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High-performance liquid-chromatographic determination of 5-aminosalicylic acid and its metabolites in blood plasma

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Abstract

Mesalazine (5-aminosalicylic acid, 5-ASA), an anti-inflammatory agent for the treatment of inflammatory bowel diseases, is metabolized in organism to the principal biotransformation product, *N*-acetyl-5-ASA. Some other phase II metabolites (*N*-formyl-5-ASA, *N*-butyryl-5-ASA, *N*-β-D-glucopyranosyl-5-ASA) have also been described. 5-ASA is a polar compound and besides it exhibits amphoteric properties. The extraction of this compound from biomatrices and its chromatographic analysis is complicated. In order to improve the reliability of the determination of parent 5-ASA, a derivatization of 5-ASA together with 4-ASA (added to samples as a precursor of I.S.-2) was involved into the method. More lipophilic *N*-propionyl-5-ASA and *N*-propionyl-4-ASA (I.S.-2) were obtained using propionic anhydride. These derivatives were well extractable together with *N*-acyl-5-ASAs (metabolites) and *N*-acetyl-4-ASA (I.S.-1). As the first internal standard (I.S.-1) was used for the evaluation of extracted *N*-acyl-metabolites, the second internal standard (I.S.-2) served for the evaluation of both derivatization and extraction steps of parent drug 5-ASA. Based on these reasonings, new HPLC bioanalytical method for the determination of 5-ASA and its metabolites in blood plasma was developed and validated. The sample preparation step consists of the deproteination of plasma by $HClO_4$ and the above-mentioned derivatization of ASAs followed by liquid–liquid extraction of all *N*-acyl-ASA-derivatives. Chromatographic analyses were performed on a 250-4 mm column containing Purospher RP-18 e, 5 μm (Merck, Darmstadt, Germany) with a precolumn (4-4 mm). The column effluent was monitored using both UV photodiode-array ($\lambda = 313$ nm) and fluorescence detectors ($\lambda_{exc.} = 300$ nm/$\lambda_{emiss.} = 406$ nm) in tandem. The identity of individual *N*-acyl-ASAs in the extracts from biomatrices was verified by characteristic UV-spectra and by HPLC/MS experiments. The whole analysis lasted 23 min at the flow rate of 1 ml min^{-1}. LLOQ (LOD) was estimated 126 (20) pmol ml^{-1} of plasma for *N*-acetyl-5-ASA and 318 (50) pmol ml^{-1} of plasma for *N*-propionyl-5-ASA. The validated HPLC method was applied to pharmacokinetic studies of mesalazine in humans and animals.

Keywords: Mesalazine (5-aminosalicylic acid, 5-ASA); *N*-acyl derivatives of 5-ASA; HPLC with UV photodiode-array, fluorescence and mass-spectrometric detections; Pharmacokinetics

1. Introduction

5-Aminosalicylic acid (5-ASA, mesalazine, CAS 89-57-6) is widely used for the treatment of inflammatory bowel diseases (IBDs), especially for ulcerative colitis and Crohn's disease [1]. This compound is administered as a parent drug (mesalazine), or in the form of its prodrug—salicylazosulfapyridine (salazosulfapyridine, sulphasalazine, CAS 599-79-1). Salicylazosulfapyridine was introduced into therapeutic use by Svartz in 1941 [2], but the clinical evidence that 5-ASA represents the therapeutic moiety of salicylazosulfapyridine [3,4] in the treatment of inflammatory bowel diseases was given much later.

The mode of mesalazine action is uncertain but, at least in part, may consist in its ability to inhibit local prostaglandin and leukotriene synthesis in the gastrointestinal mucosa [5]. In addition, 5-ASA was found to be a very potent free radical scavenger. The compound readily supplies its electrons to reactive oxygen species (ROS), thus suppressing their toxicity [6–8].

As shown in Fig. 1, the prodrug salicylazosulfapyridine is cleaved in the colon by bacterial azoreductases to 5-ASA, an active compound, which is furthermore metabolized in the gut wall and in the liver [9] to phase II biotransformation products, *N*-acetyl-5-ASA (the principal metabolite), *N*-formyl-5-ASA, *N*-butyryl-5-ASA and *N*-β-D-glucopyranosyl-5-ASA [10–12].

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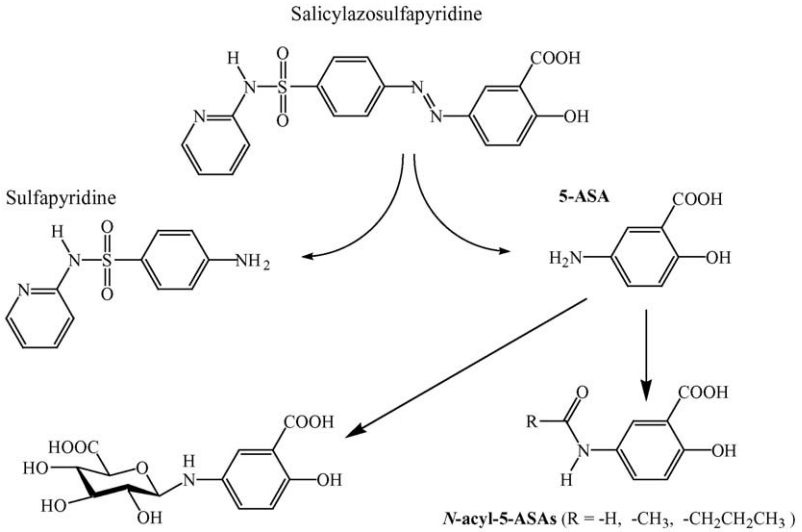

Fig. 1. Metabolic cleveage of salicylazosulfapyridine into active 5-aminosalicylic acid (5-ASA) and subsequent phase II biotransformation of 5-ASA.

The acetylated metabolites are excreted mainly in urine by tubular secretion together with traces of 5-ASA. The elimination half-life of mesalazine is reported to be about 1 h and the drug is bound to plasma proteins by 40–50%. *N*-Acetyl-5-ASA has a half-life of up to 10 h and is bound to plasma proteins by 80% [1,4]. Certain part of 5-ASA is eliminated in faeces.

Various analytical approaches to the sample preparation of biomatrices and to HPLC determination of 5-ASA and its metabolites in biological samples have been described [12–34].

In order to remove the interfering ballasts from the biomatrices and to increase the selectivity and sensitivity of the analytical method, a sample handling step was usually used prior to the instrumental analysis of 5-aminosalicylic acid and its metabolites.

Acetonitrile [12,18], methanol [15,21,27] or perchloric acid [14,19,28] were used for the deproteinization of the fluid samples (plasma, serum) or tissue homogenates. After centrifugation, a supernatant containing the analytes was obtained. Direct injection of the supernatant onto an HPLC column was often used [15,19,21,27]. In most of the methods, ASA-derivatives contained in the supernatant were derivatized and extracted before the HPLC analysis.

