Journal of Chromatography B, 872 (2008) 99–106

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a HPLC-ESI-MS/MS method for the determination of 5-aminosalicylic acid and its major metabolite *N*-acetyl-5-aminosalicylic acid in human plasma

Elisabetta Pastorini[a], Marcello Locatelli[a], Patrizia Simoni[b], Giulia Roda[b], Enrico Roda[b], Aldo Roda[a,*]

[a] *Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy*
[b] *Department of Internal Medicine and Gastroenterology, University of Bologna, Via Massarenti 9, I-40138 Bologna, Italy*

ARTICLE INFO

Article history:
Received 14 February 2008
Accepted 17 July 2008
Available online 29 July 2008

Keywords:
Mesalazine
HPLC-ESI-MS/MS
Method validation
Pharmacokinetic
IBD

ABSTRACT

A new HPLC method for the determination of 5-aminosalicylic acid (5-ASA) and *N*-acetyl-5-aminosalicylic acid (*N*-Ac-5-ASA) in human plasma was developed and validated. Plasma samples were analyzed after protein precipitation with methanol and the two analytes were separated using a C18 column with a mobile phase composed of 17.5 mmol/L acetic acid (pH 3.3):acetonitrile = 85:15 (v/v) at 0.2 mL/min flow rate. 4-ASA and *N*-Ac-4-ASA were used as internal standards. Selective detection was performed by tandem mass spectrometry with electrospray source, operating in negative ionization mode and in multiple reaction monitoring acquisition (m/z 152 → 108 for 5-ASA; m/z 194 → 150 and 194 → 107 for *N*-Ac-5-ASA). The limit of quantification (LOQ) was 50 ng/mL for both analytes (0.2 ng injected) and matrix-matched standard curves showed linearity up to 4000 ng/mL. In the entire analytical range the within- and between-batch precision (R.S.D.%) values were respectively ≤6.3% and ≤11% for 5-ASA and ≤8.0% and ≤10% for *N*-Ac-5-ASA. For both analytes the within- and between-batch accuracy (bias%) values ranged respectively from −8.4% to 7.9% and from −7.9% to 8.0%. The overall recoveries ($n = 6$) at three tested concentration levels (i.e. 100, 1000 and 4000 ng/mL) were respectively >90% for 5-ASA and >95% for *N*-Ac-5-ASA (R.S.D.% ≤ 10%). The method was applied to evaluate the pharmacokinetic of 5-ASA after a single oral dose administration of this compound (1200 mg) to 24 healthy volunteers. The mean maximum concentration levels were 680 ng/mL for 5-ASA and 1240 ng/mL for *N*-Ac-5-ASA and the kinetic profiles were in agreement with previous studies.

1. Introduction

5-Aminosalicylic acid (mesalazine, 5-ASA) is a drug widely used in the treatment of inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's diseases [1–3]. Its mechanism of action is not yet fully understood: it seems to act locally, at the colonic mucosa level, since systemic concentrations following oral dosing are very low [4]. 5-ASA inhibits local prostaglandin and leukotriene synthesis in the gastrointestinal mucosa [5]. Other studies have proved that 5-ASA is a potent-free radical scavenger, thus suppressing toxicity of reactive oxygen species [6–8]. All these properties seem to play an important role in reducing the acute inflammatory response. When orally administered, 5-ASA is rapidly absorbed, although with low efficiency, from the upper gastrointestinal tract [9]. In the gut wall and in the liver 5-ASA is metabolized by the *N*-acetyltransferase I enzyme mainly to its *N*-acetyl-5-ASA (*N*-Ac-5-ASA) derivative [10–12]. This compound, considered therapeutically inert [13], is the major metabolite present in blood [14,15]. In plasma, both 5-ASA and *N*-Ac-5-ASA are found 40–50% and 80%, respectively bound to proteins [16]. The knowledge of pharmacokinetic and metabolism of 5-ASA from mesalazine-containing drugs is mandatory when new drug formulations are developed for the treatment of IBDs, and therefore validated analytical methods are needed for bioequivalence studies.

Due to the presence of the primary aromatic amino group ($-NH_3^+$ $pK_a = 6$), carboxylic group (–COOH $pK_a = 3$) and phenolic group (–OH $pK_a = 13.9$) in the molecule [17], 5-ASA exhibits amphoteric properties, which, together with its high polarity, complicate its extraction, separation and detection [16]. A number of analytical methods have been developed for the analysis of 5-ASA and its metabolite in many biological matrices, e.g. plasma [16,18–23], serum [24], feces [24], urine [18,19,22–24], stones [25] and rectal biopsies [23,24]. These methods include HPLC combined with

* Corresponding author. Tel.: +39 051 343398; fax: +39 051 343398.
E-mail address: aldo.roda@unibo.it (A. Roda).
URL: http://www.anchem.unibo.it (A. Roda).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.07.026

UV [16,25–27], fluorescence (FL) [14–16,18,20,28,29], and electrochemical (EC) [21] detections. The preanalytical step for human plasma analysis includes protein precipitation with acetonitrile [15], methanol [21,26,28,30] or perchloric acid [14,16,29]. Most of the previously developed HPLC methods required a preanalytical derivatization step [16,18,28,29], consisting in acylation of the primary amino group followed by liquid–liquid or solid phase extraction [16], resulting in time-consuming procedures potentially affected by variability of reaction yield. Separation of 5-ASA and *N*-Ac-5-ASA derivative is usually performed by reversed-phase chromatography [16,18,28,29]. The direct analysis were carried out using ion pair methods [21,27] and, although affected by poor retention of 5-ASA, reversed-phase chromatography [20,25].

