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Note

Simplified high-performance liquid chromatographic method for 5-aminosalicylic acid in plasma and urine

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During the last few years interest in salicylazosulfapyridine (SASP; Azulfidine®) has been focussed on 5-aminosalicylic acid (5AS) [1—3]. Gut bacteria split the azo bond of SASP forming 5AS and sulfapyridine (SP). Several groups reported the superiority of 5AS and SASP over SP in the therapy of Crohn's disease and ulcerative colitis [1—3], indicating that 5AS may be the therapeutic active moiety of SASP, an established drug for the therapy of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.

SASP has to be taken for long periods of time even after achieving remission phases to prevent a relapse (for review see refs. 4 and 5). Thereby the sulfonamide SP is responsible for the side effects which are reported with an incidence of 20—30% [6—10]. Since 5AS has been proven as the active moiety of SASP, SP could be excluded by the direct administration of 5AS, and thus side-effects might be minimized.

On treating patients with 2—4 g of SASP daily, 5AS and its major acetylated metabolite in plasma and urine show maximal concentrations of about 1 μg/ml [11]. These levels require a method of high sensitivity. We will describe a specific high-performance liquid chromatographic (HPLC) assay with increased sensitivity compared to our previously published measurements [12]. Its application to monitoring plasma and urine of patients receiving SASP or 5AS will be reported.

EXPERIMENTAL

Reagents and material

5-Amino-2-hydroxybenzoic acid (5AS) and 4-amino-2-hydroxybenzoic acid

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(PAS) were purchased from Merck (Darmstadt, G.F.R.). The internal standard AcPAS was synthesized by acetylation of PAS with acetic anhydride and purified by recrystallization. All other reagents were of analytical grade (Merck).

Apparatus

The chromatographic separations were performed on a high-performance liquid chromatograph (Model SP 740, Spectra-Physics, Darmstadt, G.F.R.) equipped with a self-packed 250 mm × 4.6 mm I.D. analytical column packed with reversed-phase material (Nucleosil 10 C-18, 10 μm; Macherey and Nagel, Düren, G.F.R.) and a 120 mm × 4.6 mm I.D. precolumn, prepacked with a reversed-phase material (LiChrosorb C-18, 5 μm, Knauer, Oberursel, G.F.R.). The detection was performed with a fluorescence monitor (Spectra-Physics, Model FS 970 M-A 1), excitation at 300 nm, cut-off filter at 418 nm.

Sample preparation

Table I summarizes the extraction procedure for plasma or urine. All samples were run in duplicate, one with and one without acetylation prior to extraction.

Chromatographic conditions

The mobile phase consisted of deionized water adjusted to pH 3 by concentrated perchloric acid—methanol—acetonitrile (75:12.5:12.5). A flow-rate of 0.5—0.6 ml/min with a resulting pressure of about 300 bar was established and samples of 100 μl were injected.

TABLE I

EXTRACTION PROCEDURE FOR 5AS AND 5AcAS FROM PLASMA AND URINE

A. Acetylation procedure
500 μl of plasma or urine (diluted 1:100 to 1:1000) in duplicate (one with and one without acetylation prior to extraction)
20 μl of internal standard (0.1 mg AcPAS per ml double-distilled water)
10 μl of acetic acid anhydride in one of the duplicates
Shake for 15 min
B. Deproteinization
Add 50 μl of concentrated perchloric acid
Shake immediately for 5 min
Separate from proteins by centrifugation
C. Extraction
Take 400 μl of supernatant
Add 500 μl of 1 *N* HCl and 9 ml of diethyl ether
Extract by shaking for 10 min
Separate by centrifugation for 5 min
Evaporate the organic phase under nitrogen to dryness
Dissolve residue in 200 μl of the mobile phase

