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Note

Simple and sensitive high-performance liquid chromatographic assay for 5-aminosalicylic acid and acetyl-5-aminosalicylic acid in serum

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Sulphasalazine has been used extensively in the treatment of inflammatory bowel disease [1] and rheumatoid arthritis [2]. It consists of sulphapyridine linked to 5-aminosalicylic acid (5-ASA) by an azo bond. After oral administration it is hydrolysed by colonic bacteria to release its two main components. Since the realization that 5-ASA is the main moiety responsible for the antiinflammatory effect [3], much emphasis has been placed on the pharmacokinetics of 5-ASA and its main metabolite, acetyl-5-aminosalicylic acid (Ac-5-ASA). Whereas the formation of N-acetylsulphapyridine is under genetic control, the formation of Ac-5-ASA appears to be independent of the acetylator status [4].

The development of an efficient high-performance liquid chromatographic (HPLC) assay for 5-ASA and Ac-5-ASA has been hampered by the need for high sensitivity and the difficulty in extracting 5-ASA from biological fluids. The assay reported by Fischer and Klotz [5] was unable to measure plasma 5-ASA concentrations below 0.3 μg/ml. An improved method [6] required the prior acetylation of 5-ASA before assay. Hansen [7] reported an assay utilizing dynamically modified silica and an ion-pair agent in the mobile phase, which was sensitive enough to measure Ac-5-ASA at 0.02 μg/ml in plasma. Unfortunately, the limit of detection for 5-ASA in plasma was not stated. The assay was also limited by the need for long assay times when salicylates were present in the sample. A more recent assay [8] was able to measure 5-ASA and Ac-5-ASA simultaneously down to 0.5 μg/ml in plasma and also sulphapyridine and acetylsulphapyridine. This method utilized a column packed with short alkyl chain bonded silica and a mobile phase containing an ion-pair agent. Detection of the four compounds was by means of UV and fluorescence detectors connected in series. However, despite the advan-

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tages provided by such a system, there is need for a method specific only for 5-ASA and its metabolite, in view of the current tendency towards the administration of pure 5-ASA that is free from sulphapyridine. Our intention was therefore to develop a simple efficient assay for the sensitive measurement of 5-ASA and Ac-5-ASA in serum.

EXPERIMENTAL

Materials

Tetrahydrofuran and acetonitrile were of HPLC grade. 5-ASA and Ac-5-ASA were gifts from Pharmacia (Uppsala, Sweden).

Apparatus

Separations were performed on a 250 mm × 4 mm I.D. RP-18 column (5 μm) (Merck, Darmstadt, F.R.G.). A 2-μm column inlet filter and a 7.5 mm × 2.1 mm I.D. guard column filled with RP-18 (10 μm) material was placed before the analytical column. The pump was part of the Varian Vista 5560. Detection was accomplished using a Varian 2070 spectrofluorimeter (emission wavelength 485 nm, excitation wavelength 300 nm). The detector signals were recorded on a Varian Vista 402 data system.

Chromatographic conditions

The mobile phase was a combination of tetrahydrofuran–acetonitrile–potassium phosphate buffer (0.067 *M*, pH 3.2) (5:10:85) and was pumped through the column at 0.8 ml/min. The back-pressure generated was approximately 100 bar. All assays were performed at ambient temperature.

Sample preparation

Blood samples were collected in unheparinized tubes. Serum was separated immediately by centrifugation and stored at −20°C until taken for analysis. A volume of 50 μl of concentrated perchloric acid was added to a 1-ml aliquot of serum to precipitate the serum proteins. The sample was centrifuged at 1200 *g* for 10 min, 400 μl of the clear supernatant were removed, 35 μl of 4 *M* sodium hydroxide solution were added and 50 μl of the resulting mixture (pH 2) were injected on to the column. No internal standard was necessary for the assay.