HPLC combined with mass spectrometry (MS) using electrospray interface is now recognized as a powerful tool for both confirmatory and quantitative analyses, owing to its high sensitivity and selectivity, and therefore highly recommended for pharmacokinetic studies in complex matrices [31]. Multiple reaction monitoring (MRM) mode with triple quadrupole tandem mass spectrometry (MS/MS) enables selective and accurate analyses over a wide linear range. In recent years, GC coupled with electron impact ionization-ion trap MS [25] and LC with electrospray (ESI) source-ion trap MS [16] were used for confirmation of 5-ASA and *N*-Ac-5-ASA in biological samples. Moreover, for these analytes very few applications of the HPLC-MS/MS technique for quantitative purpose are reported in clinical journals, although experimental details and data regarding analytical aspects are not described at all [19,22,23].

In this paper, a new HPLC-ESI-MS/MS method for the simultaneous determination of 5-ASA and *N*-Ac-5-ASA in human plasma samples is described. Sample treatment required a simple and rapid protein precipitation step, followed by analysis of underivatized compounds, using 4-ASA and *N*-Ac-4-ASA as internal standards. The method was validated according to criteria reported in internationally accepted recommendations for drug analysis in clinical studies [32–33], by evaluating linearity, recovery, precision, accuracy, and stability.

The method was applied for the analysis of human plasma samples collected from 24 healthy volunteers after a single-dose oral administration of 1200 mg of mesalazine in order to evaluate the pharmacokinetic of both 5-ASA and *N*-Ac-5-ASA.

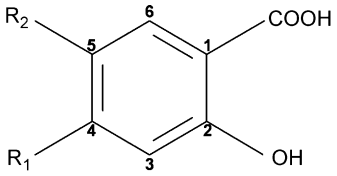
2. Experimental

2.1. Chemicals and reagents

5-ASA ($C_7H_7NO_3$) and its internal standard 4-ASA, both approx. 99%, were purchased from Sigma–Aldrich (Milan, Italy) while *N*-Ac-5-ASA and *N*-Ac-4-ASA were synthesized in laboratory. Chemical structures of the four compounds are shown in Fig. 1.

N-Acetylated derivatives were synthesized from 5-ASA or 4-ASA using acetic anhydride as acetylating agent (Carlo Erba, Milan, Italy), following a previously described procedure [16]. Briefly, 5-ASA or 4-ASA (2 g, 0.013 mol) were dissolved in equimolar acetic anhydride. The reaction mixture was maintained at room temperature under stirring for 3 h, and then allowed to stand overnight. The crude product was filtered off, washed with water, and dried. Then, products were purified and characterized. The identity and purity of individual products were confirmed by NMR, HPLC-DAD, and HPLC/MS. *N*-Acetylated derivatives were found more than 99.5% pure.

All other reagents, acetonitrile (HPLC-grade, RS plus), methanol (HPLC-grade, RS plus), and glacial acetic acid (≥99.8%), were of analytical grade (Carlo Erba Reagents, Milan, Italy). HPLC-grade water was prepared using a Milli-Q Synthesis A10 system (Millipore, Molsheim, France).



	R_1	R_2
5-ASA	- H	- NH_2
4-ASA	- NH_2	- H
N-Ac-5-ASA	- H	- $NHCOCH_3$
N-Ac-4-ASA	- $NHCOCH_3$	- H

Fig. 1. Chemical structures and acronyms of the investigated compounds.

2.2. Calibration standards and quality control samples

Stock solutions of 5-ASA, *N*-Ac-5-ASA, 4-ASA and *N*-Ac-4-ASA were prepared separately by dissolving the crystalline pure powders in methanol in order to achieve a primary concentration of 100 μg/mL, and stored in aliquots at −20 °C.

Working standard solutions were prepared by appropriate dilutions of the 100 μg/mL stock solutions to obtain solutions at concentration levels in the 1.25–50 μg/mL range and stored at −20 °C for a period of time not longer than 4 weeks, as reported by Palumbo et al. [21].

Composite working solution of internal standards (5 μg/mL for 4-ASA and 20 μg/mL for *N*-acetyl-4-ASA) was obtained by diluting 4-ASA stock solution to 10 μg/mL and *N*-Ac-4-ASA stock solution to 40 μg/mL and then mixing equal volumes of these solutions. This solution was stored in 4 mL aliquots at −20 °C for the duration of the study. All stock and working standard solutions were stored in glass tubes. Analyte-free human plasma was collected and pooled to get a sufficient volume to prepare matrix-matched calibration standards and quality control (QC) samples. Separate solutions were used to prepare calibration standards and QC samples. Plasma calibration standards of analytes at concentrations of 50, 100, 200, 400, 1000, 2000, 3000 and 4000 ng/mL, and QC samples at 150, 800 and 2500 ng/mL were prepared by spiking each analyte working standard solution into the human plasma pool aliquots. Volume of working standard solution spiked into plasma was less than 5% of plasma volume.

2.3. Sample preparation

An aliquot of 490 μL plasma was put in disposable plastic tubes and mixed with 10 μL of the internal standard composite working solution to achieve final concentrations of 100 and 400 ng/mL of 4-ASA and *N*-Ac-4-ASA, respectively. For protein precipitation, 1.0 mL of methanol was added to the mixture. The sample was stirred and centrifuged at 12,000 × *g* for 10 min. Then 1.2 mL of the supernatant was transferred into plastic vials and dried under vacuum. The residue was dissolved in 500 μL of water containing 50 mmol/L acetic acid, the solution was centrifuged for a few minutes and transferred into disposable glass autosampler vials. Finally, 4 μL of this solution was injected into the HPLC-ESI-MS/MS system for analysis.

2.4. HPLC-ESI-MS/MS analysis

Liquid chromatography was performed using a 2695 Alliance system from Waters (Milford, MA, USA) equipped with a built-in

autosampler for 120 samples. The analytical column was a Synergi Hydro-RP (4 μm, 150 mm × 2.0 mm i.d.) protected by a guard column (4 μm, 10 mm × 2.0 mm i.d.), both supplied by Phenomenex (Torrance, CA, USA).