The derivatization step consisted in an acylation of the primary aromatic amino group by acetic anhydride [14,28], propionic anhydride [17,24,27] or benzyl chloroformate [18]. When acetic anhydride was used for the derivatization, each individual sample had to be divided into two portions of the same volume. One portion was acetylated and extracted, the other was just extracted without acetylation prior to HPLC analyses. The difference in the amounts of *N*-acetyl-5-ASA from both analyses enabled to discriminate between the authentic 5-ASA metabolite present in the biomatrix and the 5-ASA derivative formed during the sample preparation.

A liquid–liquid extraction of *N*-acyl-ASA-derivatives into diethyl ether [14,28], dichloromethane [13], ethyl acetate [18], acetonitrile [12,18,24], methanol [34], 1,1,1-trichlorethane [12] was the last step in the sample preparation, solid phase extraction has also been used [17].

A number of high-performance liquid chromatography (HPLC) methods with a fluorescence [12–20,22–25,27,28,32], ultraviolet [15,23], electrochemical [21,23,26,29,31,34] and mass spectrometry detection [33] were described. Micellar electrokinetic chromatographic (MEKC) method involving UV detection was also developed and validated [30].

4-Aminosalicylic acid (4-ASA) [13,27], *N*-acetyl-4-ASA [14,18], *N*-propionyl-4-ASA [17,24,34], *N*-acetyl-anthranilic acid [28] were employed as internal standards for the determination of 5-ASA and its metabolites in biological samples.

5-ASA and its metabolites were identified and determined in various biomatrices: in plasma [10–15,28,34], serum [17,19,21], cerebrospinal fluid [21], urine [10,11,14,15,17,28], faeces [17], endoscopic intestinal and rectal tissue biopsies [24,26,27]. Some of the described methods were used in pharmacokinetic studies [34–37] or therapeutical drug monitoring [34].

The stability of 5-ASA and its metabolites in plasma samples was also evaluated [22,25]. ASA-derivatives were found to be stable in plasma samples stored at −20 °C for 8 months.

This communication describes a bioanalytical HPLC method involving a deproteination of blood plasma samples and the lipophilicity-increasing derivatization using propionic anhydride followed by a liquid–liquid extraction into ethyl acetate. The separation conditions proposed enabled a simultaneous analysis of eight aminosalicylic acid (ASA) derivatives within 20 min. The influence of the sample preparation steps (the deproteination, derivatization and extraction) on the reliability of the HPLC determination was checked using two internal standards. Three detection principles were used in the identification and determination of 5-ASA and its metabolites. A high-performance liquid chromatography with tandem ultraviolet photodiode-array and fluorescence detections was used for routine analyses. HPLC with mass spectrometric detection was employed in the confirmation of the structure of the

synthesized standards and 5-ASA metabolites found in the biomatrices.

The developed and validated analytical method was applied to pharmacokinetic studies of various mesalazine dosage forms (tablets, suppositories).

2. Experimental

2.1. Chemicals, preparations, solutions, materials

Mesalazine (5-aminosalicylic acid, 5-ASA, $C_7H_7O_3N$, 153.14 g/mol) and PAS (4-amino-salicylic acid, 4-ASA, $C_7H_7O_3N$, 153.14 g/mol) were purchased from Aldrich. The standards of the mesalazine derivative (*N*-propionyl-5-ASA, $C_{10}H_{11}O_4N$, 209.2 g/mol), mesalazine metabolites—*N*-formyl-5-aminosalicylic acid (*N*-formyl-5-ASA, $C_8H_7O_4N$, 181.04 g/mol), *N*-acetyl-5-aminosalicylic acid (*N*-acetyl-5-ASA, $C_9H_9O_4N$, 195.17 g/mol), *N*-butyryl-5-aminosalicylic acid (*N*-butyryl-5-ASA, $C_{11}H_{13}O_4N$, 223.08 g/mol) and two internal standards used in HPLC determination, IS-1 (*N*-acetyl-4-ASA, $C_9H_9O_4N$, 195.17 g/mol) and IS-2 (*N*-propionyl-4-ASA, $C_{10}H_{11}O_4N$, 209.2 g/mol), were synthesized in our laboratories.

Acetonitrile (HPLC grade), ammonium acetate (Fractopur®), ethyl acetate (LiChrosolv® for liquid chromatography; all from Merck, Darmstadt, Germany), perchloric acid 70% (puriss. p.a., Fluka, Buchs, Switzerland), formic acid (85%), hydrochloric acid (35%), phosphoric acid (85%), methanol, potassium dihydrogenphosphate (KH_2PO_4), sodium hydrogenphosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium hydroxide (all analytical grade, Lachema, Brno, Czech Republic), ultra-high quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga Ltd., Bucks, England) were used in the sample preparation and chromatography of aminosalicylic acid (ASA) derivatives. Acetic anhydride (p.a., LACHEMA a.s., Neratovice, Czech Republic), propionic anhydride (99+%) and butyric anhydride (98%, both Aldrich-Chemie, Steinheim, Germany) were applied for the preparation of *N*-acyl-ASA-derivatives.

An ammonium acetate buffer (0.005 M, pH 3) was prepared by the dissolution of ammonium acetate (0.385 g) in UHQ water (990 ml), adjusting to pH 3 with 85% formic acid (approximately 2 ml) and filling to a total volume of 1000 ml with UHQ water.

A phosphate buffer (0.01 M, pH 3) was made by the dissolution of sodium hydrogenphosphate dodecahydrate (3.58 g) in UHQ water (990 ml), adjusting to pH 3 with a solution of phosphoric acid (2 mol l^{-1}) and filling to a total volume of 1000 ml with UHQ water.

A phosphate buffer (pH 7.4) was mixed from 800 ml of 0.067 M sodium hydrogenphosphate dodecahydrate (23.976 g in 1000 ml of UHQ water) and 200 ml of 0.067 M potassium dihydrogenphosphate (2.2695 g in 250 ml of UHQ water).

Two mesalazine formulations (suppositories or coated tablets containing 500 mg of 5-ASA) were tested in this study.

The stock solutions (10^{-3} M) of all aminosalicylic acid (ASA) compounds under study were prepared using the equimolar amounts of sodium hydroxide to increase their solubility in water. Lower concentrations (10^{-4} M) were prepared by diluting the stock solutions with UHQ water.

A mixture of internal standards [300 μl of 10^{-3} M aqueous *N*-acetyl-4-ASA (I.S.-1), 300 μl of 10^{-3} M aqueous 4-ASA (precursor of I.S.-2) and 2400 μl of UHQ water] was used for spiking of the analyzed biomatrix samples containing 5-ASA and its metabolites.

2.2. NMR and HPLC/MS analyses

A Varian Mercury-Vx BB 300 NMR spectrometer was employed for the NMR analyses of the synthetic standards of *N*-acyl derivatives of 5-ASA and 4-ASA, respectively. The NMR spectra were recorded at 300 MHz for ^1H, and 75 MHz for ^{13}C. Chemical shifts are given as δ-values in ppm, the coupling constants are given in Hz. Analytical sample (15–20 mg) was dissolved in deuterated methanol (0.7 ml) and the solution was transferred via pipette into an NMR tube (203 mm length, 5 mm diameter).

