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Validation of a LC method for the determination of 5-aminosalicylic acid and its metabolite in plasma and urine

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Abstract

The choice of a proper analytical method for the quantification of drugs and/or their metabolites in biological samples plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. The aim of this study was validation of a method for the identification and quantitative determination of 5-aminosalicylic acid (5-ASA) and its metabolite *N*-acetyl-5-aminosalicylic acid in human plasma and urine. According to previous studies on the disposition of 5-ASA (mesalazine) in a patient with inflammatory bowel diseases, we have developed a rapid, sensitive method for the determination of 5-ASA and its acetylated metabolite, *N*-acetyl-5-aminosalicylic acid (Ac-5-ASA). The advantage of this method is that it measures both compounds, and is more rapid, reproducible and credible than the previous studies [C. Fischer, K. Maier, U. Klotz, J. Chromatogr. Biomed. Appl. 225 (1981) 498–503; P.N. Shaw, A.L. Sivner, L. Aarons, J.B. Houston, J. Chromatogr. Biomed. Appl. 274 (1983) 393–397; B. Norlander, R. Gotthard, M. Strom, Aliment. Pharmacol. Ther. 3 (1989) 333–342; U. Klotz, G.L. Stracciari, Arzneim.-Forsch. Drug Res. II 43 (12) (1993) 1357–1359]. 5-ASA was quantitatively determined in human plasma and urine samples by liquid chromatography following prior derivatization to its acetylated metabolite (Ac-5-ASA). *N*-Acetyl-anthranilic acid was used as the internal standard. The detection was performed with a spectrofluorimetric detector, excitation at 311 nm, cut-off at 449 nm. The method was validated for the following parameters: linearity, recovery, sensitivity, precision, accuracy, selectivity and stability, limits of quantification and of detection. It showed good linearity ($r^2 \geq 0{,}996$) in the range 0.1 ng/ml to 8 μg/ml using a Lichrospher 60 RP-select B column. The lower limit of detection was 20 ng/ml in plasma and urine. The within-run relative standard deviations (R.S.D.) were below 6.7% at all concentration levels and the between-run R.S.D. were below 25.4% at all concentration levels.

Keywords: 5-Aminosalicylic acid; 5-Amino-2-hydroxybenzoic acid; Mesalazine; *N*-acetyl-5-aminosalicylic acid; High-performance liquid chromatography; Validation

1. Introduction

Mesalazine, beside sulphasalazine and olsalazine, is most often used in the treatment of inflammatory bowel diseases (IBD) such as

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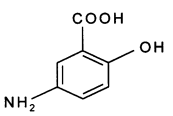
Crohn's disease and ulcerative colitis [1,2]. Pharmaceuticals generally differ in the type of carrier, which protects the active substance against breaking down in the upper alimentary tract. Sulphasalazine is a pro-drug comprising 5-aminosalicylic acid (5-ASA) and sulphapyridine joined by an azo bond. Azo-bond linkage with the carrier molecule sulphapyridine enables 5-ASA to reach the colon, where bacterial enzymes split the azo bond, liberating the two components. The sulphapyridine moiety of sulphasalazine is an effective carrier molecule for mesalazine, but it is considered to be responsible for the most cases of intolerance to sulphasalazine. Consequently, research has been directed to develop a mesalazine formulation that would successfully deliver the active substance to the colon and be devoid of toxic effects exerted by the carrier [1–3]. The knowledge of the bioavailability, bioequivalence and the pharmacokinetic profile of 5-ASA from the different 5-ASA-containing drugs is important for the effective therapy of IBD.



Acetyl-5-aminosalicylic acid (Ac-5-ASA) was extracted from the acidified medium into diethyl ether. 5-ASA as an amphoteric compound can be extracted into organic solvents after addition of ion-pair reagent to the aqueous medium, but still only in poor yield. Thus, 5-ASA has been determined indirectly as its acetylated derivative Ac-5-ASA. The concentration of 5-ASA has been calculated from the differences between the Ac-5-ASA concentrations in the acetylated and non-acetylated samples [3,4]. For determination, the method of Fischer et al. with our own modifications was applied [3]. The solubility of 5-ASA in pure methanol was increased by acidifying the solvent with a drop of 1 M HCl. The mobile phase consisted of a mixture of deionized water (adjusted to pH 3 with perchloric acid):methanol:acetonitrile (60:20:20 v/v/v). *N*-Acetyl-anthranilic acid was used as the internal standard. The analysis time and the chromatographic separations took 10 min. The detection was performed with a spectrofluorimetric detector, excitation at 311 nm, cut-off at 449 nm.