Mobile phase composition was 17.5 mmol/L acetic acid water (solvent A) and acetonitrile (solvent B). Separation was achieved under isocratic elution conditions (8 min at 15% B) followed by column purge (5 min at 80% B) and column re-equilibration (12 min at 15% B) at 0.2 mL/min flow rate. HPLC effluent was introduced directly to the electrospray source operating in negative ionization (NI) mode connected to a triple quadrupole mass spectrometer (Quattro LC, Micromass, UK). In order to find the tuning parameters for each compound and then to acquire MS spectra, 5 μg/mL standard solutions in 17.5 mmol/L acetic acid:acetonitrile = 85:15 (v/v) were infused at 40 μL/min by connecting an infusion pump (11Plus, Harvard, Holliston, MA, USA) to the interface.

Nitrogen was used as nebulizer gas at 100 L/h flow rate and as desolvation gas at 675 L/h. Ion source block and desolvation temperatures were set respectively at 120 and 230 °C. Capillary voltage was 2.5 kV and cone voltage was 20 V. Total ion current chromatograms (TIC) were acquired using mass spectrometer in multiple reaction monitoring mode, selecting the following m/z ion transitions: 152 → 108 for 5-ASA and 4-ASA; 194 → 150, 194 → 107 for *N*-Ac-5-ASA; 194 → 108 for *N*-Ac-4-ASA. For quantitative analysis of *N*-Ac-5-ASA, transition m/z 194 → 107 was selected because this transition was more specific and gave higher signal-to-noise ratio (S/N) values than m/z 194 → 150. Dwell time and inter-channel delay values were set respectively to 250 and 30 ms for each selected ion transition. Relative collision energies (RCE), expressed as percent of the maximum instrument voltage difference value (200 V) were 7.5% for 5-ASA and 4-ASA, and 9% for *N*-Ac-5-ASA and *N*-Ac-4-ASA.

Mass-Lynx Version 4.0 software (Micromass) was employed for instrument control, data acquisition, and processing.

2.5. Human plasma samples

5-ASA and *N*-Ac-5-ASA concentrations were measured in human plasma from 24 healthy volunteers (15 females and 9 males, mean age 27.3 ± 3.4 years) using the developed method. Subjects were considered to be appropriate for study after medical examination and clinical laboratory analysis to assess general health. The study was approved by the State authority and the Institutional Ethics Committee (Saint Orsola-Malpighi Hospital, University of Bologna, Italy). Smoking, medication, alcoholic beverage and coffee assumption were restricted 24 h before dosing and during sample collections. The study was conducted in accordance with the current revision of the Declaration of Helsinki concerning medical research in human and with current Good Clinical and Laboratory Practice Guidelines. In addition, volunteers gave written informed consent.

Drug treatment consisted of single oral dose mesalazine administration (1200 mg) as three tablets (400 mg each) of Pentacol 400® from SOFAR (Milan, Italy).

For all subjects involved in the study, venous blood samples (9 mL) were withdrawn via an in-dwelling cannula or by a venepuncture into K_3EDTA-Vacuette® tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) at 0 h (predose), 1, 2, 4, 6, 8, 12, 18, 24, 32, and 48 h post-dose. The samples were centrifuged at 2000 × *g* for 10 min. The resulting plasma fraction was frozen as three separate aliquots in polypropylene tubes at −80 °C together with QC samples.

2.6. Quantification and statistical evaluation

Calibration curves were obtained by analyzing the standards prepared in freshly spiked human plasma according to the above described procedure. Quantitative analysis was performed by extracting from HPLC-ESI-MS/MS dataset the ion currents of the following transitions: m/z 152 → 108 for 5-ASA and 4-ASA; m/z 194 → 107 for *N*-Ac-5-ASA; m/z 194 → 108 for *N*-Ac-4-ASA. Calibration curves were plotted using weighted linear least-squares regression analysis (weighting factor $1/x$) according to the equation $y = a + bx$, where y is the analyte/internal standard peak area ratio, x the concentration (ng/mL) of 5-ASA or *N*-Ac-5-ASA in the calibration samples, a the intercept, and b is the slope of the regression line. The weighting factor was chosen to minimize deviation of back-calculated values from theoretical concentrations. Concentrations of the QC samples and unknown samples were calculated by interpolating their analyte/internal standard peak area ratios on the calibration curve.

Recovery was assessed by comparing peak area values obtained by analyzing analyte-free pool plasma samples spiked before sample preparation with those obtained from pool plasma samples spiked after sample treatment. Matrix effect was evaluated by comparing signals obtained from pool plasma samples spiked after sample treatment with those obtained from standard solutions.

3. Results and discussion

3.1. MS/MS detection optimization

MS optimization was performed in both NI and positive ionization (PI) modes and for different mobile phase compositions to find optimal ESI and fragmentation conditions compatible with the separation step. Although both 5-ASA and *N*-Ac-5-ASA could be detected in both NI and PI modes [16], NI mode provided wider linear range and was therefore selected. Moreover, adducts with sodium ions affecting sensitivity were observed when operating in PI mode. Higher S/N values were obtained in solvents containing weak acids. This is in agreement with studies performed by Wu et al. [34], that described how ionization efficiency in negative ESI is influenced by mobile phase pH. In particular, it was observed that the intensity of the deprotonated ion signal of acidic substances is sometimes enhanced by acidification. The formation of charged droplets in ESI is, indeed, achieved mainly through ion reduction on capillary surface. Positive ions produced by protonation in an acidic environment increase the reduction process rate and enable the spray to carry more easily a negative charge excess, which is then transferred to the analyte. In the present study, acetic acid was preferred to formic acid because of better sensitivity. As an example, MS and MS/MS full scan NI spectra of 5-ASA and *N*-Ac-5-ASA are shown in Fig. 2. Fragments detected were in agreement with those reported by Nobilis et al. [16]. The only fragment formed in MS/MS for both 5-ASA and 4-ASA is m/z 108 $[M–CO_2–H]^-$ (Fig. 2A). In the case of acetyl derivatives two fragments were observed in MS/MS spectra: m/z 150 $[M–CO_2–H]^-$ for both compounds (Fig. 2C) and m/z 108 $[C_6H_6NO]^-$ for *N*-Ac-4-ASA and m/z 107 $[C_6H_5NO]^{\bullet -}$ for *N*-Ac-5-ASA (Fig. 2C). Probably, *N*-acetyl group in *meta* or *para* position with respect to –OH group is the reason for the appearance of different energetically favored product ions for *N*-Ac-4-ASA and *N*-Ac-5-ASA, respectively.