High-performance liquid chromatography–mass spectrometry (HPLC/MS) analyses were performed on an Esquire3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) and a liquid chromatograph consisting of a Model 616 pump, a Model 717+ autosampler, a thermostated column compartment and a Model 996 photodiode-array detector (all from Waters, Milford, MA, USA). Electrospray ionization (ESI) mass spectra were recorded in the mass range *m/z* 50–1200 using both positive-ion and negative-ion modes and the following setting of tuning parameters: target mass *m/z* = 200, compound stability = 100%, pressure of the nebulizing gas = 70 psi, the drying gas flow rate = 12 l/min and temperature of ion source = 365 °C. UV-spectra of all chromatographic peaks were recorder in the range 200–400 nm using a diode-array UV detector with the highest resolution 1.2 nm. The standards and extracts from biomatrices were dissolved in the mobile phase and 100 μl of the solution was injected into HPLC/MS system. Mobile phase for HPLC/MS experiments consisted of acetonitrile–0.005 M ammonium acetate buffer pH 3 (15: 85, v/v), the chromatographic column, length of analysis and flow rate were identical as described in Section 2.6.

2.3. Syntheses of the analytical standards

5-ASA or 4-ASA (1 g, 0.0065 mol) was dissolved in corresponding acyl anhydride (acetic anhydride, propionic anhydride, butyric anhydride) and the reaction mixture was stirred for 3 h at ambient temperature. The mixture was then allowed to stand overnight. The crude crystalline product was filtered off and washed with water. The pure product [*N*-acyl-5(4)-ASA] was dried over P_2O_5.

For the preparation of *N*-formyl-5-ASA, the following procedure was used:

A solution of 5-ASA (1.53 g, 0.01 mol) and 85% formic acid (0.65 g, 0.012 mol) in toluene (30 ml) was heated under reflux using the Dean-Stark trap for azeotropic distillation in order to remove the water released during the reaction. Toluene was then removed by distillation and the crude crystalline product was washed with water. The pure product (*N*-formyl-5-ASA) was dried over P_2O_5.

The identity and purity of individual products were confirmed by NMR, HPLC-PDA and HPLC/MS.

2.3.1. NMR-results

2.3.1.1. N-acetyl-4-ASA. ^{1}H NMR (300 MHz, CD_3OD) δ 7.95 (1H, d, J = 8.8 Hz, H6), 7.53 (1H, d, J = 1.9 Hz, H3), 7.40 (1H, dd, J = 8.8 Hz, J = 1.9 Hz, H5), 2.27 (3H, s, CH_3), 2.13 (3H, s, CH_3), ^{13}C NMR (75 MHz, CD_3OD) δ 172.0, 171.5, 167.2, 153.1, 145.2, 133.7, 119.5, 117.2, 115.1, 24.0, 21.1.

2.3.1.2. N-propionyl-4-ASA. ^{1}H NMR (300 MHz, CD_3OD) δ 7.76 (1H, d, J = 8.7 Hz, H6), 7.32 (1H, d, J = 1.9 Hz, H3), 7.02 (1H, dd, J = 8.7 Hz, J = 1.9 Hz, H5), 2.39 (2H, q, J = 7.7 Hz, CH_2), 1.18 (3H, t, J = 7.7 Hz, CH_3), ^{13}C NMR (75 MHz, CD_3OD) δ 175.7, 173.1, 164.2, 146.6, 132.2, 111.5, 109.2, 107.8, 31.2, 10.0.

2.3.1.3. N-acetyl-5-ASA. ^{1}H NMR (300 MHz, CD_3OD) δ 8.04 (1H, d, J = 2.6 Hz, H6), 7.57 (1H, dd, J = 8.9 Hz, J = 2.6 Hz, H4), 6.86 (1H, d, J = 8.9 Hz, H3), 2.09 (3H, s, CH_3), ^{13}C NMR (75 MHz, CD_3OD) δ 173.2, 171.5, 159.8, 131.3, 129.6, 123.2, 118.2, 113.5, 23.5.

2.3.1.4. N-propionyl-5-ASA. ^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (1H, d, J = 2.7 Hz, H6), 7.58 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H4), 6.86 (1H, d, J = 8.8 Hz, H3), 2.35 (2H, q, J = 7.4 Hz, CH_2), 1.18 (3H, t, J = 7.4 Hz, CH_3), ^{13}C NMR (75 MHz, CD_3OD) δ 175.3, 173.2, 159.8, 131.4, 129.6, 123.2, 118.2, 113.5, 30.8, 10.3.

2.3.2. ESI mass spectra

2.3.2.1. 4-ASA, MW = 153. Positive-ion ESI-MS: *m/z* 154 $[M+H]^+$ (100%), 136 $[M+H–H_2O]^+$.

Positive-ion ESI-MS/MS of *m/z* 154: *m/z* 136 $[M+H\text{-}H_2O]^+$ (100%).

Negative-ion ESI-MS: *m/z* 327 $[2M–2H+Na]^-$, 152 $[M\text{-}H]^-$ (100%), 108 $[M–H–CO_2]^-$.

2.3.2.2. N-acetyl-4-ASA, MW = 195. Positive-ion ESI-MS: *m/z* 218 $[M+Na]^+$, 196 $[M+H]^+$ (100%), 178 $[M+H–H_2O]^+$, 136 $[M+H–H_2O–CH_2CO]^+$.

Positive-ion ESI-MS/MS of *m/z* 196: *m/z* 178 $[M+H–H_2O]^+$ (100%), 136 $[M+H–H_2O–CH_2CO]^+$.

Negative-ion ESI-MS: *m/z* 411 $[2M–2H+Na]^-$, 194 $[M–H]^-$ (100%), 150 $[M–H–CO_2]^-$, 108 $[M–H–CO_2–CH_2CO]^-$.

Negative-ion ESI-MS/MS of *m/z* 194: *m/z* 150 $[M–H–CO_2]^-$ (100%), 108 $[M–H–CO_2–CH_2CO]^-$.

2.3.2.3. N-propionyl-4-ASA, MW = 209. Positive-ion ESI-MS: *m/z* 232 $[M+Na]^+$, 210 $[M+H]^+$ (100%), 192 $[M+H–H_2O]^+$, 136 $[M+H–H_2O–CH_3CHCO]^+$.

Positive-ion ESI-MS/MS of *m/z* 210: *m/z* 192 $[M+H–H_2O]^+$ (100%), 136 $[M+H–H_2O–CH_3CHCO]^+$.

Negative-ion ESI-MS: *m/z* 439 $[2M–2H+Na]^-$, 208 $[M–H]^-$ (100%), 164 $[M–H–CO_2]^-$, 108 $[M–H–CO_2–CH_3CHCO]^-$.

Negative-ion ESI-MS/MS of *m/z* 208: *m/z* 164 $[M–H–CO_2]^-$ (100%), 108 $[M–H–CO_2–CH_3CHCO]^-$.

2.3.2.4. 5-ASA, MW = 153. Positive-ion ESI-MS: *m/z* 154 $[M+H]^+$ (100%), 136 $[M+H–H_2O]^+$.

Positive-ion ESI-MS/MS of *m/z* 154: *m/z* 136 $[M+H–H_2O]^+$ (100%).

