric mean ratios of C_{max}, $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$ lying within the acceptable criteria of 80-125%. The test and reference formulations had very similar $t_{1/2}$ at approximately 9.5 h for mesalamine and 15.5h for N-Acetyl mesalamine. Period, sequence and treatment had no significant effects on C_{max}, $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$.

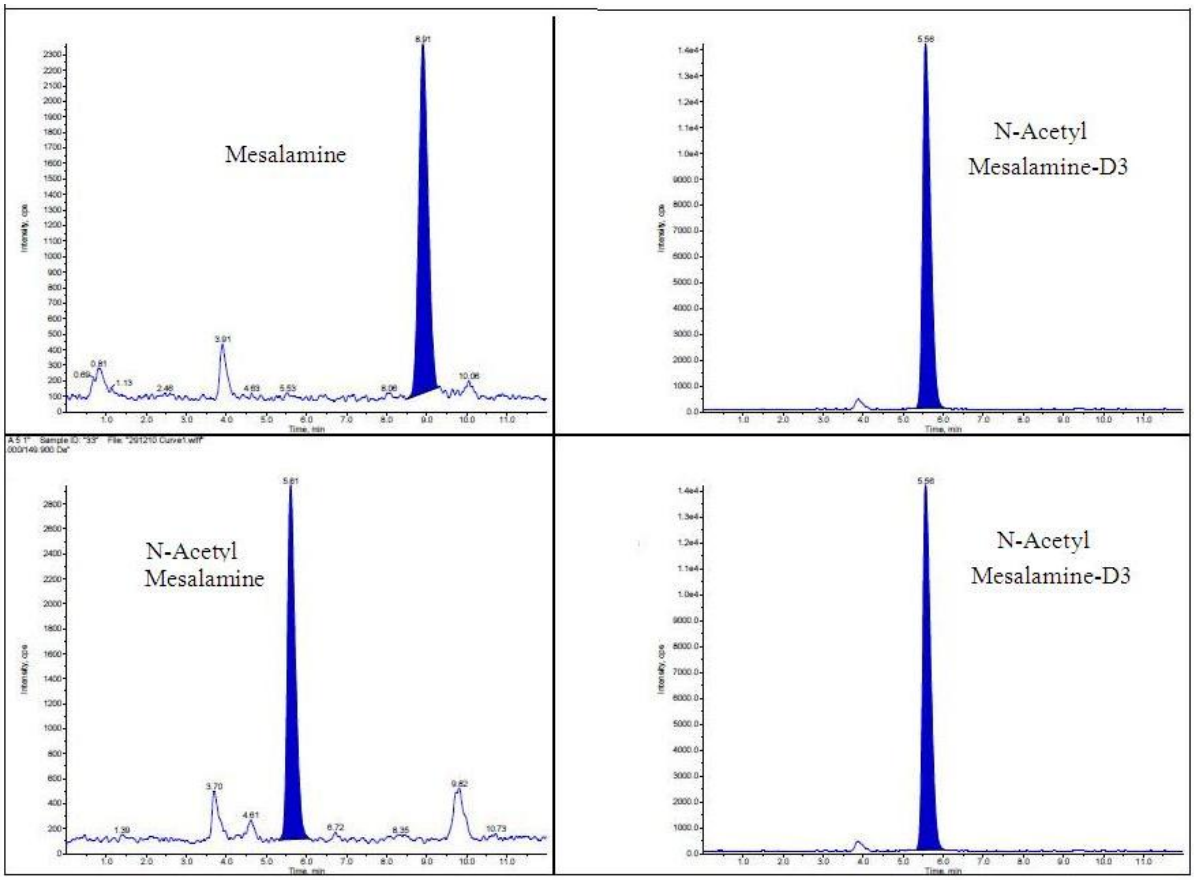

Figure 6. Post dose chromatograms of mesalamine and N-Acetyl mesalamine at 24h

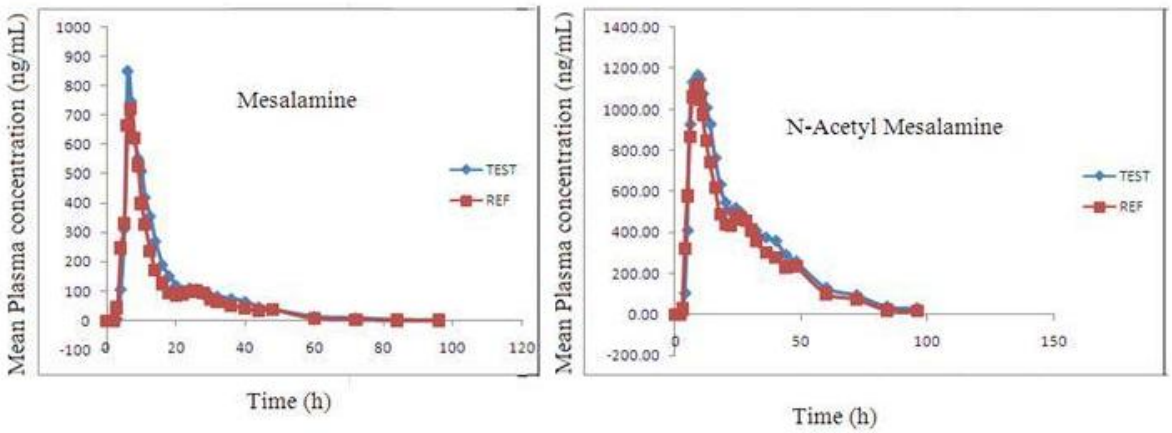

Figure 7. Mean plasma concentrations versus time graph of milnacipran and N-Acetyl mesalamine

Table5. Mean pharmacokinetic parameters for (a) mesalamine, and (b)N-Acetyl mesalamine. T and R were treatment sequence assignments.

(a)

	Cmax(ng/mL)		Tmax(h)		$AUC_{0\text{-}tlast}$		$AUC_{0\text{-}\infty}$	
	T	R	T	R	T	R	T	R
Mean	1275.96	1155.74	9.18	10.43	8192.16	8210.63	8469.62	8553.22
SD	861.52	730.30	4.46	5.93	3589.37	3583.30	3644.14	3813.59
Min	113.35	210.18	4.00	4.00	1691.96	1619.39	1807.40	1653.72
Median	999.43	913.96	8.00	9.00	8135.24	8270.01	8871.14	8399.75
Max	3704.27	2910.73	24.00	32.00	17442.71	19011.11	17460.41	19032.36
CV(%)	67.50	63.20	48.60	56.90	43.80	43.60	43.00	44.60

(b)

	C_{max}(ng/mL)		T_{max}(h)		$AUC_{0\text{-}tlast}$		$AUC_{0\text{-}\infty}$	
	T	T	T	T	T	R	T	R
Mean	1777.03	11.32	11.32	13.91	27773.79	27773.79	30537.83	30072.30
SD	1247.60	6.36	6.36	8.22	13154.38	13154.38	16038.56	17043.46
Min	75.85	5.00	5.00	5.00	318.43	318.43	389.94	4081.43
Median	1587.65	9.00	9.00	11.00	28911.73	28911.73	30813.78	27667.55
Max	5463.41	30.00	30.00	40.00	56070.22	56070.22	68121.11	83529.93
CV(%)	70.20	56.20	56.20	59.10	47.40	47.40	52.50	56.70