3.2. Optimization of LC separation

Several HPLC method variables influencing separation of 5-ASA, its metabolite, and internal standards were investigated, due to

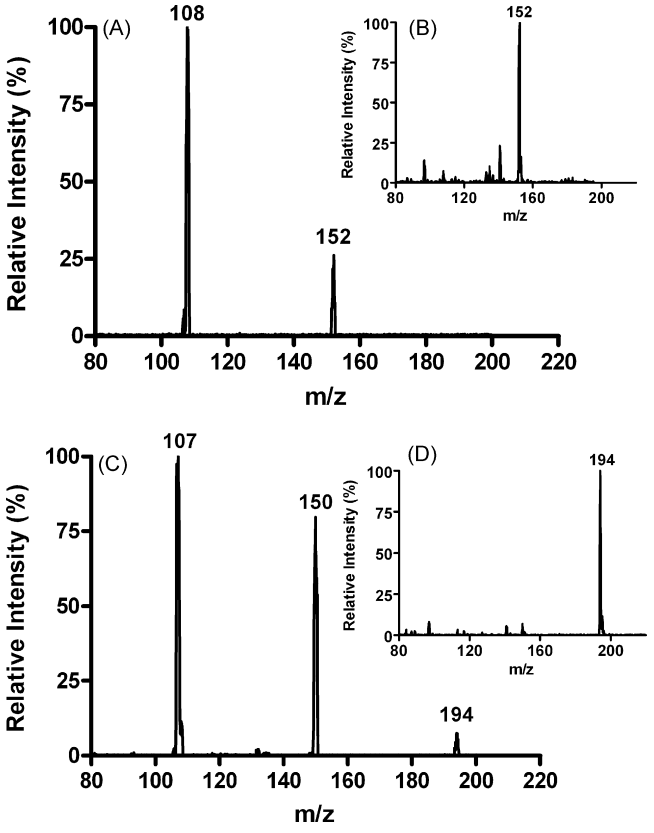


Fig. 2. Fragmentation spectra for (A) 5-ASA and (C) *N*-Ac-5-ASA; ESI-MS spectra for (B) 5-ASA and (D) *N*-Ac-5-ASA. All spectra obtained by direct infusion of a 5 μg/mL solution in 17.5 mmol/L acetic acid water:acetonitrile = 85:15 (v/v).

great difference in the physical properties of these analytes, such as *N*-Ac-5-ASA lipophilicity and 5-ASA polarity and amphoterism. 5-ASA is eluted as a sharp and symmetric peak from reversed-phase columns and separated from *N*-Ac-5-ASA and internal standards under acidic chromatographic conditions (pH < 4.0) with small percentages of organic modifier [16,20,21,25]. In our extensive preliminary experiments a set of different columns, with different lengths and particle size, packed with C18 reversed phase, polar reversed phase or –CN phase, was tested to optimize 5-ASA retention. However, as also reported by other authors [16,20,25], 5-ASA retention time was slightly shorter than twice the column dead time. A series of aqueous mobile phases containing different additives (weak acids or ion pair reagents) with different pH values, in combination with acetonitrile or methanol as organic modifier, were also tested. The best result in terms of ESI response, peak shape, resolution and overall run time was obtained with Sinergi Hydro RP C18 column, using 17.5 mmol/L acetic acid (pH 3.3) and 15% acetonitrile. We found that, at pH > 4.0 or in the presence of NH_4^+ ions, retention times were shortened until coelution of the compounds, with broadened peaks and weakened responses. The addition to water of trifluoroacetic acid, triethylamine or tripropylamine as ion pair agents slightly increased 5-ASA retention time, but strong signal suppression together with irreproducibility was observed. The use of acetonitrile at a percentage lower than 15% did not increase the 5-ASA retention and response was weakened. Use of a more polar solvent, such as methanol, did not allow the increase of 5-ASA retention. In addition, *N*-Ac-5-ASA and both internal standards were strongly retained to require a gradient elution, thus providing different chromatographic conditions for each analyte and its internal standard.

In the optimized analytical conditions, mean retention times ($n = 50$) were as follows: 2.13 ± 0.01 min (R.S.D.% = 0.46%) for 5-ASA; 4.49 ± 0.09 min (R.S.D.% = 2.0%) for *N*-Ac-5-ASA; 6.06 ± 0.08 min (R.S.D.% = 1.3%) for 4-ASA; 6.36 ± 0.12 min (R.S.D.% = 1.9%) for *N*-Ac-4-ASA. Conditions set for column purge and re-equilibration ensured column pressure and chromatogram background stability together with reproducible retention times for at least 100 consecutive injections. Width peak, S/N and system stability during an analytical batch (42 h long) were considered to find the optimal injection volume (4 μL). As an example, a typical MRM chromatogram obtained from analysis of human plasma spiked at 50 ng/mL of both analytes is shown in Fig. 3C–F.

3.3. Selectivity

The selectivity of the method was evaluated by analyzing blank human plasma samples from 10 different subjects. A representative MRM chromatogram obtained for a drug-free human plasma sample is shown in Fig. 3A. No interfering peaks due to endogenous species were observed at the elution time expected for 5-ASA (Fig. 3A), whereas blank plasma extract showed an interfering peak at a retention time close to, but resolved from, *N*-Ac-5-ASA (Fig. 3B). This indicates that the HPLC method with MRM acquisition is suitable for the selective detection of these compounds in human plasma.