2. Experimental

2.1. Chemicals and reagents

5-ASA and *N*-acetyl-5-ASA in substantio were provided by GLAXO Wellcome Poznań S.A. Methanol, acetonitrile, diethyl ether and perchloric acid used in this determination were of analytical grade (Merck). The internal standard *N*-acetyl-anthranilic acid was synthesized by acetylation of anthranilic acid with acetic acid and purified by recrystallization.

2.2. Chromatographic conditions

The chromatographic separations and quantitative determination were performed on a high-performance liquid chromatograph equipped with a pump model 64 (KNAUER), and Shimadzu RF-551 fluorescence detector was used for detection. A MERCK analytical column (LiChrospher 60 RP-select B; 5 μm particle size, 250 × 4.6 mm) was used as the stationary phase. Precolumn (LiChroCART 4-4; 5 μm, 4 × 4 mm) was used to protect the analytical column. An injector was the Rheodyne 7125 with a noose of 100 μl. The data was collected by using an integrator D-7500 (MERCK HITACHI). The mobile phase consisted of deionized water (adjusted to pH 3 by perchloric acid):methanol:acetonitrile (60:20:20 v/v/v). The flow rate was 1.2 ml/min. Fluorescence detection was monitored at an excitation wavelength of 311 nm and an emission wavelength of 449 nm, range × 32 or × 64, sensitivity high.

2.3. Sample preparation

Plasma and urine, used in this study, were collected from volunteers (men between 20 and 40 years old, non-smoking, healthy). The urine samples were collected for 24 h. Samples were stored at the temperature of about −40°C. 'Stock solutions' of 5-ASA, Ac-5-ASA and the internal stan-

dard were prepared in methanol and stored for about 1 week at 4°C, without light access. Solubility of 5-ASA in the pure methanol was increased by acidifying with 1 M HCl. The quality control (QC) samples were prepared in human plasma and urine free from 5-ASA and Ac-5-ASA, and stored under the same conditions as the analytical samples. The calibration samples were prepared immediately before analysis.

All samples were run in duplicate, one with and the other without acetylation prior to extraction. A 600 μl volume of plasma and urine (only the urine's samples were diluted with distilled water, 1:100) was pipetted onto the test tube. Next, 10 μl acetic acid anhydride was added to one of the duplicates and the samples were shaken for 15 min (acetylation procedure). After acetylation, 100 μl internal standard (20 μg *N*-acetyl-anthranilic acid per ml pure methanol) was added to all samples. Next, samples of plasma or urine were precipitated with 50 μl concentrated perchloric acid and shaken immediately for 5 min (deproteinization). After shaking, the solution was centrifuged at 4000 rpm for 10 min.

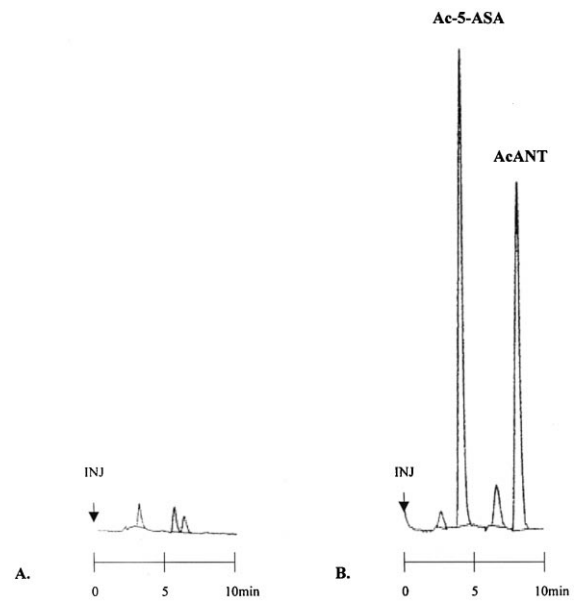


Fig. 1. HPLC separation of Ac-5-ASA and *N*-acetyl-anthranilic acid in human plasma after extraction. A, Blank.

