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AN IMPROVED STABLE ISOTOPE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF N-ACETYL-5-AMINOSALICYLIC ACID AND ITS DERIVATIZED PARENT, 5-AMINOSALICYLIC ACID IN HUMAN PLASMA

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ABSTRACT

A new stable isotope LC-MS/MS for the determination of 5-aminosalicylic acid (5-ASA) and N-acetyl-5-ASA in human plasma was developed and validated. The objective of this study was to develop and validate an improved selective LC-MS/MS method for the simultaneous determination method of 5-ASA as its derivatized product, N-propionyl-5-ASA along with its principal phase II acetylated metabolite, N-acetyl-5-ASA using 5-ASA D3 and N-acetyl-5-ASA D3 as internal standards. Derivatization step is necessary to convert the ionizable amino group to non ionizable N-propionyl amino moiety in 5-ASA and 5-ASA D3 by using propionic anhydride, followed by liquid-liquid extraction with ethyl acetate (acidified medium) from human plasma. Lower limit of quantification was found to be 5 ng/mL and 7.5 ng/mL for 5-ASA and N-acetyl-5-ASA respectively. The temperature of column and autosampler were maintained at 40°C and 4°C, respectively. The method validation were carried out as per the USFDA guidelines as described, showing a linearity system ($r^2 > 0.99$) over a range of 5 ng/mL to 1000 ng/mL for 5-ASAand 7.5 ng/mL to 1500 ng/mL concentrations for N-acetyl-5-ASAand a recovery shows 98.8% and 78.8% for 5-ASA and N-acetyl-5-ASA respectively. Intra-assay and inter-assay precision and accuracy data were within the acceptable range. Stability studies indicate that both 5-ASA and N-acetyl-5-

ASA were reasonably stable within the given test condition. The deuterated internal standards used in the study helped in overall assay performance and the accuracy of the data This validated method can be successfully applied for the quantification of 5-ASA and its acetylated metabolite to support pharmacokinetic or bio-studies.

KEYWORDS: 5-ASA, N-acetyl-5-ASA, LC-MS/MS Method validation.

INTRODUCTION

5-ASA, the drug most often used in the treatment of inflammatory bowel diseases (IBD), especially for ulcerative colitis and crohn's disease. Its mechanism of action is uncertain but atleast it inhibits local prostaglandin and leukotriene synthesis in the gastro intestinal mucosa. Sulphasalazine is a prodrug comprising 5-aminosalicylic acid (5-ASA) and sulphapyridine joined by an azo bond. Azo-bond linkage with the carrier molecule sulphapyridine enables 5-ASA to reach the colon, where bacterial enzymes split the azo bond liberating two components. Orally administered 5-ASA is rapidly absorbed, though with low efficiency from the upper gastro intestinal tract. In the gut wall and in the liver 5-ASA is metabolized by the enzyme, N-acetyl transferase I mainly to its N-acetyl-5-ASA derivative, which is the major metabolite present in the blood and plasma.[1-3]

A simple less sensitive LC-MS/MS method was reported with lower limit of quantification of 50 ng/ml for both 5-ASA and its metabolite, N-acetyl-5-ASA in human plasma. HPLC methods with ultraviolet, electrochemical detection were also reported. HPLC-FLD method for the simultaneous determination of 5-ASA and its metabolite in human plasma with 23 min run time analysis was reported which would be costly affair for less selective HPLC method. 5-ASA derivatized to N-acetyl-5ASA was studied along with its principle metabolite where the analysis was carried in two aliquots to differentiate the exogenous and endogenous N-acetyl-5-ASA. Chromatographic methods for 5-ASA in human bile was reported using HPLC-FLD, which was complicated and time consuming sample extraction procedure, for which LLOQ was found to be 100 ng/ml.[4-12]

This work explains about bioanalytical LC-MS/MS method which involves deproteination and derivatization of plasma samples using propionic anhydride in methanol. Before extracting the samples by using ethyl acetate, 20% formic acid was added to the reaction mixture as both the derivatized parent (N-propionyl-5-ASA) and its acetylated metabolite, N-acetyl-5-ASA extraction would be facilitated in acidified medium into ethyl acetate. The

reliability of sample preparation was confirmed by the presence of 5-ASA-D3 as an internal standard, where derivatized products, N-propionyl-5-ASA and N-propionyl-5-ASA-D3 (internal standard) were detected along with the principle metabolite, N-acetyl-5-ASA and its internal standard N-acetyl-5-ASA-D3 by simultaneous LC-MS/MS determination.