Negative-ion ESI-MS: *m/z* 152 $[M–H]^-$ (100%), 108 $[M–H–CO_2]^-$.

2.3.2.5. N-formyl-5-ASA, MW = 181. Positive-ion ESI-MS: *m/z* 204 $[M+Na]^+$, 182 $[M+H]^+$, 164 $[M+H–H_2O]^+$ (100%).

Positive-ion ESI-MS/MS of *m/z* 182: *m/z* 164 $[M+H–H_2O]^+$ (100%).

Negative-ion ESI-MS: *m/z* 383 $[2M–2H+Na]^-$ (100%), 180 $[M–H]^-$, 136 $[M–H–CO_2]^-$.

2.3.2.6. N-acetyl-5-ASA, MW = 195. Positive-ion ESI-MS: *m/z* 218 $[M+Na]^+$, 196 $[M+H]^+$ (100%), 178 $[M+H–H_2O]^+$, 152 $[M+H–CO_2]^+$, 136 $[M+H–H_2O–CH_2CO]^+$.

Positive-ion ESI-MS/MS of *m/z* 196: *m/z* 178 $[M+H–H_2O]^+$ (100%).

Negative-ion ESI-MS: *m/z* 411 $[2M–2H+Na]^-$ (100%), 194 $[M–H]^-$, 150 $[M–H–CO_2]^-$.

Negative-ion ESI-MS/MS of *m/z* 194: *m/z* 150 $[M–H–CO_2]^-$ (100%).

2.3.2.7. N-propionyl-5-ASA, MW = 209. Positive-ion ESI-MS: *m/z* 232 $[M+Na]^+$, 210 $[M+H]^+$ (100%), 192 $[M+H–H_2O]^+$, 136 $[M+H–H_2O–CH_3CHCO]^+$.

Positive-ion ESI-MS/MS of *m/z* 210: *m/z* 192 $[M+H–H_2O]^+$ (100%), 166 $[M+H–CO_2]^+$, 136 $[M+H–H_2O–CH_3CHCO]^+$.

Negative-ion ESI-MS: *m/z* 439 $[2M–2H+Na]^-$, 208 $[M–H]^-$ (100%), 164 $[M–H–CO_2]^-$.

Negative-ion ESI-MS/MS of *m/z* 208: *m/z* 164 $[M–H–CO_2]^-$ (100%).

2.3.2.8. N-butyryl-5-ASA, MW = 223. Positive-ion ESI-MS: *m/z* 246 $[M+Na]^+$, 224 $[M+H]^+$ (100%), 206 $[M+H–H_2O]^+$, 136 $[M+H–H_2O–CH_3CH_2CHCO]^+$.

Positive-ion ESI-MS/MS of *m/z* 224: 206 *m/z* $[M+H–H_2O]^+$ (100%), 180 $[M+H–CO_2]^+$, 136 $[M+H–H_2O–CH_3CH_2CHCO]^+$.

Negative-ion ESI-MS: *m/z* 467 $[2M–2H+Na]^-$, 222 $[M–H]^-$ (100%), 178 $[M–H–CO_2]^-$.

Negative-ion ESI-MS/MS of *m/z* 222: *m/z* 178 $[M–H–CO_2]^-$ (100%).

2.4. Volunteers and biological material

Fourteen healthy volunteers entered the pharmacokinetic study. All the subjects were healthy according to their medical history, physical examination, haematology, clinical chemistry and urinalysis. The study was approved by the state authority and the Institutional Ethics Committee. Smoking, medication and alcohol, methylxanthine and chinine containing beverages were restricted 5 days before dosing and during the experiment.

Single rectal dose of mesalazine preparation (suppository containing 500 mg of 5-ASA) was administered to every volunteer.

Blood samples of 5 ml volume were taken in the following time intervals: 0 (predose), 0.33, 0.67, 1, 2, 3, 4, 5, 6, 8, 12, 24 and 32 h after the rectal administration of suppository. The samples were withdrawn from the cubital vein into the heparinized Monovette® syringes (Sarstedt, Germany) and centrifuged immediately at 2000 × *g* for 10 min. The plasma was separated and stored in polypropylene tubes at −70 °C until the analysis.

2.5. Sample preparation

The frozen plasma samples were thawed out and centrifuged (2000 × *g* for 10 min at 25 °C). To 1 ml of plasma in a 9-ml glass tube equipped with a ground-glass stopper, 30 μl of the mixture of internal standards [10^{-4} M aqueous *N*-acetyl-4-ASA (I.S.-1) and 4-ASA (precursor of I.S.-2); see Section 2.1] were added and the plasma was shortly vigorously shaken. Concentrated perchloric acid (20 μl) was added to the precipitation of proteins and the content of the tube was vortexed-mixed for 1 min. After centrifugation (2000 × *g*, 12 min), the supernatant (600 μl) was transferred into a clean tube and 600 μl of phosphate buffer pH 7.4 was added. Twenty microlitres of propionic anhydride were added and the content of the tube was shortly vigorously shaken and then allowed to stand at 25 °C for 20 min. Concentrated hydrochloric acid (50 μl) was added, the content of the tube was shaken and acyl derivatives of ASA were extracted into 3 ml of ethyl acetate. After centrifugation (2000 × *g*, 12 min), the tubes were stored in a deep freezer (−34 °C for 60 min) until the water layer froze to ice. The organic layer containing the analytes was