The clear supernatant was subjected to extraction. Five hundred microlitres of 1 M HCl and 9 ml diethyl ether was added to the supernatant and extracted by shaking for 10 min (extraction procedure). The mixture was separated by centrifugation for 5 min. The organic phase was evaporated to dryness and the residue was dissolved in 300 μl mobile phase. A 100 μl volume of this solution was injected directly onto the batcher. The chromatographic separation took 10 min.

2.4. Calibration curves

Calibration curves were constructed every day after the analysis of drug free plasma or urine samples containing known amounts of 5-ASA and Ac-5-ASA, with and without the acetylation procedure. The plasma or urine samples were spiked with 5-ASA and Ac-5-ASA to the following concentration: blank, 0.10, 0.50, 1.00, 1.50, 2.00 μg/ml. *N*-Acetyl-anthranilic acid was used as the internal standard. These samples were analysed according to the procedure described for sample preparation (Section 2.3). The number of QCs per batch was six (three concentration levels in duplicate). They were assessed according to 4-6-20 rule [5].

3. Results

3.1. Specificity/selectivity

The specificity/selectivity of the method can be illustrated by comparing the chromatograms obtained after analysis of a test solution of the pure compound with the chromatograms of the independent blank plasma and urine samples. No interfering peaks can be seen if blank plasma or urine are used (Figs. 1 and 4). The peak of the Ac-5-ASA was well resolved and showed no interferences with endogenous or exogenous materials. In urine, only the acetylated metabolite of 5-ASA can be analysed.

The retention time for Ac-5-ASA was 4 min and that for the internal standard (*N*-acetyl-anthranilic acid) was 8 min.

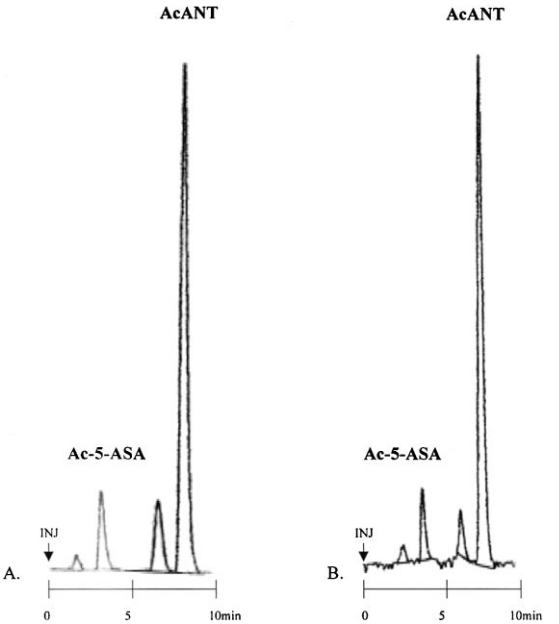


Fig. 2. Chromatograms. (A) Human plasma after acetylation. This chromatogram show LLOQ for 5-ASA (concentration, 100 ng/ml). (B) Human plasma without acetylation. This chromatogram show LLOQ for Ac-5-ASA (concentration, 100 ng/ml).

3.2. Sensitivity

The lower detection limit depends on several parameters such as the mobile phase and the fluorescence monitor used. Under the experimental conditions described, the detection limit (defined as three times the baseline noise) was approximately 20 ng/ml. The lowest concentration of the calibration graph was 100 ng/ml and it was the lower limit of quantification (LLOQ). The LLOQ is shown in Figs. 2 and 3.

3.3. Linearity

Detector response for 5-ASA and Ac-5-ASA was linear to a concentration of at least 8 μg/ml. The resulting data was plotted as peak height (measured electronically by the integrator) versus concentration and studied by the linear regression analysis. Tables 1–3 give the results for the assessment of the goodness of fit/lack of fit for the both compounds (5-ASA and Ac-5-ASA) in plasma and for Ac-5-ASA in urine assay. The goodness of fit was highly significant. A test for lack of fit indicated that the linear model is appropriate for establishing a relationship between the concentration and the response. In general, correlation coefficients above 0.996 were observed during the validation experiments.