Thus the objective of the present study was to develop and validate an improved selective stable isotope LC-MS/MS method for the simultaneous determination of 5-ASA ant its metabolite N-acetyl-5-ASA in human plasma. This method provides good sensitivity with 5 ng/ml and 7.5 ng/ml for 5-ASA in its derivatized form as 5-propionyl-ASA and its metabolite N-acetyl-5-ASA. Finally the developed method was validated as per the regulatory guidelines.[13]

MATERIALS AND METHODS

Working standards of Mesalamine, N-Acetyl Mesalamine were procured from Sigma, India, 5-ASA D3 and N-Acetyl-5-ASA D3 was procured from Clearsynth Labs, India. HPLC grade Acetonitrile and methanol was purchased from JT Baker chemicals, Mumbai, India. Ammonium acetate GR grade and Ethyl acetate GR grade were supplied from Merck chemicals, Mumbai, India. Propionic anhydride was procured from Rankem chemicals Mumbai, India. Human plasma was procured from Life care voluntary bank, Bangalore. All aqueous solutions and buffers were prepared using deionized and doubly distilled water from a Milli-Q-System throughout the experiment.

MASS SPECTROMETRY AND CHROMATOGRAPHIC CONDITIONS

The LC system, SHIMADZU consists of isocratic pump (LC-20AD Prominence liquid chromatography pump), autosampler (SIL-HTc) and column thermostat. The mobile phase consists of acetonitrile: 2mM ammonium acetate (60:40 v/v) and chromatographic separation was performed at 40°C temperature with flow rate 1.0 ml/min by using Inertsil C8, (4.6 x 125 mm) 3.5 µm column. Mass detection was carried out on a (API 4000, AB SCIEX, USA) equipped with a source of electrospray ionization. The LC-MS/MS detector was operated at unit resolution in Multiple Reaction Monitoring (MRM) mode. The data were acquired using the Analyst 1.5 software. The transitions of molecular ions were optimized as 208.00/164.00, 194.00/107.00, 211.00/167.00 and 197.00/106.80 for 5-ASA, N-acetyl-5-ASA, 5-ASA D3 and N-acetyl-5-ASA D3 respectively.

PREPARATION OF WORKING STANDARD/QUALITY CONTROL (QC) SOLUTIONS

The stock solution preparation of 5-ASA and N-acetyl-5-ASA (Fig 1A & 1B) were prepared in 5.00% ammonia solution (v/v) & water at concentration of 1000 µg/mL each. The mixed calibration curve of working solutions were prepared from above stock solutions using the diluent of Acetonitrile: Water (50:50 v/v) and ranges from 50 ng/mL to 10000 ng/mL concentrations range for 5-ASA and 75 ng/mL to 15000 ng/mL concentrations range for N-acetyl-5-ASA. The mixed quality control working solutions were prepared at concentrations of 50 ng/mL (LLOQQC), 150 ng/mL (LQC), 5000 ng/mL (MQC) and 7500 ng/mL (HQC) respectively using the diluents [Acetonitrile: Water (50:50 v/v)] for 5-ASA and 75 ng/mL (LLOQQC), 225 ng/mL (LQC), 7500 ng/mL (MQC) and 11250 ng/mL (HQC) respectively using diluent [Acetonitrile: Water (50:50 v/v)] for N-acetyl-5-ASA. All solution was stored at 2-8°C and was brought to room temperature as when required. The 5-ASA D3 and N-acetyl-5-ASA D3 (Fig 2A & 2B) stock solutions (1 mg/ml each) were used to prepare IS mixture to achieve concentration 500 ng/mL for 5-ASA D3 and 250 ng/mL for N-acetyl-5-ASA D3 using diluent [Acetonitrile: Water (50:50 v/v)]. All solution was stored at 2-8°C and was brought to room temperature as when required.