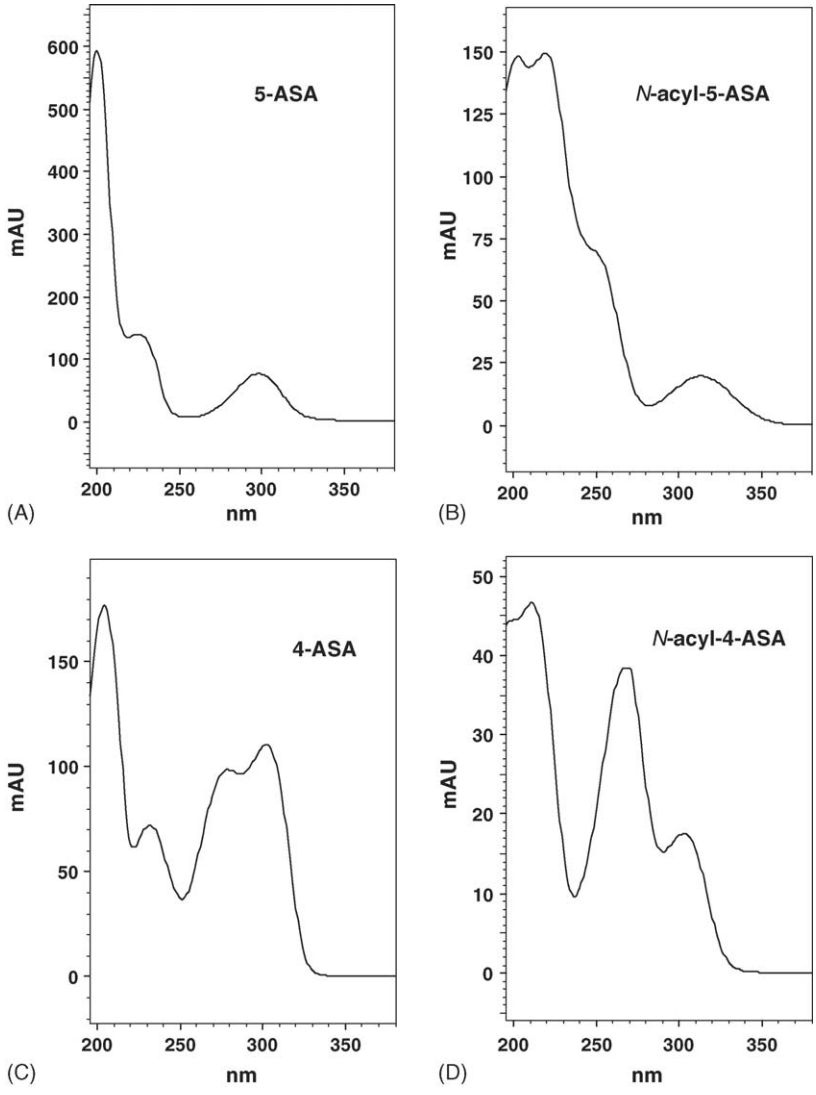

Fig. 2. UV-spectra of aminosalicylic acids (ASA-derivatives) obtained using a UV6000 photodiode-array detector during the HPLC analysis. (A) 5-ASA (t_R = 2.84 min, λ_{max} = 200, 225 and 298 nm); (B) *N*-formyl-5-ASA (t_R = 4.54 min, λ_{max} = 203, 219 and 313 nm), *N*-acetyl-5-ASA (t_R = 5.14 min), *N*-propionyl-5-ASA (t_R = 9.05 min), *N*-butyryl-5-ASA (t_R = 18.72 min); (C) 4-ASA (t_R = 6.9 min, λ_{max} = 204, 232, 279 and 302 nm); (D) *N*-acetyl-4-ASA (t_R = 7.5 min, λ_{max} = 211–216, 268, 303 nm), *N*-propionyl-4-ASA (t_R = 16.37 min).

decanted into another clean 3-ml tube and the solvent was evaporated (water bath 45 °C, stream of nitrogen). The dry extract in the glass tube was reconstituted in 600 μl of the mobile phase and transferred into the vial of the autosampler. Hundred microlitres of the sample were injected into the chromatographic column.

2.6. HPLC with tandem ultraviolet photodiode-array and fluorescence detection

Routine chromatographic analyses were performed using a Thermo Electron (formerly Thermo Finnigan) chromatograph (San Jose, CA, USA). The chromatographic system was composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100-μl sample loop, UV6000 LP photodiode-array detector (UV-PDA) with Light Pipe Technology, FL3000 fluorescence detector (FL), SN4000 system controller and a data station (Intel-Pentium 4CPU 1.6 GHz, RAM 256 MB, HDD 40GB) with ChromQuest 4 analytical software (Thermo Electron Inc., San Jose, CA, USA) working under the Windows 2000 operating system (Microsoft Corporation).

A LiChroCART® 250 mm × 4 mm column packed with Purospher RP-18e, 5 μm and precolumn LiChroCART® 4-4 with the same stationary phase (Merck, Darmstadt, Germany) was employed for 5-ASA derivative analyses.

A mobile phase containing acetonitrile–0.01 M Na_2HPO_4 buffer pH 3 in the 15:85 ratio (v/v) was used for routine analyses, the length of the isocratic analysis was 23 min at the flow rate of 1 ml min^{-1}.

A tandem UV-PDA detector → FL detector was used for monitoring of the column effluent.

UV detection was performed at 313 nm. For the collection of the characteristic UV-spectra (see Fig. 2), a photodiode-array scan mode (in the range 195–365 nm with 1 nm distance) was employed.

Fluorescence detector was set up at $\lambda_{exc.}$ = 300 nm and $\lambda_{emiss.}$ = 406 nm; these wavelengths were chosen according to the fluorescence spectra of individual ASA-derivatives. The lamp flash rate was adjusted to 100 Hz and PMT voltage was 600 V.

2.7. Calibrations

A 10-level calibration series of *N*-acetyl-5-ASA + 5-ASA/*N*-acetyl-4-ASA + 4-ASA (I.S.-1 + precursor of I.S.-2) mixtures with the plasmatic concentrations 10, 43.34, 86.7, 166.7, 966.7, 1766.7, 2566.7, 3366.7, 4166.7 and 4966.7 pmol ml^{-1} for 5-ASA and its metabolite and with the same 4-ASA and *N*-acetyl-4-ASA concentrations (3000 pmol ml^{-1}) was prepared in the following way: the proportional volumes (3, 13, 26, 50, 290, 530, 770, 1010, 1250, 1490 μl) of 10^{-4} M solutions of *N*-acetyl-5-ASA and 5-ASA were mixed and filled up to the total volume of 3 ml with UHQ water for each of the 10 calibration levels. To 100 μl of this mixture, 900 μl of drug-free plasma was added. The plasma samples containing the above-mentioned concentrations of the analytes were spiked by 30 μl of the mixture of two internal standards (see Section 2.1). The next sample preparation procedures were identical as described in Section 2.5. Six individual samples were prepared at each calibration level.

For the evaluation of the extraction recovery and the yield of the derivatization procedure, two additional 10-level aqueous calibration series (with the identical concentrations as described above) were prepared and compared mutual and with the plasmatic calibration.

The first aqueous calibration series contained *N*-acetyl-5-ASA + 5-ASA/*N*-acetyl-4-ASA + 4-ASA mixtures and the individual samples were derivatized only (no liquid–liquid extraction step was performed).

The second aqueous calibration series containing *N*-acetyl-5-ASA + *N*-propionyl-5-ASA/*N*-acetyl-4-ASA + *N*-propionyl-4-ASA mixtures was analyzed without any sample preparation step.

2.8. Validation of the analytical procedure

Statistical evaluation of the calibration analyses (see Section 2.7) by the least-squares method was performed using the ChromQuest software. The linearity of the calibration curves was tested and evaluated for aqueous solutions of *N*-acetyl-5-ASA + *N*-propionyl-5-ASA/*N*-acetyl-4-ASA + *N*-propionyl-4-ASA series and for the plasmatic calibration series based on the *N*-acetyl-5-ASA + 5-ASA/*N*-acetyl-4-ASA + 4-ASA samples, which were deproteinized, derivatized and extracted. Regression coefficients were calculated [$y = kx + q$, where x was the concentration ratio of *N*-acetyl-5-ASA to *N*-acetyl-4-ASA (I.S.-1) or *N*-propionyl-5-ASA to *N*-propionyl-4-ASA (I.S.-2) and y was the corresponding peak-area ratio *N*-acetyl-5-ASA to *N*-acetyl-4-ASA (I.S.-1) or *N*-propionyl-5-ASA to *N*-propionyl-4-ASA (I.S.-2), respectively]. The coefficient of the determination (r^2) was also expressed. The accuracy was determined as a relative error bias [accuracy (%) = $100 \times (C_{real} - C_{determined})/C_{real}$] calculated from the corresponding calibration curve equation. The intra-day precision was calculated as the relative standard deviation [RSD (%) = 100 SD/mean] from six identically prepared plasma calibration samples measured during 1 day and evaluated at various concentration levels. The set of plasma samples of eight concentration levels prepared at each of 14 following days was used for estimating of the inter-day variability (precision and accuracy). The range of the applicability of HPLC method was enclosed within the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). The lower limit of quantification (LLOQ) was determined as the lowest concentration in the standard calibration curve which was measured with a precision of 20% and accuracy of ±20% [38,39]. Upper limit of quantification (ULOQ) was equal to the highest concentration in 10-level human plasma calibration. Limit of detection (LOD) was calculated as the concentration at a signal-to-noise ratio of 3. The recovery for *N*-propionyl-5-ASA (the derivatized form of 5-ASA) and for *N*-acetyl-5-ASA (the metabolite) was calculated and the stability studies were performed [39].