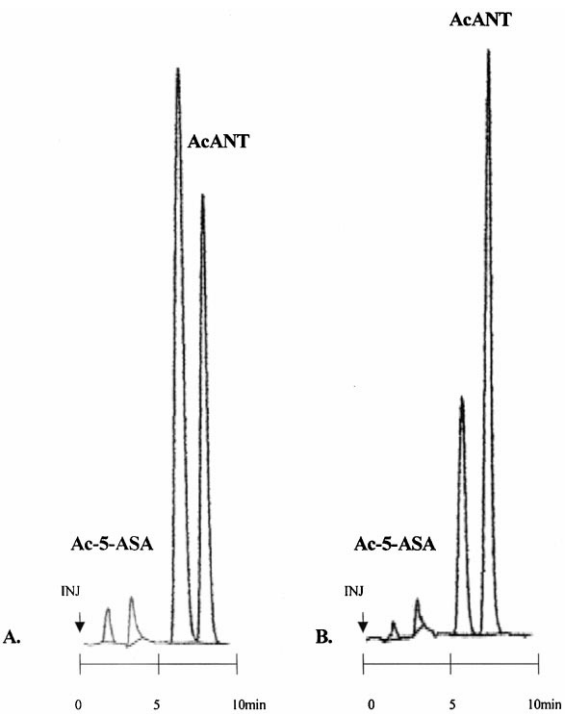


Fig. 3. Chromatograms. (A) Human urine with acetylation. This chromatogram show LLOQ for 5-ASA (concentration, 100 ng/ml). (B) Human urine without acetylation. This chromatogram show LLOQ for Ac-5-ASA (concentration, 100 ng/ml).

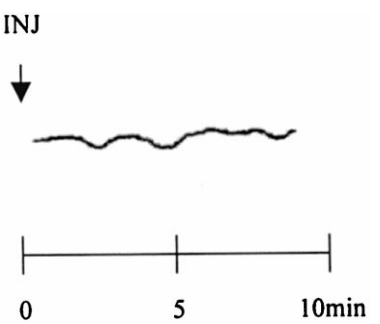


Fig. 4. Chromatogram of blank urine sample.

Table 1
Goodness of fit and lack of fit for 5-ASA with acetylation procedure (concentration range, 0.1–2.0 μg/ml; $n = 15$)

Concentration (μg/ml)	X_1	X_2	X_3	Mean	S.D.	R.S.D. (%)
0.10	0.098	0.142	0.087	0.109	0.03	26.70
0.50	0.373	0.378	0.343	0.365	0.02	5.19
1.00	0.649	0.669	0.733	0.684	0.04	6.42
1.50	1.208	1.113	1.371	1.231	0.13	10.60
2.00	1.609	1.542	1.557	1.569	0.04	2.24

Table 2
Goodness of fit and lack of fit for Ac-5-ASA (concentration range, 0.1–2.0 μg/ml; $n = 15$)

Concentration (μg/ml)	X_1	X_2	X_3	Mean	S.D.	R.S.D. (%)
0.10	0.092	0.084	0.074	0.083	0.01	10.82
0.50	0.339	0.340	0.344	0.341	0.00	0.78
1.00	0.681	0.722	0.683	0.695	0.02	3.32
1.50	0.974	1.058	1.026	1.019	0.04	4.16
2.00	1.395	1.793	1.709	1.632	0.21	12.85

Table 3
Goodness of fit and lack of fit for Ac-5-ASA in urine (concentration range. 0.1–2.0 μg/ml; $n = 25$)

Concentration (μg/ml)	X_1	X_2	X_3	X_4	X_5	Mean	S.D.	R.S.D. (%)
0.10	0.082	0.076	0.085	0.094	0.055	0.078	0.015	18.6
0.50	0.339	0.34	0.344	0.313	0.375	0.342	0.022	6.4
1.00	0.681	0.722	0.683	0.709	0.687	0.696	0.018	2.6
1.50	1.299	1.106	1.208	1.113	1.371	1.219	0.116	9.5
2.00	1.609	1.793	1.709	1.712	1.669	1.698	0.067	4.0