PREPARATION OF CALIBRATION CURVE STANDARDS AND QUALITY CONTROL SAMPLES

The calibration curve standards and quality control samples were prepared by spiking blank plasma with above working solutions at 5% to preserve the integrity of plasma sample. The mixed calibration curve standards were prepared at concentration of 5 ng/mL to 1000 ng/mL for 5-ASA and 7.5 ng/mL to 1500 ng/mL for N-acetyl-5-ASA. The mixed quality control samples for 5-ASA were prepared at concentrations of 5 ng/mL (LLOQQC), 15 ng/mL (LQC), 200 ng/mL (MQC) and 750 ng/mL (HQC) respectively using pooled human plasma. The quality control samples for N-acetyl-5-ASA were prepared at concentrations of 7.5 ng/mL (LLOQQC), 22.5 ng/mL (LQC), 300 ng/mL (MQC) and 1125 ng/mL (HQC) respectively using pooled human plasma. All aliquots of spiked plasma were transferred to RIA tube and stored at -80 ± 5°C until analysis.

SAMPLE PREPARATION AND EXTRACTION

To 250 µL aliquot of sample in RIA vial, 50 µL (5-ASA D3 and N-acetyl-5-ASA D3) of internal standard solution (Except calibration blank) was added and vortexed for 10 seconds.

Then 250 µL of water was added and vortexed for 30 seconds. To derivatize the aromatic amino group, 25 µL of 10% propionic anhydride in methanol was added and vortexed for 3 mins and the samples were kept for 15 mins at room temperature to yield N-acyl-ASA. Then the reaction mixture was acidified by adding 20 µL of 20% formic acid in methanol and vortexed for 3 mins. Finally 3 ml of ethyl acetate was added and vortexed for 5 min. Samples were centrifuged for 5 min at 4000 rpm at 5°C. Plasma layer was flash freezed and the organic layer was transferred into another freshly labelled RIA vials. The supernatant was dried under nitrogen for 20 min at 50°C and the residue was reconstituted with 200 µL of mobile phase [(acetonitrile: 2mM ammonium Acetate (60:40 v/v)]. By injecting 10 µL of the sample volume, analysis was performed in LC-MS/MS.

RESULTS AND DISCUSSION

Mass spectrometry

Initially, the precursor and product ions were optimized by infusing 500 ng/mL solutions in the mass spectrometer between m/z 50 and 400 range in the positive as well as negative mode. However, sensitivity with protonated precursor ion in the positive mode was less than the deprotonated precursor ion in the negative mode. Hence negative ionization mode was selected. Further, the use of mobile phase improved the response of precursor [M-H] + ions at m/z 208.0, 194.0, 211.0 and 197.0 for 5-ASA (derivatized to N-propionyl-5-ASA), N-acetyl-5-ASA, 5-ASA D3 (derivatized to N-propionyl-5-ASA D3) and N-acetyl-5-ASA D3 respectively. Most intense and consistent product ions for 5-ASA (derivatized to N-propionyl-5-ASA), N-acetyl-5-ASA, 5-ASA D3 (derivatized to N-propionyl-5-ASA D3) and N-acetyl-5-ASA D3 were found at m/z 164.0, 107.0, 167.0 and 1006.8 respectively by applying 25 eV collision energy. The MRM parameters like nebulizer gas, heater gas flow, ion spray voltage and source temperature were suitably optimized to obtain a consistent and adequate response for both the parent and metabolite. A dwell time of 200 ms for 5-ASA (derivatized to N-propionyl-5-ASA), N-acetyl-5-ASA, 5-ASA D3 (derivatized to N-propionyl-5-ASA D3) and N-acetyl-5-ASA D3 was adequate and no cross talk was observed between their MRMs.