3. Results and discussion

3.1. Preparation of N-acyl-aminosalicylic acid derivatives and selection of the optimal internal standards

Six derivatives of *N*-acyl-aminosalicylic acid were used as analytical standards in this study. Mesalazine (5-ASA) and PAS (4-ASA) were commercially available; the standards of all expected phase II mesalazine metabolites (*N*-formyl-5-ASA, *N*-acetyl-5-ASA, *N*-butyryl-5-ASA), the derivative of 5-ASA (*N*-propionyl-5-ASA) and two internal standards [*N*-acetyl-4-ASA (I.S.-1) and *N*-propionyl-4-ASA (I.S.-2)] were synthesized in our laboratory (see Section 2.3).

5-ASA and 4-ASA were acylated using the corresponding acetic, propionic and butyric anhydrides. The nucleophilic nitrogen of the primary amino groups in 5-ASA and 4-ASA reacted readily with the deficient acyl anhydrides at ambient temperature. An increase of the temperature (over 40 °C) led to the parallel formation of a by-product, which was identified as *N*-acyl-*O*-acyl-aminosalicylic acid.

In the case of *N*-formyl-5-ASA, 5-ASA was acylated by concentrated formic acid with azeotropic removal of water (as described in Section 2.3).

The identity and purity of the analytical standards of *N*-acyl derivatives are documented by NMR and HPLC/MS analyses in Section 2.3.

As indicated above, two of the prepared *N*-acyl-4-ASA-derivatives were chosen as the internal standards for HPLC analyses of 5-ASA and its metabolites. *N*-Acetyl-4-ASA (I.S.-1) was added to the biomatrices as a suitable internal standard for the determination of all *N*-acyl-5-ASA metabolites. *N*-Propionyl-4-ASA (I.S.-2) was formed proportionally together with *N*-propionyl-5-ASA during the derivatization of a 5-ASA containing sample spiked with 4-ASA (precursor of I.S.-2) as demonstrated in Fig. 3. The concentration of 5-ASA (which was evaluated in the form of *N*-propionyl-5-ASA) was thus calculated using I.S.-2.

3.2. Derivatization of 5(4)-aminosalicylic acids and the extraction of N-acyl derivatives from biomatrices

In our study, two types of the analytes (amphoteric 5-ASA and 4-ASA versus acidic *N*-acyl-5-ASA) were present in plasma samples.

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; $pK_a = 13.9$) [40] in the molecule of 5-ASA, this compound exhibits amphoteric properties, which complicate, together with its considerable polarity, the extraction and HPLC analysis. Hence, it appeared reasonable to derivatize the amino group by the acylation in order to suppress the amphoteric properties of 5-ASA. The ionizable amino group was masked as a non-ionizable *N*-acylamino moiety and the resultant *N*-acyl-5-ASA possessed higher lipophilicity and was well extractable at low pH.

Of the acyl anhydrides used for the derivatization, only propionic anhydride was an acceptable agent for *N*-acylation with a view to the fact 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 (see Section 1).

A study of the influence of various reaction conditions on the yield of the derivatization reaction was based on the evaluation of aqueous and plasmatic calibration series described in Section

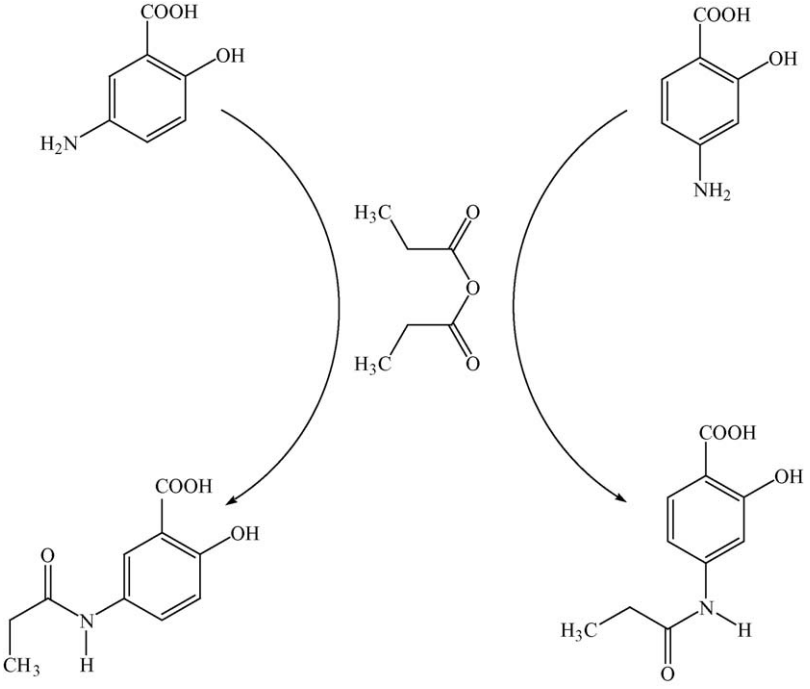

Fig. 3. Derivatization of amphoteric 5-ASA and 4-ASA (precursor of I.S.2) by propionic anhydride to the corresponding acids, *N*-propionyl-5-ASA and *N*-propionyl-4-ASA.

2.7. The volume of 20 μl of propionic anhydride added to 1 ml of human plasma containing 5-ASA in the range of applicability (see Section 3.3.1) was found to be sufficient for a linear response in the calibration curve. The derivatization of the samples was performed at 25 °C and accomplished in 20 min with high yields (86–105%) of the desired *N*-propionyl-5-ASA and *N*-propionyl-4-ASA.

Various extraction media (*t*-butylmethyl ether, diethyl ether, ethyl acetate, dichloro-methane) for the liquid–liquid extraction of *N*-acyl-ASAs were tested in our prevalidation experiments. The best results were achieved with ethyl acetate (see the recovery values in Table 3).

3.3. Chromatography

3.3.1. Routine HPLC with UV photodiode-array and fluorescence detections

Use of a simple isocratic mobile phase based on a mixture of acetonitrile and phosphate buffer of pH 3 (15: 85, v/v) in a combination with the above-mentioned stationary phase, Purospher RP-18e enabled an efficient separation of all eight compounds under study within 20 min, as shown in Fig. 4A. All compounds including the amphoteric 5-ASA and 4-ASA were eluted from the chromatographic column in narrow symmetrical concentration zones. The peak of 5-ASA had a very short retention time (t_R = 2.89 min) and a low response, which substantiates its derivatization to the more lipophilic and more easily extractable *N*-propionyl-5-ASA (t_R = 9.16 min).