3.4. Recovery and matrix effect

Recoveries of 5-ASA and *N*-Ac-5-ASA were assessed at three concentration levels (100, 1000 and 4000 ng/mL). They were >90% for 5-ASA and >95% for *N*-Ac-5-ASA. In addition, recoveries of 4-ASA and *N*-Ac-4-ASA at concentration levels of 100 and 400 ng/mL, respectively, were also evaluated. More details are reported in Table 1.

It is well known and documented in HPLC-ESI-MS analysis that during the ESI process coextracted and coeluted matrix components can decrease the yield of analyte ion production by competition processes [35]. Then, matrix effect was also studied. *N*-Ac-5-ASA was recovered at 92 ± 5%, whereas 5-ASA was recovered at 30 ± 3% ($n = 6$). The latter result can be explained with the poor retention time of 5-ASA in chromatographic elution, which possibly determines coelution with interferences. Intra-matrix variability was also tested by processing plasma samples from 10 different healthy subjects and spiking them with both analytes at 150 ng/mL. Bias values were within the ±15% range, whereas R.S.D.% values were less than 7%. Thus, matrix effect on 5-ASA, even thought present, did not significantly affect the accuracy and precision of this HPLC-ESI-MS/MS method, if matrix-matched calibration procedure was adopted.

3.5. Limit of detection

Limits of detection (LODs) were estimated by measuring S/N values obtained in human plasma spiked at 50 ng/mL level and extrapolating the corresponding values to S/N = 3. LOD value was 15 ng/mL for both 5-ASA and *N*-Ac-5-ASA.

3.6. Linearity and limit of quantification

Calibration curve parameters, derived by the statistical analysis of six independently obtained eight-point calibration curves in plasma, are reported in Table 2. The calibration curves showed a good linearity in the concentration range between 50 and 4000 ng/mL ($R^2 \geq 0.988$ for 5-ASA and $R^2 \geq 0.995$ for *N*-Ac-5-ASA). The back-calculated calibration standard points showed R.S.D.%

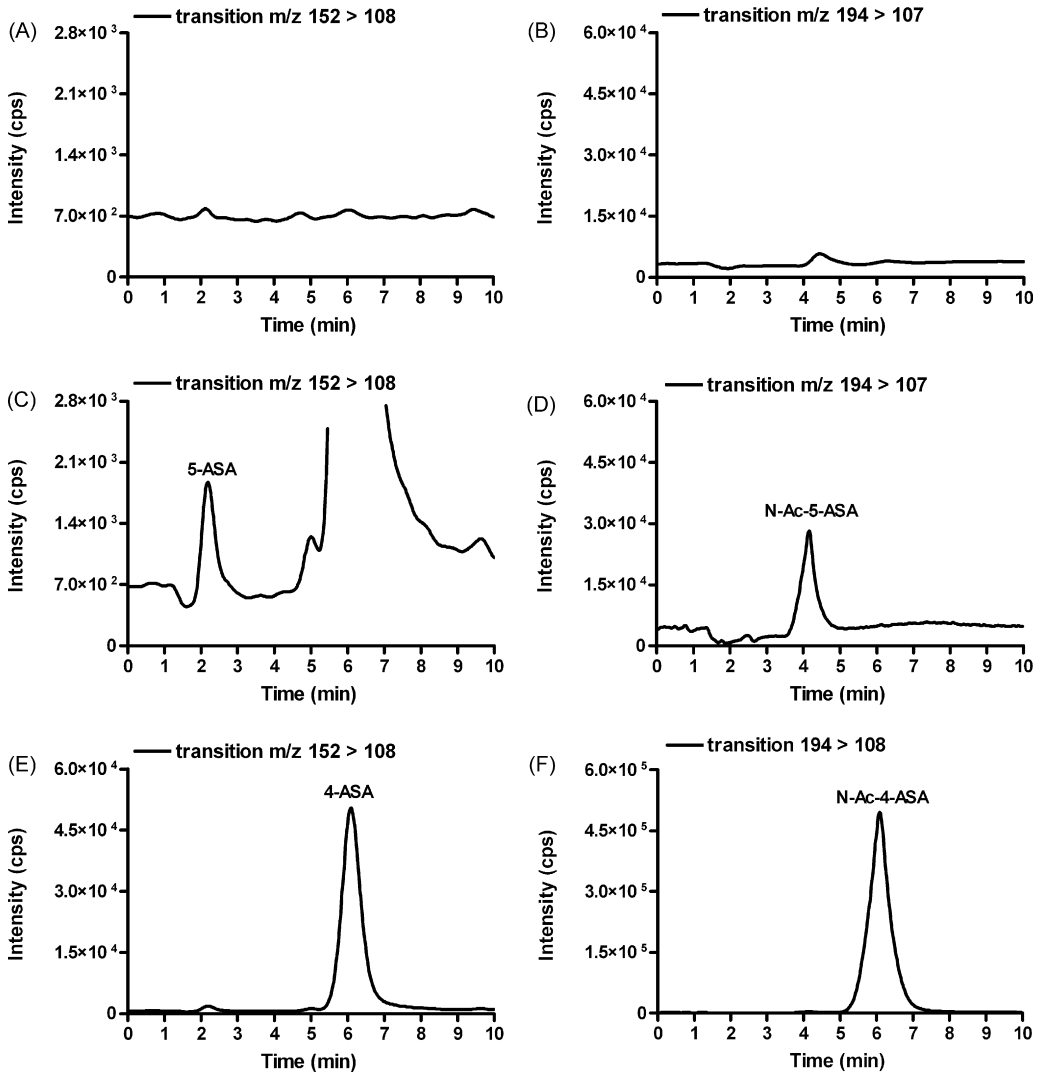


Fig. 3. Typical MRM chromatograms (transition m/z 152 → 108 for 5-ASA and 4-ASA, m/z 194 → 107 for *N*-Ac-5-ASA, 194 → 108 for *N*-Ac-4-ASA) obtained for (A–B) drug-free control human plasma samples, and for human plasma spiked with (C) 50 ng/mL of 5-ASA; (D) 50 ng/mL of *N*-Ac-5-ASA; (E) 100 ng/mL of 4-ASA; and (F) 400 ng/mL of *N*-Ac-4-ASA.

values ranging from 1.7% to 6.5% for both analytes. The percent difference between the standard concentrations calculated from the calibration curve and theoretical ones for both tested analytes ranged from −14% to 10%. Limit of quantification (LOQ) was evaluated according to the guidance for industry on the validation of bioanalytical methods, i.e. as the lowest analyte concentration corresponding to a response at least 5 times higher than blank response and which can be determined with 80–120% accuracy and 20% precision [33]. The back-calculated concentration data obtained from calibration curves allowed to assess 50 ng/mL as the validated LOQ of the analytical method (Table 3) for 5-ASA and *N*-Ac-5-ASA. This confirms that the method showed sufficient sensitivity to support clinical trials and pharmacokinetic studies, even if signal was partially suppressed.