Optimization of extraction procedure

The derivatization step consisted of an acylation of the primary aromatic amino group of 5-ASA, in order to suppress the amphoteric properties {(-NH_3^+; pK_a=6), carboxylic group (-COOH; pK_a=3) and phenolic group (-OH; pK_a=13.9) of 5-ASA. The ionizable amino group

was masked as a non-ionizable N-acylamino moiety which possessed higher lipophilicity and as well as extractable at lower pH. Propionic anhydride was selected as N-acylation agent, with a view that other derivatives of the homologous series (N-formyl-, N-acetyl and N-butyryl-5-ASA) could be present in the biomatrices as authentic metabolites of 5-ASA. 25 µl of 10% propionic anhydride added to 500 µL of plasma mixture containing 5-ASA was found to be linear enough to obtain derivatized product in the plasma and aqueous samples. The derivatization of the samples was performed at 25°C and accomplished within 15 mins to yield desired N-propionyl-5-ASA and N-propionyl-5-ASA D3 in the reaction mixture. Extraction solvents like ethyl acetate, t-butylmethyl ether, diethyl ether were tested for the liquid-liquid extraction of N-acyl-ASA derivatives from the plasma mixture. Ethyl acetate (in acidified medium) was selected as suitable solvent due to its better consistency across the concentrations. Deuterated internal standards had similar extraction recovery as the non-labelled analytes.

Chromatographic Separation

Separation of 5-ASA (derivatized to N-propionyl-5-ASA) and its metabolite N-acetyl-5-ASA was tried in different columns from the available resources like Inertsil C8 (4.6 x 125 mm, 3.5 µm), Symmetry C18 (100 mm × 4.6 mm, 5 µm), ACE CN (100 mm × 4.6 mm, 5 µm), Cosmosil C18 (100 mm × 4.6 mm, 5 µm) and Alltima C18 (150 mm × 4.6 mm, 5 µm). Initial chromatographic conditions were optimized using different mobile phase combinations such as 10 mM ammonium formate/acetonitrile, 5 mM ammonium acetate/acetonitrile and 2 mM ammonium acetate/acetonitrile in order to attain adequate retention and separation, short run time, symmetric peak shape and sufficient response for both the analytes. The best chromatographic conditions as a function of analyte peak intensity, peak shape, adequate retention and analysis run time were achieved on Inertsil C8 (4.6 x 125 mm, 3.5 µm) column using 2 mM ammonium acetate (PH 4-0) and acetonitrile (30:70, v/v) as mobile phase under isocratic conditions.

Method Validation

Selectivity

Representative MRM chromatograms of extracted K_2-EDTAblank human plasma with IS (Fig 3A & 4A), 5-ASA and N-acetyl-5-ASA at LLOQ concentration (Fig. 3B & 4B) demonstrate the selectivity of the method. No endogenous compounds were found to interfere at the retention time of 5-ASA and N-acetyl-5-ASA respectively.

Sensitivity

The individual standard curve data from three runs met all of the preset criteria: i) <20% deviation from the nominal concentration at the limit of quantification (LLOQ), which was defined as the lowest standard, ii) <15% deviation of standards from their back calculated concentration, other than LLOQ from nominal concentrations, iii) at least six out of eight nonzero standards of each nominal concentration meeting the above criteria, including the LLOQ and the calibration standard at the highest concentration. This quantification method for the simultaneous determination of 5-ASA and N-acetyl-5-ASA was found to be sensitive with LLOQ of 5 ng/mL and 7.5 ng/mL respectively.

Linearity

Linearity was used to confine the performance of the method. A linear least squares regression with a three calibration curves were prepared using eight non-zero standards ranging from 5 ng/mL to 1000 ng/mL for 5-ASA and 7.5 ng/mL to 1500 ng/mL for 5-ASA and N-acetyl-5-ASA respectively. Peak area ratios of each drug to IS were used for regression analysis. A linear regression model (y=mx+c) was evaluated, using $1/X^2$ as weighting factor, where X is the concentration of each drug and y corresponds to the areas ratio. The regression coefficients (r^2) for the three runs were greater than 0.98 for 5-ASA and N-acetyl-5-ASA respectively (Table 1A & 1B).

Accuracy and precision

The precision and accuracy were assessed by analyzing method validation samples over three runs. The percentage accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values. Precision was calculated in terms of coefficient of variation (CV %) The intra-assay precision and accuracy was assessed by analyzing six replicates at each QC level, while the inter-assay precision and precision was determined over three runs conducted on two days by analyzing as many samples. At each concentration level a deviation within ± 15.0% from the nominal concentration was acceptable except LLOQ, for which it should be within ± 20.0%. Minimum 67% (4 out of 6) of the quality control samples at each level should meet the acceptance criteria. Accuracy and precision data for the parent, 5-ASA and its metabolite N-acetyl-5-ASA were presented in Tables 2A and 2B respectively. Inter-day precision and accuracy data was presented in Table 3 for 5-ASA and N-acetyl-5-ASA respectively.