Table 4
Accuracy and precision of the measurement of 5-ASA (with acetylation procedure) and Ac-5-ASA (between-run) in plasma and urine ($n = 45$)

	Nominal concentration (μg/ml)	Measured concentration, mean (μg/ml)	S.D.	R.S.D. (%)
5-AS plasma	0.1	0.201	0.03	15.87
	1.0	1.755	0.35	19.81
	2.0	3.384	0.64	18.93
AcAS plasma	0.1	0.242	0.04	15.42
	1.0	1.623	0.41	25.41
	2.0	3.393	0.70	20.65
AcAS urine	0.1	0.087	0.023	25.86
	1.0	0.745	0.090	12.05
	2.0	1.749	0.075	4.27

3.4. Precision and accuracy

A summary of the results on precision and accuracy as derived from the measured concentration for the validation samples is given in Tables 4 and 5. The within-run relative standard devia-

Table 5
Accuracy and precision of the measurement of 5-ASA (with acetylation procedure) and Ac-5-ASA (within-run) in plasma and urine ($n = 45$)

	Nominal concentration (μg/ml)	Measured concentration, mean (μg/ml)	S.D.	R.S.D. (%)
5-AS plasma	0.1	0.158	0.01	6.70
	1.0	1.160	0.07	5.86
	2.0	2.811	0.06	2.23
AcAS plasma	0.1	0.171	0.01	5.57
	1.0	0.942	0.06	6.62
	2.0	2.223	0.03	1.31
AcAS urine	0.5	0.084	0.008	8.9
	1.5	0.696	0.018	2.61
	5.0	1.698	0.067	3.96

tion (R.S.D.) was below 6.7% at all concentration levels in plasma and below 8.9% in urine. The between-run R.S.D. was below 25.8% at all concentration levels in plasma and urine. Criteria of acceptance are $\pm 15\%$ at all level concentrations, except at the LLOQ where they are $\pm 20\%$ [6,7].

3.5. Recovery

For determination of recovery of the analytes, the mean peak heights obtained for triplicate measurements were compared with the mean peak heights obtained from triplicate direct injections performed in the same run. The five recoveries thus obtained were used to calculate the mean recovery. Data on the absolute analytical recovery of Ac-5-ASA are given in Table 6. The recovery is relatively low, about 50%. The reason for the low recovery is probably poor solubility of Ac-5-ASA into the organic solvents, but the achieved sensitivity is sufficient for plasma and urine level.

4. Discussion

Ac-5-ASA was extracted from the acidified medium into diethyl ether. Because 5-ASA as an amphoteric compound can be extracted into organic solvents, but in poor yield even after addition of ion-pair reagent to the aqueous medium, it was determined indirectly in the form of its acetylated derivative Ac-5-ASA. The efficiency of the acetylation process was about 98% (acetic anhydride was used in excess). The concentration value of 5-ASA has been calculated from the difference between the Ac-5-ASA concentration in samples with and without the acetylation procedure [3,4]. Solubility of 5-ASA in pure methanol was increased by acidifying the solvent. Volumetric composition of the mobile phase was changed and new internal standard was used. This enabled to reduce time of analysis and chromatographic separations to 10 min. The detection was performed with a spectrofluorimetric detector, excitation as set at 311 nm, cut-off at 449 nm. Application of such analytical conditions, for example the new excitation and emission wavelengths, minimized the level of noise. The chromatographic conditions applied allowed for better determination of the Ac-5-ASA content in plasma and urine samples. The advantage of this method is its high sensitivity and specificity.

Table 6
Recovery of *N*-acetyl-5-aminosalicylic acid

Value h_{AcAS}/h_{AcANT} after extraction	Value h_{AcAS}/h_{AcANT} from direct injection	Recovery (%)
1.476	2.95	50.03
1.369	2.828	48.41
1.351	2.658	50.83
1.564	3.111	50.27
1.453	2.674	54.34
Total		50.78

5. Conclusion

The described method can be used to monitor the level of 5-aminosalicylic acid and its major metabolite *N*-acetyl-5-aminosalicylic acid. The knowledge of pharmacokinetics of 5-ASA is important in bioequivalence studies, when a new drug formulation is prepared.

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