Analysis of the extracts from the human plasma samples showed that only the parent drug 5-ASA and its principal metabolite *N*-acetyl-5-ASA were present in this biomatrix (see Fig. 4B and C and compare the results in Section 3.3.2). Hence, validation of the bioanalytical HPLC method involving the deproteinization, derivatization and extraction of the plasmatic samples containing these two analytes was performed using an internal standard method (see Sections 2.7 and 2.8).

The selectivity of the method was tested by the analysis of blank human plasma samples from 10 different subjects. No interferences were observed in the extracts from these samples.

Table 1
Intra-day precision and accuracy of *N*-acetyl-5-ASA and *N*-propionyl-5-ASA determination in human plasma calibration samples (nine various concentration levels with six individually prepared samples at each calibration level were measured in 1 day using fluorescence detection)

Added	Found (mean ± SD)	Precision (RSD) (%)	Accuracy (%)
Concentration of *N*-acetyl-5-ASA (pmol ml^{-1})			
43.3	33.9 ± 1.9	5.6	−15.3
86.7	86.4 ± 5.3	6.2	−0.3
166.7	183.8 ± 11.1	6	9.3
966.7	952.5 ± 36.1	3.8	−1.4
1766.7	1765.9 ± 63.9	3.6	0
2566.7	2592.0 ± 101.5	3.9	1
3366.7	3366.6 ± 71.6	2.1	0
4166.7	4099.7 ± 131.8	3.2	−1.6
4966.7	5012.5 ± 73.6	1.5	0.9
Concentration of *N*-propionyl-5-ASA (pmol ml^{-1})			
43.3	42.3 ± 4.1	9.6	−3.1
86.7	93.5 ± 5.5	5.9	8.7
166.7	183.6 ± 3.9	2.1	10.7
966.7	963.6 ± 24.4	2.5	−0.3
1766.7	1743.4 ± 69.6	4	−1.3
2566.7	2589.3 ± 83.6	3.2	0.9
3366.7	3292.4 ± 52.7	1.6	−2.2
4166.7	4153.6 ± 178.8	4.3	−0.3
4966.7	5024.5 ± 133.9	2.7	1.2

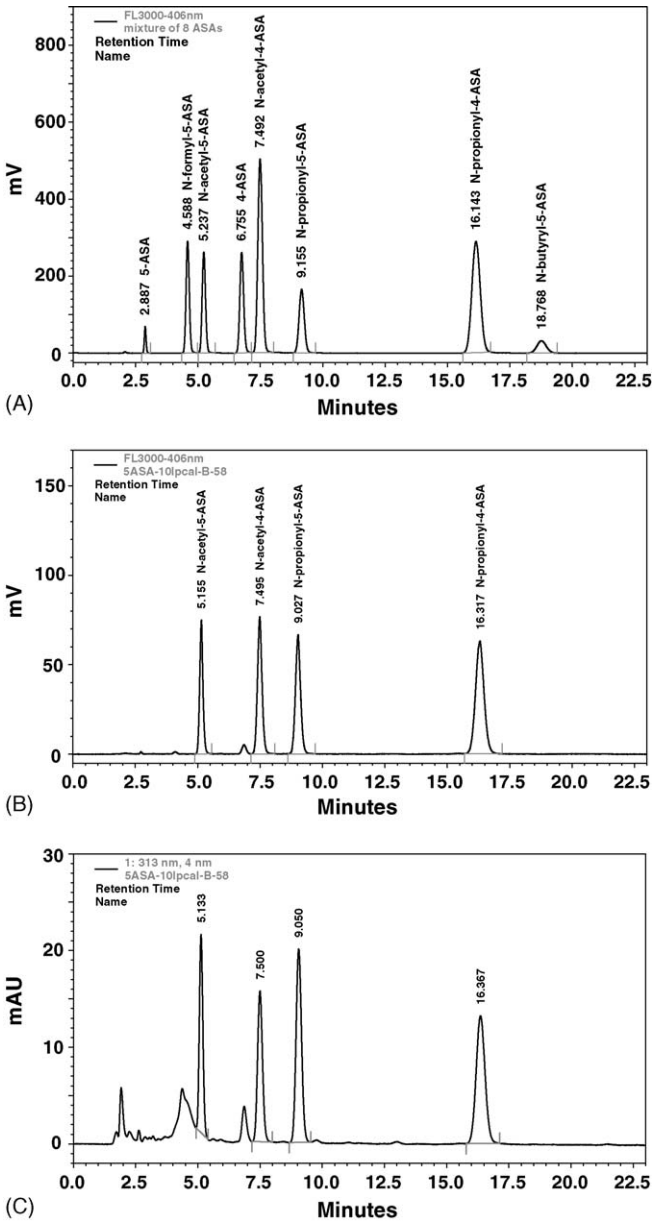

Fig. 4. (A) A chromatogram of the separation of a 10^{-5} M mixture of eight aminosalicylic acids (ASA-derivatives) under study. The chromatogram was acquired using a FL3000 fluorescence detector. (B and C) Chromatograms of the derivatized extract from the human plasma containing the principal phase II metabolite *N*-acetyl-5-ASA, *N*-acetyl-4-ASA (I.S.-1), the parent compound 5-ASA and 4-ASA (precursor of I.S.-2), both in the form of their *N*-propionyl-derivatives. Upper chromatogram (B) was acquired using a fluorescence detector (at $\lambda_{(exc.)}$ = 300 nm and $\lambda_{(emiss.)}$ = 406 nm), the lower chromatogram (C) using an ultraviolet photodiode-array detector (λ = 313 nm). See Section 2.6 for the chromatographic conditions.

Table 2
Inter-day precision and accuracy of *N*-acetyl-5-ASA and *N*-propionyl-5-ASA determination in human plasma samples containing eight various concentrations of both compounds, which were prepared and measured in 14 following days

Added	Found (mean ± SD)	Precision (RSD) (%)	Accuracy (%)
Concentration of *N*-acetyl-5-ASA (pmol ml^{-1})			
86.7	87.3 ± 24	27.5	−0.7
166.7	158.9 ± 21.6	13.6	4.9
966.7	977.8 ± 43.8	4.5	−1.1
1766.7	1762.2 ± 46.9	2.7	0.3
2566.7	2567.9 ± 72.6	2.8	0.0
3366.7	3360.8 ± 83.3	2.5	0.2
4166.7	4180.4 ± 70.3	1.7	−0.3
4966.7	4958.4 ± 84.2	1.7	0.2
Concentration of *N*-propionyl-5-ASA (pmol ml^{-1})			
86.7	92.6 ± 45.8	49.4	−6.4
166.7	170.6 ± 40.6	23.8	−2.3
966.7	962.4 ± 32.2	3.3	0.5
1766.7	1758.3 ± 90.8	5.2	0.5
2566.7	2605.7 ± 112.3	4.3	−1.5
3366.7	3307.1 ± 132.1	4.0	1.8
4166.7	4162.8 ± 110.5	2.7	0.1
4966.7	4993.7 ± 135.0	2.7	−0.5

Fluorescence detection was used.