Recovery and ion suppression

Absolute recovery percentage was determined by comparing the mean peak area obtained by injecting six extracted samples of LQC, MQC and HQC with the mean peak area obtained by injection of respective aqueous standard solutions reconstituted in extracted blank matrix. The mean extraction recovery for 5-ASA and N-acetyl-5-ASA was 98.8 and 78.8 respectively. Moreover, the internal standard normalized matrix factors were obtained ranging from 0.99 to 1.02 for 5-ASA and 0.99 for N-acetyl-5-ASA respectively (Table 4). All the values were close to 1.0, which indicates minimum matrix interference with stable isotope labelled IS which would efficiently compensate for any possible ion suppression or enhancement.

Stability studies

The stability studies were evaluated using LQC-5 ng/mL and HQC-750 ng/mL for 5-ASA and LQC-7.5 ng/mL and HQC-1125 ng/mL for N-acetyl-5-ASA respectively. To evaluate the freeze–thaw stability six aliquots of LQC and HQC samples were freezed at -80°C and thawed followed by again freezing and thawing. Samples were analyzed after the third cycle, along with fresh reference samples of the same concentration. Back-calculated concentrations of third freeze–thaw cycle quality control samples versus fresh QC samples were within the acceptance limit (Table 5).

To evaluate the Bench top stability, six aliquots of LQC and HQC were maintained at room temperatures for approximately 10 h without processing (which exceeds the time that samples normally remain at room temperature). After a stipulated period of storage over bench, stability samples were processed and analyzed against fresh CC and QC and found to be stable for both the derivatized parent and its metabolite (Table 5).

Autosampler stability was tested by keeping the processed QC samples in the autosampler for approximately 50 h at 5°C. After a stipulated period, samples were analyzed along with the fresh QC samples and found to be stable for both the derivatized parent and its metabolite. The autosampler stability (5°C) in the above tested condition was within their acceptable limits (Table 5).

Table 1 A: Concentration response data of precision and accuracy batch: Linearity of 5-ASA.

5-ASA	STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H
	5.00	**10.00**	**50.00**	**100.00**	**200.00**	**400.00**	**800.00**	**1000.00**
1	4.93	10.19	53.86	93.45	211.40	390.58	727.63	1040.84
2	4.99	9.88	54.15	99.93	205.75	398.20	742.73	979.50
3	5.22	9.06	51.51	98.53	207.42	408.93	755.15	1031.37
Mean	**5.04**	**9.71**	**53.17**	**97.31**	**208.19**	**399.23**	**741.84**	**1017.24**
S.D (+/-)	**0.15**	**0.58**	**1.45**	**3.41**	**2.90**	**9.22**	**13.78**	**33.02**
C.V. (%)	**3.06**	**6.02**	**2.72**	**3.50**	**1.39**	**2.31**	**1.9**	**3.2**
% Nominal	**100.89**	**97.10**	**106.35**	**97.31**	**104.10**	**99.81**	**92.7**	**101.7**
N	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

Table 1 B: Concentration response data of precision and accuracy batch: Linearity of N-acetyl-5-ASA.

N-acetyl-5-ASA	STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H
	7.50	**15.00**	**75.00**	**150.00**	**300.00**	**600.00**	**1200.00**	**1500.00**
1	7.49	14.97	76.86	147.37	305.86	596.78	1134.34	1554.45
2	7.26	15.82	78.39	149.44	311.73	614.39	1085.39	1452.78
3	7.75	13.94	76.60	147.38	310.13	637.18	1151.41	1468.02
Mean	**7.50**	**14.91**	**77.28**	**148.06**	**309.24**	**616.12**	**1123.71**	**1491.75**
S.D (+/-)	**0.24**	**0.94**	**0.96**	**1.19**	**3.03**	**20.25**	**34.27**	**54.83**
C.V. (%)	**3.25**	**6.31**	**1.25**	**0.81**	**0.98**	**3.29**	**3.0**	**3.7**
% Nominal	**99.99**	**99.40**	**103.04**	**98.71**	**103.08**	**102.69**	**93.6**	**99.5**
N	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

Table 2 A: Intra-Run Precision and Accuracy (PA) (Batch 01, 02 and 03) of 5-ASA.