3.7. Precision and accuracy

Table 3 reports the within- and between-batch accuracy and precision results for both 5-ASA and *N*-Ac-5-ASA, obtained analyzing QC samples prepared in human plasma at three different

Table 1
Recovery and standard deviation (S.D.) values obtained by analyzing human plasma samples spiked at three concentration levels (100, 1000 and 4000 ng/mL) of 5-ASA and *N*-Ac-5-ASA, and at 100 ng/mL of 4-ASA and 400 ng/mL of *N*-Ac-4-ASA ($n = 6$)

Theoretical concentration (ng/mL)	5-ASA		*N*-Ac-5-ASA		4-ASA		*N*-Ac-4-ASA	
	Recovery (%)	S.D. (%)	Recovery (%)	S.D. (%)	Recovery (%)	S.D. (%)	Recovery (%)	S.D. (%)
100	97.6	8.5	97.5	6.4	86.8	4.8	–	–
400	–	–	–	–	–	–	94.3	3.0
1000	97.5	8.9	98.3	6.1	–	–	–	–
4000	90.8	4.4	95.2	6.1	–	–	–	–

Table 2
Linear calibration parameters obtained by least-square fitting of six independent eight-point curves in the 50–4000 ng/mL concentration range for 5-ASA and *N*-Ac-5-ASA

Curve	5-ASA			*N*-Ac-5-ASA		
	Slope	Intercept	R^2	Slope	Intercept	R^2
1	0.000715	0.0124	0.996	0.000525	0.0225	0.996
2	0.000765	0.0112	0.988	0.000501	0.0218	0.997
3	0.000732	0.0119	0.993	0.000533	0.0210	0.995
4	0.000735	0.0169	0.995	0.000572	0.0243	0.999
5	0.000622	0.0151	0.999	0.000538	0.0214	0.999
6	0.000692	0.0172	0.994	0.000560	0.0218	0.996
Mean	0.000710	0.0141	0.994	0.000538	0.0222	0.997
S.D.	0.000049	0.0026	0.004	0.000025	0.0012	0.002
R.S.D. (%)	7.0	19	0.4	4.7	5.2	0.2

concentration levels of 150, 800 and 2500 ng/mL. The three concentration values were chosen because they were the most representative of a typical pharmacokinetic profile.

For within- and between-batch precisions, R.S.D.% values calculated for all the tested levels ($n = 6$ each) did not exceed 8% and 10%, respectively. For within- and between-batch accuracies, bias values ranged from −3.6% to 7.9% and from −4.3% to 8.0%, respectively.

3.8. Stability of stock solutions

The stability of 5-ASA and *N*-Ac-5-ASA stock solutions (100 μg/mL) in methanol was evaluated by analyzing refrigerated (+4 °C) and frozen (−20 °C) aliquots during the validation of the method for a period of time of 30 and 120 days, respectively.

For storage at 4 °C no significant variations were observed during the first 6 days, while the measured concentration decreased to about 50% after about 15 days for 5-ASA and to about 75% for *N*-Ac-5-ASA after 20 days. Stability of each analyte stock solution (100 μg/mL) stored at −20 °C was tested for a period of 120 days. No significant deviations were observed during this time. Bias% for the analytes was between −1.0% and 1.5% after 120 days of storage.

3.9. Stability of samples

Laboratory tests were made to assay behavior of 5-ASA and *N*-Ac-5-ASA in plasma under different experimental conditions, as recommended by FDA Guideline. During validation stored samples were re-run against fresh calibrants. Results confirmed that both analytes were stable for at least 4 months during plasma sample storage at −80 °C, for 4 h at room temperature, and during three freeze/thaw cycles in agreement with data from other authors [20,30].

To test stability of compounds during a typical analysis cycle, plasma samples fortified at three concentration levels (150, 800 and 2500 ng/mL) of both 5-ASA and *N*-Ac-5-ASA were processed and analyzed at the beginning (reference) and during a 42 h storage period at 7 °C in the autosampler carousel. Under the above conditions, analytes resulted stable, since no significant decrease in concentrations nor modification of the chromatographic traces were observed (data not shown). After 42 h, bias values ranged from 7.2% to 3.1%.

3.10. Application to a pharmacokinetic study

The validated HPLC-ESI-MS/MS method was employed to measure plasma levels of 5-ASA and its major metabolite, *N*-Ac-5-ASA, in 24 healthy volunteers, treated with 1200 mg of Pentacol (three tablets of 400 mg each) in single oral dose. Fig. 4 shows a typical MRM chromatogram obtained by analyzing a human plasma sample collected 8 h after mesalazine administration. 5-ASA and *N*-Ac-5-ASA mean maximum concentrations ±S.E.M. (standard error of the mean) were 680 ± 150 and 1240 ± 220 ng/mL, respectively, with average t_{max} of 8 h. The 5-ASA and *N*-Ac-5-ASA concentration profiles in plasma were in agreement with those reported by other authors for similar formulations and in proportion to the dosages administered (e.g. 1500 mg [14] and 800 mg [22]). Representative 5-ASA and *N*-Ac-5-ASA mean plasma concentration-time profiles obtained in this study are shown in Fig. 5. Further statistical evaluation on the obtained profiles with validated pharmacokinetic software packages was beyond the aim of the present paper. When the method will be applied for bioequivalence studies, data will be