RESULTS

5AS as an amphoteric compound can be extracted into organic phases only

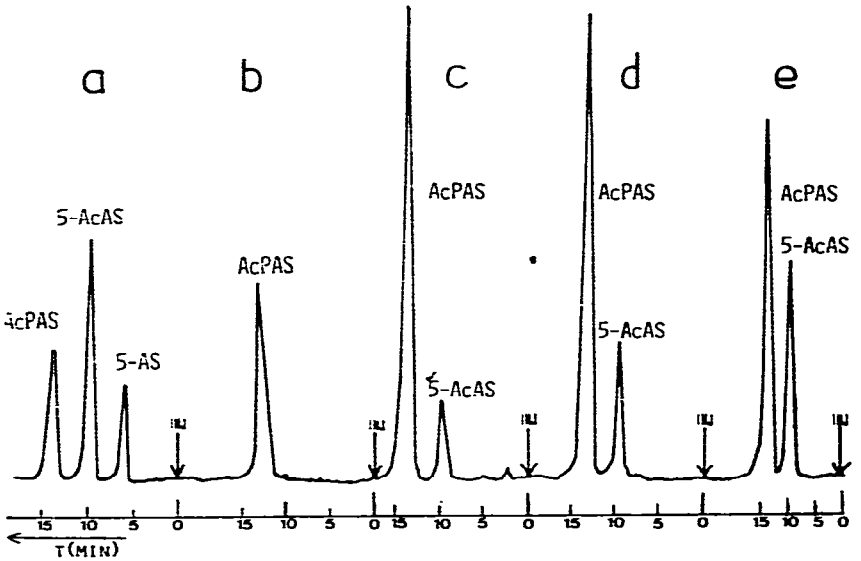

Fig. 1. HPLC separation of (a) 50 ng each of 5AS, 5AcAS and AcPAS, injected directly into the HPLC system; (b) extract of 500 μl of blank plasma spiked with internal standard; (c, d) extract of 500 μl of blank plasma spiked with 1 μg of 5AS, 0.1 μg of 5AcAS and 1 μg of AcPAS, without (c) and with (d) acetylation procedure; (e) extract of plasma of patient treated with 3 g of SASP and spiked with internal standard before acetylation and extraction procedure.

in poor yield by addition of an ion-pair reagent to the aqueous medium [12]. The major metabolite of 5AS, the N-acetylated 5AS, is more lipophilic and can be extracted easily from the acidified aqueous phase into organic solvents such as ethyl acetate, dichloromethane or diethyl ether. In addition, 5AcAS possesses better fluorescence characteristics than 5AS. We used these favourable properties of 5AcAS to obtain a more sensitive detection.

The N-acetylated isomer of 4-aminosalicylic acid (AcPAS) was added as internal standard prior to the acetylation procedure. Since only the 5AcAS has to be extracted no addition of an ion-pair reagent is necessary. In contrast to dichloromethane, no interfering peaks appear after HPLC separation, and the extraction yield is increased by a factor of 2 if diethyl ether is used as solvent.

Comparing peak heights after direct injection into the HPLC system, 5AcAS is extracted in 50% yield into diethyl ether. This result was achieved with two different concentrations (0.1 and 0.4 μg/ml). The recovery is relatively low but the achieved sensitivity (lower limit 0.02 μg/ml) is sufficient for plasma level monitoring.

Some typical HPLC chromatograms are reproduced in Fig. 1. By injecting 50 ng each of 5AS, 5AcAS and AcPAS directly into the HPLC system, three well-separated peaks appear with retention times of 5.8, 9.6 and 13.8 min, respectively. The peak heights indicate about a two-fold higher fluorescence sensitivity of 5AcAS than 5AS. Fig. 1c (without acetylation) and Fig. 1d (with acetylation) show the chromatograms obtained after extraction of 500 μl of blank plasma spiked with 0.1 μg of 5AS, 0.1 μg of 5AcAS and 1 μg of AcPAS. Only the acetylated compounds are extracted. No interfering peaks can be seen (Fig. 1b) if blank plasma is used.

TABLE II

ACCURACY AND PRECISION OF THE MEASUREMENT OF 5AS AND 5AcAS IN PLASMA

Spiked concentration μg/ml		Acetylation procedure	n	Measured concentration (mean ± S.D. μg/ml 5AcAS)		
5AS	5AcAS					
0.4	0.4	—	6	0.40	±	0.02
0.4	0.4	+	6	0.80	±	0.03
0.8	0.8	—	6	0.80	±	0.02
0.8	0.8	+	6	1.59	±	0.05
0.4	—	+	5	0.40	±	0.01
1.6	—	+	6	1.60	±	0.05
0.1	0.1	—	8	0.10	±	0.01
0.1	0.1	+	8	0.20	±	0.01

The HPLC system involves a ternary solvent system (aqueous phase, methanol, acetonitrile). Using an ion-pair reagent like trimethylcetylammonium bromide we observed a decrease in resolution after several injections.