5-ASA	Concentration (ng/mL)			
	LLOQ QC	**LQC**	**MQC**	**HQC**
	5	**15**	**500**	**750**
PA BATCH-01				
Mean	4.95	15.07	493.26	733.31
SD	0.20	0.47	6.51	11.03
C.V.%	4.08	3.13	1.32	1.50
% Nominal	98.98	100.50	98.65	97.77
N	6	6	6	6
PA BATCH-02				
Mean	5.27	15.56	494.88	739.95
SD	0.23	0.73	6.93	27.35
C.V.%	4.40	4.69	1.40	3.70
% Nominal	105.41	103.73	98.98	98.66
N	6	6	6	6
PA BATCH-03				
Mean	4.91	14.57	502.42	756.51
SD	0.30	0.35	6.10	10.88
C.V.%	6.18	2.43	1.22	1.44
% Nominal	98.27	97.13	100.48	100.87
N	6	0	6	6

Table 2 B: Intra-Run Precision and Accuracy (PA) (Batch 01, 02 and 03) of N-acetyl-5-ASA.

N-acetyl-5-ASA	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	7.5	22.5	750	1125
PA BATCH-01				
Mean	8.30	24.40	816.75	1221.29
SD	0.07	0.32	19.45	20.94
C.V.%	0.85	1.30	2.38	1.71
% Nominal	110.70	108.43	108.90	108.56
N	6	6	6	6
PA BATCH-02				
Mean	8.02	24.00	829.57	1236.64
SD	0.25	0.65	8.01	20.18
C.V.%	3.07	2.72	0.97	1.63
% Nominal	106.92	106.67	110.61	109.92
N	6	6	6	6
PA BATCH-03				
Mean	7.54	23.57	810.83	1198.66
SD	0.39	0.31	14.88	12.51
C.V.%	5.21	1.34	1.83	1.04
% Nominal	100.54	104.75	108.11	106.55
N	6	0	6	6

Table 3: Inter-Run Precision and Accuracy (PA) (Batch 01, 02 and 03) of 5-ASA and N-acetyl-5-ASA.

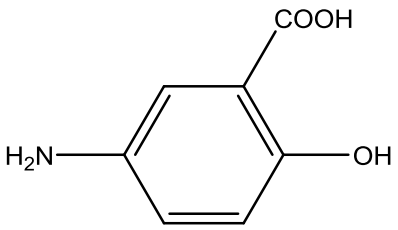
5-ASA	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	5	15	500	750
Mean	5.04	15.07	496.86	743.26
SD	0.29	0.66	7.38	19.78
C.V.%	5.69	4.36	1.48	2.66
% Nominal	100.80	100.47	99.37	99.10
N	18	18	18	18
N-acetyl-5-ASA	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	7.5	22.5	750	1125
Mean	7.95	23.99	819.05	1218.86
SD	0.41	0.55	16.12	23.50
C.V.%	5.18	2.31	1.97	1.93
% Nominal	106.00	106.62	109.21	108.34
N	18	18	18	18

Table 4: Recovery and matrix factor of 5-ASA and N-acetyl-5-ASA.

QC	% Recovery	
	5-ASA	N-acetyl-5-ASA
LQC	110.3	83.5
MQC	96.2	80.3
HQC	89.9	78.8
Mean Recovery	98.8	80.8
QC	**Matrix Factor**	
	5-ASA	**N-acetyl-5-ASA**
LQC	1.03	0.99
HQC	0.99	0.99

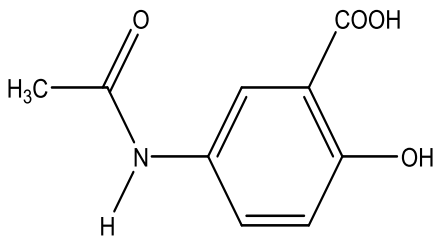
Table 5: Stability studies of 5-ASA and N-acetyl-5-ASA.