Also, the resolution among individual ASA peaks was satisfactory (see Fig. 4A). As it is apparent from Fig. 4B and C, the fluorescence chromatograms were much clearer, fewer ballast peaks were observed and better response was achieved in comparison with the UV detection. On the other hand, the UV photodiode-array detector played an important role in the identification of the individual ASA-derivatives according to their characteristic UV-spectra and retention times (see Fig. 2).

Validation results using fluorescence detection are summarized in Tables 1–3. The intra-day variability (mean ± SD, precision and accuracy) was calculated from human plasma calibration for both *N*-acetyl-5-ASA and *N*-propionyl-5-ASA (Table 1). Acceptable intra-day results for the precision and accuracy were found in the range of 43.3–4967 pmol ml^{-1}. The evaluation of inter-day variability is shown in Table 2. The precision exceeded the limit of 20% at the concentration of 86.7 pmol ml^{-1} for *N*-acetyl-5-ASA and at the concentrations of 86.7 and 166.7 pmol ml^{-1} for *N*-propionyl-5-ASA. The LLOQ values for *N*-acetyl-5-ASA and *N*-propionyl-5-ASA were calculated from Table 2 by a linear interpolation method. Acceptable inter-day results for the precision and accuracy were achieved in the ranges of 126–4967 pmol ml^{-1} for *N*-acetyl-5-ASA and 318–4967 pmol ml^{-1} for *N*-propionyl-5-ASA. The parameters of calibration curve and the extraction recoveries for both compounds are presented in Table 3. Limits of detection (LODs) were also determined (20 pmol ml^{-1} for *N*-acetyl-5-ASA and 50 pmol ml^{-1} for *N*-propionyl-5-ASA).

The stock solutions of ASA-derivatives were found to be stable for 20 days (storage at 5 °C) and 5-ASA and *N*-acetyl-5-ASA in plasma samples stored at −70 °C were stable at least for 90 days.

3.3.2. HPLC with mass spectrometric detection

All synthetic standards (see Sections 2.1 and 2.3) were measured using HPLC/MS and MS/MS and the mass spectra were interpreted in detail. The molecular weights of all compounds were confirmed on the basis of $[M+H]^+$ and $[M+Na]^+$ ions observed in the positive-ion ESI mode and $[M–H]^-$ ions in the negative-ion ESI mode. The peaks of the (de)protonated molecules were the base peaks of all mass spectra except for *N*-formyl-5-ASA, where these peaks were the second most abundant. Logical neutral losses (e.g., H_2O, CO_2, CH_2CO, CH_3CHCO and CH_3CH_2CHCO) correlate with the structures of individual compounds. In the studied human plasma samples, the following analytes were positively identified, based on the interpretation of their mass spectra and the agreement of their retention times and UV-spectra with the synthetic standards: *N*-acetyl-5-ASA, *N*-propionyl-5-ASA, *N*-acetyl-4-ASA and *N*-propionyl-4-ASA.

3.4. Pharmacokinetic studies

The validated bioanalytical HPLC method was applied to biotransformation and disposition studies of mesalazine in humans and animals.

As an example, the results from a pharmacokinetic study performed in 14 healthy human volunteers are demonstrated. The particulars of this experiment were described in Section 2.4. The average plasma concentration–time profiles of 5-ASA and its principal metabolite *N*-acetyl-5-ASA in human volunteers after the rectal administration of one suppository containing 500 mg of mesalazine are shown in Fig. 5.

The main pharmacokinetic variables were estimated from the measured pharmacokinetic data: Maximal concentration (C_{max}) achieved in the time t_{max} after the administration of mesalazine form was found directly from the measured concentrations (without any interpolation). The area under the curve from $t = 0$–48 h ($AUC_{0–48}$) was determined via the linear trapezoidal rule. The average values of these three pharmacokinetic variables for parent compound (5-ASA) and its metabolite (*N*-acetyl-5-ASA) were found or calculated:

5-ASA: $C_{max} = 1758$ pmol ml^{-1}, $t_{max} = 1$ h, $AUC_{0–48} = 15397$ pmol h ml^{-1}; *N*-acetyl-5-ASA: $C_{max} = 2312$ pmol ml^{-1},

Table 3
Internal standard calibration curve parameters for *N*-acetyl-5-ASA and *N*-propionyl-5-ASA for spiked plasma samples (results based on the mean values from 14 individual calibration sequences prepared in various days)

	Slope	Intercept	Correlation coefficient	Range of applicability (pmol ml^{-1})	Recovery (%)
N-acetyl-5-ASA	0.4651 ± 0.018	0.0011	0.9999	126–4967	78
N-propionyl-5-ASA	0.4036 ± 0.046	−0.0022	0.9997	318–4967	81

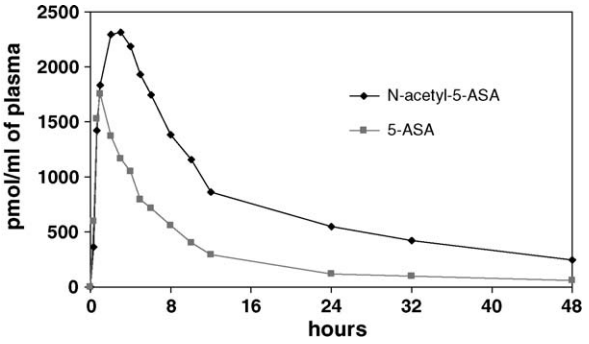

Fig. 5. Average pharmacokinetics of *N*-acetyl-5-ASA and 5-ASA (determined as *N*-propionyl-5-ASA) in 14 healthy volunteers after the single rectal administration of mesalazine preparation (suppository, 500 mg of 5-ASA).

$t_{max} = 3$ h, $AUC_{0–48} = 43110$ pmol h ml^{-1}The estimated pharmacokinetic variables were employed for the evaluation of the drug bioavailability in bioequivalence studies.

4. Conclusions

A new bioanalytical method involving a deproteinization, lipophilicity-increasing derivatization step followed by a simple liquid–liquid extraction of plasma samples and subsequent high-performance liquid-chromatographic determination of derivatized 5-ASA (in the form of *N*-propionyl-5-ASA) and its principal phase II metabolite, *N*-acetyl-5-ASA, using both UV photodiode-array and fluorescence detections, was developed and validated.

This method enables an excellent mutual separation of eight analytes: 5-ASA (mesalazine), *N*-formyl-5-ASA, *N*-acetyl-5-ASA, *N*-butyryl-5-ASA (three phase II mesalazine metabolites), *N*-propionyl-5-ASA and internal standards used (*N*-acetyl-4-ASA and *N*-propionyl-4-ASA and 4-ASA, its precursor). All compounds were unambiguously identified by UV photodiode-array detection (based on their UV-spectra and retention times), mass spectrometric detection (in both positive-ion ESI mode and negative-ion ESI mode) and two principal analytes (5-ASA and *N*-acetyl-5-ASA) were determined using a validated internal standard method employing a fluorescence detection.

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