Table 6. Test/Reference ratios of C_{max}, $AUC_{0\text{-}tlast}$, and $AUC_{0\text{-}\infty}$ for mesalamine and N-Acetyl mesalamine following the oral administration of 400 mg mesalamine enteric coated tablet formulations

Mesalamine						
Dependent	TestGeoLSM	RefGeoLSM	Ratio[%Ref]	CI_90_Lower	CI_90_Upper	Power
ln(AUCINF_obs)	7582.05	7660.48	98.98	88.91	110.19	0.96
ln(AUClast)	7327.56	7393.20	99.11	88.92	110.47	0.96
ln(Cmax)	983.14	927.88	105.96	97.77	114.83	1.00
N-Acetyl mesalamine						
Dependent	TestGeoLSM	RefGeoLSM	Ratio[%Ref]	CI_90_Lower	CI_90_Upper	Power
ln(AUCINF_obs)	23112.34	25078.13	92.16	69.88	121.55	0.37
ln(AUClast)	21346.59	23032.79	92.68	70.63	121.61	0.38
ln(Cmax)	1319.09	1236.11	106.71	82.70	137.70	0.42

4. CONCLUSION

A method has been developed and validated over the concentration range of 2 - 1500 ng/mL for mesalamine and 10 - 2000 ng/mL for N-Acetyl mesalamine in human plasma. The selectivity, sensitivity, precision and accuracy obtained with this method enabled to test the present study. The validated method was successfully applied in 34 healthy volunteers and demonstrated the bioequivalence of the 400 mg mesalamine enteric-coated tablet formulations of test product (APLRC) as well as the reference product (Mesacol®) .The results concluded that the two formulations can be used interchangeably.

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AUTHORS' STATEMENTS

Competing Interests

The authors declare no conflict of interest. Decision about design and conduct of the statistical analysis, interpretation of the results, as well as preparation and submission of the manuscript was made by the authors and was not influenced by others.

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