Stability Studies	QC	Nominal Conc (ng/mL)	Observed Conc (ng/mL)	% CV	% Stability
5-ASA					
Bench Top (10 h)	LQC	15	15.51	2.57	103.40
	HQC	750	756.81	2.10	100.91
Freeze Thaw (3 cycles)	LQC	15	15.70	1.85	104.64
	HQC	750	755.48	1.25	100.73
Autosampler (50 h)	LQC	15	15.27	2.08	101.80
	HQC	750	760.65	0.78	101.42
N-acetyl-5-ASA					
Bench Top (10 h)	LQC	22.5	23.63	1.24	105.02
	HQC	1125	1202.47	0.64	106.89
Freeze Thaw (3 cycles)	LQC	22.5	23.81	1.53	105.83
	HQC	1125	1214.04	1.15	107.91
Autosampler (50 h)	LQC	22.5	25.28	13.58	112.36
	HQC	1125	1209.38	1.49	17.50



5-Aminosalicylic acid

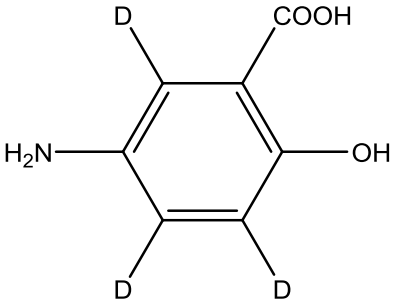
[A]



N-acetyl-5-aminosalicylic acid

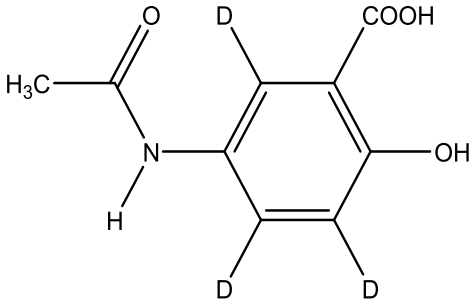
[B]

Figure 1. The chemical structure of 5-ASA [A] and its major metabolite N-acetyl-5-ASA [B].



5-aminosalicylic acid-D3

[A]



N-Acetyl-5-aminosalicylic acid-D3

[B]

Figure 2. The chemical structure of isotope labelled internal standards 5-ASA-D3 [A] and its N-acetyl-5-ASA-D3 [B].

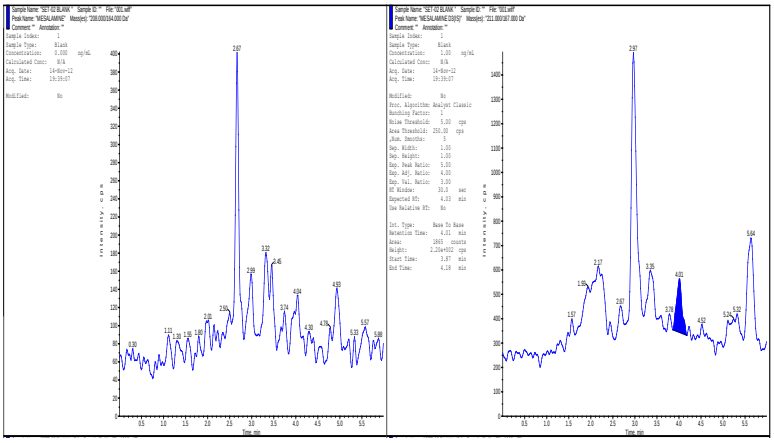


Figure 3(A). Typical MRM Chromatogram of 5-ASA and 5-ASA D3 (IS) in blank extracted K_2-EDTA human plasma.