5AcAS is quantified by measuring its peak heights related to those of the internal standard AcPAS. Standard curves are constructed after analysis of plasma or urine samples containing known amounts of 5AS and 5AcAS with and without the acetylation procedure. The standard curve is linear from 0.02 to 8 μg/ml.

Table II demonstrates the accuracy and precision for different concentrations of 5AS and 5AcAS. The lower detection limit depends on several parameters such as the mobile phase and the fluorescence monitor used. In our system we can measure 0.02 μg/ml 5AcAS either in the form of 5AcAS (without acetylation), or 5AS (with acetylation), or the sum of both.

Stability tests of 5AS and 5AcAS in plasma were performed at room temperature or at 4°C. After 7 days neither a decrease in the content of the plasma samples could be detected nor did additional peaks appear.

The application of the method to patients (see Fig. 1e) treated with 3 g of SASP revealed very low plasma levels of both 5AS and 5AcAS. The trough steady-state plasma concentrations of six patients ranged between 0.04 and

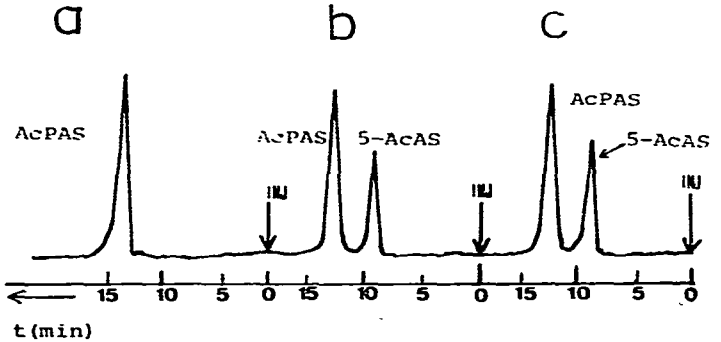

Fig. 2. HPLC chromatograms of urine samples. (a) Blank urine, diluted 1 : 1000, spiked with internal standard; (b, c) urine samples without (b) and with (c) acetylation procedure of a patient receiving 1.5 g of 5AS per day.

0.34 μg/ml 5AS and between 0.2 and 1.4 μg/ml 5AcAS. Plasma levels of patients receiving 1.5 g/day 5AS as suppositories fluctuated between 0.1 and 0.4 μg/ml 5AS and between 0.1 and 1.4 μg/ml 5AcAS.

In the urine of a patient receiving 1.5 g of 5AS in the form of suppositories we could detect only the acetylated metabolite. No difference between the acetylated and non-acetylated urine sample was seen (Fig. 2). No other peaks appeared in the HPLC chromatogram than those of 5AcAS and the internal standard. The extraction of a 100—1000-fold diluted 10-μl aliquot of an 8-h urine collection resulted in good measurable peaks.

DISCUSSION

5AS is determined indirectly in the form of its acetylated derivative 5AcAS to improve the detection limit. Samples run without the acetylation procedure will provide the endogenous 5AcAS concentration, whereas acetylated samples result in the sum of acetylated 5AS and 5AcAS; the difference between the two values will give the 5AS moiety. The plasma levels of patients receiving therapy with SASP or 5AS suppositories can be monitored by the extraction of two samples of 500 μl of plasma, one with and one without the acetylation procedure.

The ternary solvent system offers some advantage over the use of a mobile phase containing the ion-pair reagent trimethylcetylammonium bromide: quicker equilibration of the column, longer lifetime of the column, better separation of the peaks and lower cost of material. The use of a precolumn is recommended to obtain better separation and to prolong the life-span of the analytical column. Decreasing the percentage of both organic solvents from 12.5 to 7.5% in the mobile phase prolongs the retention times. This might be of advantage when interfering peaks are to be expected.

Data from Khan et al. [13] suggest that aspirin interferes with the photometric assay of Hannson and Sandberg [11]. In our more specific method this often used drug was well resolved from 5AS and its acetylated metabolite, indicating no interferences with the described method.

In conclusion, our method can be used for plasma level monitoring of 5AS, which might be helpful in guiding therapy in patients with ulcerative colitis or Crohn's disease.

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