Table 3
Within- and between-batch precisions (R.S.D.%) and accuracies (bias%) of the method, obtained from the analysis of samples spiked with 50 (LOQ level), 150, 800 and 2500 ng/mL of 5-ASA and *N*-Ac-5-ASA ($n = 6$)

Theoretical 5-ASA concentration (ng/mL)	Mean back-calculated 5-ASA concentration (ng/mL)	R.S.D. (%)	Bias (%)
Within-batch			
50	45.8	3.5	−8.4
150	161	3.6	7.6
800	863	6.3	7.9
2500	2520	3.5	0.8
Between-batch			
50	47.6	11	−4.8
150	159	10	6.1
800	852	7.0	6.6
2500	2580	7.8	3.2
Theoretical *N*-Ac-5-ASA concentration (ng/mL)	Mean back-calculated *N*-Ac-5-ASA concentration (ng/mL)	R.S.D. (%)	Bias (%)
Within-batch			
50	47.3	4.9	−5.5
150	158	8.0	5.5
800	858	2.5	7.2
2500	2410	4.1	−3.6
Between-batch			
50	46.1	9.7	−7.9
150	155	10	3.6
800	864	4.4	8.0
2500	2391	2.9	−4.3

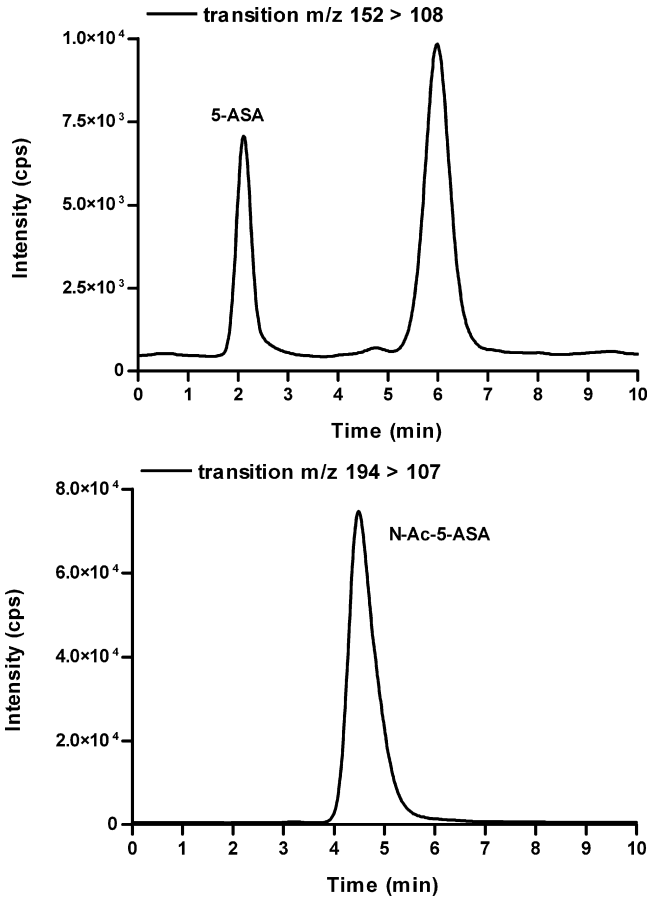


Fig. 4. Typical MRM chromatograms (transition m/z 152 → 108 for 5-ASA and m/z 194 → 107 for *N*-Ac-5-ASA) obtained for a human plasma sample collected 8 h after mesalazine administration (5-ASA concentration: 555 ng/mL and *N*-Ac-5-ASA concentration: 1170 ng/mL).

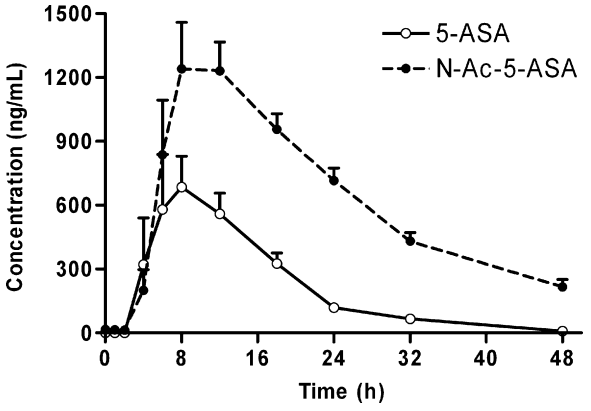


Fig. 5. Mean plasma 5-ASA and *N*-Ac-5-ASA concentration–time profiles following a single oral dose administration in healthy volunteers ($n = 24$) treated with 1.2 g of mesalazine.

analyzed in more detail to obtain pharmacokinetic parameters such as C_{max}, t_{max}, area under the curve (AUC) and all the information needed for this purpose.

4. Conclusion

The new HPLC-ESI-MS/MS method for determination of both 5-ASA and *N*-Ac-5-ASA fulfils the acceptance criteria generally established for bioanalytical assays when applied in pharmaceutical analysis. In the explored range the method is accurate, precise, selective and sensitive enough to allow the analysis of 5-ASA and *N*-Ac-5-ASA in 0.5 mL of human plasma after single oral administration of 1.2 g of mesalazine. The method does not require preanalytical derivatization and the analysis can be performed after a simple precipitation clean-up step, thus reducing analytical variability and sample handling time. Furthermore, about 60 injections/day can be performed with an autosampler system.

The use of two internal standards, 4-ASA and *N*-Ac-4-ASA, selected as structural analogues of the two analytes, and the use of matrix-matched calibration, allow to compensate signal suppression effect and reduce inaccuracy problems.

The LOQ value, estimated as 50 ng/mL for both analytes, is adequate to quantify concentration levels of 5-ASA and its main metabolite generally found in human plasma samples, collected during clinical and pharmacokinetic studies in which mesalazine drugs are administered at therapeutic dosage.