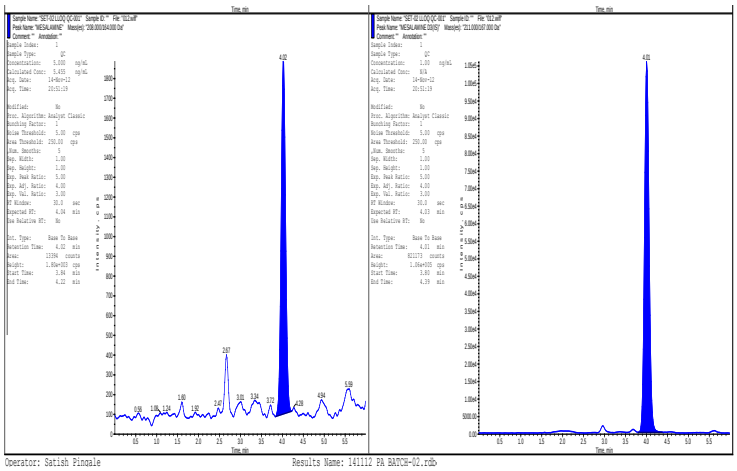


Figure 3(B). Typical MRM Chromatogram of 5-ASA and 5-ASA D3 (IS) in K_2-EDTA human plasma at LLOQ concentration (5 ng/mL).

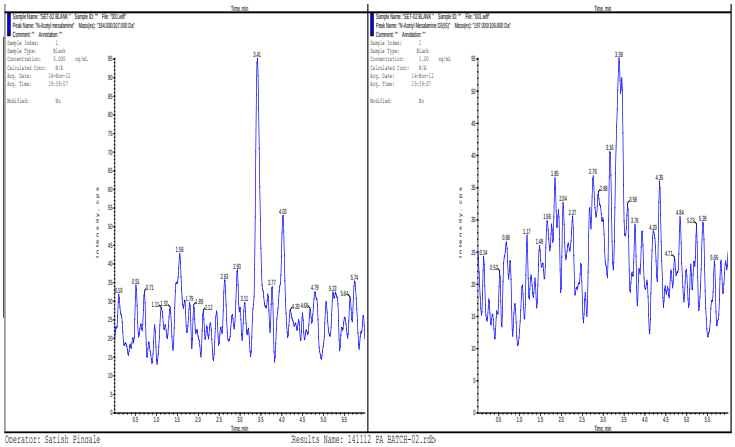


Figure 4(A). Typical MRM Chromatogram of N-acetyl-5-ASA and N-acetyl-5-ASA D3 (IS) in blank extracted K_2-EDTA human plasma.

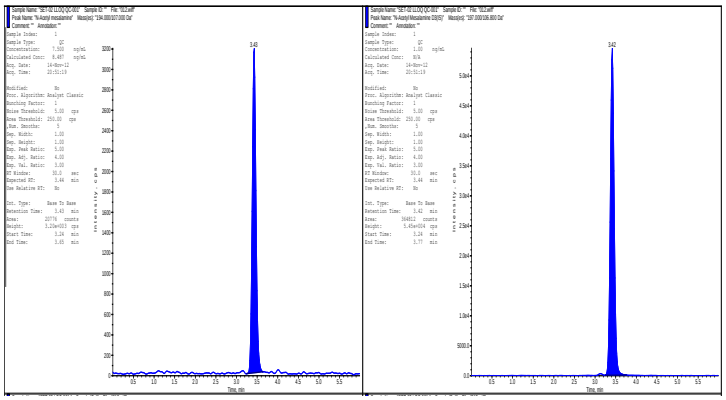


Figure 4(B). Typical MRM Chromatogram of N-acetyl-5-ASA and N-acetyl-5-ASA D3 (IS) K_2-EDTA human plasma at LLOQ concentration (7.5 ng/mL).

CONCLUSIONS

A new LC-MS/MS method was developed and validated for the simultaneous determination of 5-ASA and its principal phase II metabolite, N-acetyl-5-ASA by using stable isotopes as internal standards. This method involves a lipophilicity-increasing derivatization step followed by liquid-liquid extraction and subsequent analysis by high performance liquid chromatography coupled with tandem mass spectrometry. LLOQ was found to be 5 ng/ml and 7.5 ng/ml for 5-ASA in its derivatized form as N-propionyl-5-ASA and its metabolite N-acetyl-5-ASA. The method possessed excellent precision and accuracy and proved to be reliable as the derivatization and extraction process was found to be consistent from biological matrix. Lowest limit of quantification achieved in the present work was relatively sensitive to all previous assays Thus the present validated method would be more selective than the stand alone HPLC-UV or HPLC fluorescence method owing to its utilization of mass

spectrometer and sensitive to detect the biological concentration for supporting pharmacokinetic or bio-studies.

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