In comparison with the previously developed methods, the present one offers undoubted advantages in term of overall analytical performance, mainly related to the simple preanalytical treatment and to chromatographic separation combined with the use of ESI-MS/MS detection, thus allowing to identify and quantify the two analytes with high selectivity and sensitivity.

References

[1] D.K. Podolsky, N. Engl. J. Med. 347 (2002) 417.
[2] R. Bergman, M. Parkes, Aliment. Pharmacol. Ther. 23 (2006) 841.
[3] W.J. Sandborn, S.B. Hanauer, Aliment. Pharmacol. Ther. 17 (2003) 29.
[4] L. Stærk Laursen, M. Stokholm, K. Bukhave, J. Rask-Madsen, K. Lauritsen, Gut 31 (1990) 1271.
[5] S.M. Greenfield, N.A. Punchard, J.P. Teare, R.P.H. Thompson, Aliment. Pharmacol. Ther. 7 (1993) 369.
[6] W.H. Betts, M.W. Whitehouse, L.G. Cleland, B. Vernon-Roberts, J. Free Radic. Biol. Med. 1 (1985) 273.
[7] I. Ahnfelt-Ronne, O.H. Nielsen, A. Christensen, E. Langholz, V. Binder, P. Riis, Gastroenterology 98 (1990) 1162.
[8] P. Gionchetti, C. Guarneri, M. Campieri, A. Belluzzi, C. Brignola, P. Iannone, M. Miglioli, L. Barbara, Digest. Dis. Sci. 36 (1991) 174.
[9] B. Myers, D.N.W. Evans, J. Rodhes, B.K. Evans, B.R. Hughes, M.G. Lee, A. Richens, D. Richards, Gut 28 (1987) 196.
[10] H. Allgayer, N.O. Ahnfelt, W. Kruis, U. Klotz, K. Frank-Holmberg, H.N. Soderberg, G. Paumgartner, Gastroenterology 97 (1989) 38.
[11] A. Ireland, J.D. Priddle, D.P. Jewell, Clin. Sci. 78 (1990) 105.
[12] M.A. Peppercorn, P. Goldman, Gastroenterology 64 (1973) 240.
[13] R.A. van Hogezand, P.A.M. van Hees, J.P.W.M. van Gorp, H.J. van Lier, J.H. Bakker, P. Wesseling, U.J.G.M. van Haelst, J.H.M. van Tongeren, Aliment. Pharmacol. Ther. 2 (1988) 33.
[14] I.R. Wilding, C. Behrens, S.J. Tardif, H. Wray, P. Bias, W. Albrecht, Aliment. Pharmacol. Ther. 17 (2003) 1153.
[15] J. Tjørnelund, S.H. Hansen, J. Chromatogr. Biomed. Appl. 570 (1991) 109.
[16] M. Nobilis, Z. Vybíralová, K. Sládková, M. Lísa, M. Holcapek, J. Kvetina, J. Chromatogr. A 1119 (2006) 299.
[17] H. Allgayer, J. Sonnenbichler, W. Kruis, G. Paumgartner, Arzneim. Forsch. 35 (1985) 1457.
[18] M. Brunner, E. Lackner, P.S. Exler, H.C. de Fluiter, K. Kletter, M. Tschurlovits, R. Dudczak, H.G. Eichler, M. Müller, Aliment. Pharmacol. Ther. 23 (2006) 137.
[19] K. Dilger, D. Trenk, M. Rössle, M. Cap, A. Zähringer, V. Wacheck, C. Remmler, I. Cascorbi, W. Kreisel, G. Novacek, Eur. J. Clin. Invest. 37 (2007) 558.
[20] P. Gandia, I. Idier, G. Houin, J. Clin. Pharmacol. 47 (2007) 334.
[21] G. Palumbo, S. Bacchi, L. Primavera, P. Palumbo, G. Carlucci, Biomed. Chromatogr. 19 (2005) 350.
[22] W.J. Sandborn, S.B. Hanauer, A. Buch, Aliment. Pharmacol. Ther. 19 (2004) 1089.
[23] G. Aumais, M. Lefebvre, C. Tremblay, A. Bitton, F. Martin, A. Giard, M. Madi, J. Spénard, Aliment. Pharmacol. Ther. 17 (2003) 93.
[24] F.N. Hussain, R.A. Ajjan, K. Kapur, M. Moustafa, S.A. Riley, Aliment. Pharmacol. Ther. 15 (2001) 53.
[25] M. Orioli, C. Marinello, R. Cozzi, L.P. Piodi, M. Carini, J. Pharm. Biomed. Anal. 35 (2004) 1263.
[26] S.H. Hansen, J. Chromatogr. 226 (1981) 504.
[27] V.S. Chungi, G.S. Rekhi, L. Shargel, J. Pharm. Sci. 78 (1989) 235.
[28] F.N. Hussain, R.A. Ajjan, M. Moustafa, J.C. Anderson, S.A. Riley, J. Chromatogr. B: Biomed. Sci. Appl. 716 (1998) 257.

[29] B. Bystrowska, J. Nowak, J. Brandys, J. Pharm. Biomed. Anal. 22 (2000) 341.
[30] J. Tjørnelund, S.H. Hansen, J. Chromatogr. Biomed Appl. 570 (1991) 224.
[31] A. Roda, L. Sabatini, A. Barbieri, M. Guardagli, M. Locatelli, F.S. Violante, L.C. Rovati, S. Persiani, J. Chromatogr. B 844 (2006) 119.
[32] R. Causon, J. Chromatogr. B 689 (1997) 175.
[33] CDER and CVM Guidance for Industry, Bioanalytical Method Validation. Food and Drug Administration, May 2001. http://www.fda.gov/cder/guidance/4252fnl.pdf.
[34] Z. Wu, W. Gao, M.A. Phelps, D. Wu, D.D. Miller, J.T. Dalton, Anal. Chem. 76 (2004) 839.
[35] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972A.