Verification of the assay

Calibration standards were prepared for the concentration range of 0.05–5 μg/ml 5-ASA and Ac-5-ASA in serum. The peak heights of 5-ASA and Ac-5-ASA were calibrated against their prepared concentrations. Additional standards were prepared (0.2 μg/ml 5-ASA and Ac-5-ASA in plasma) and 1-ml aliquots of these were repeatedly assayed to calculate the within-day and between-day variability of the performance of the method.

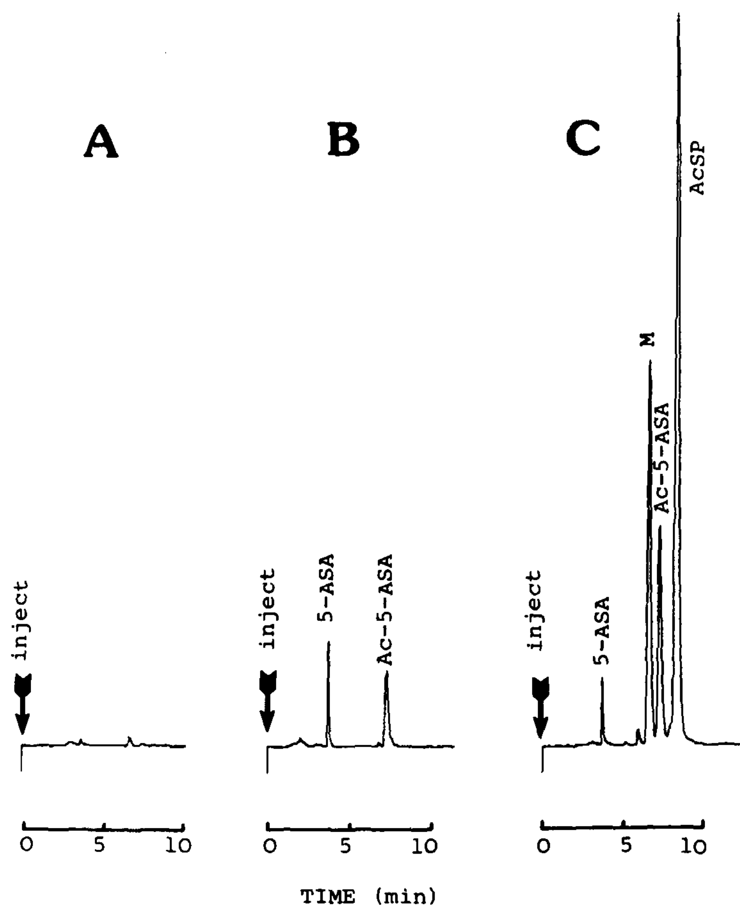

Fig. 1. Typical chromatograms showing (A) blank serum, (B) 0.5 μg/ml 5-ASA and Ac-5-ASA in control serum and (C) 5-ASA (0.87 μg/ml), Ac-5-ASA (2.4 μg/ml), acetylsulphapyridine (AcSP) and an unidentified peak (M) in the serum of patient 3.

Clinical study

Sera from thirteen patients who were being treated with sulphasalazine were obtained. These samples were assayed using a single calibration standard containing 0.5 μg/ml 5-ASA and Ac-5-ASA in serum.

RESULTS

Under the above chromatographic conditions, the retention times of 5-ASA and Ac-5-ASA were 3.7 and 7.2 min, respectively (Fig. 1B). N-Acetylsulphapyridine, the main metabolite of sulphapyridine, has significant fluorescence but was well resolved (8.2 min) from Ac-5-ASA. Sulphapyridine could not be resolved from Ac-5-ASA and at therapeutic concentrations did not interfere with the assay. Salicylic acid and propranolol also did not interfere with the assay. An additional peak was observed only in the sera of patients who had been treated with sul-

TABLE I

ACCURACY AND REPEATABILITY OF THE HPLC ASSAY FOR 5-ASA AND Ac-5-ASA IN SERUM ($n=5$)

Compound	Prepared concentration (μg/ml)	Measured concentration (μg/ml)	Coefficient of variation (%)
Within-day			
5-ASA	0.20	0.20	3.9
Ac-5-ASA	0.20	0.20	0.5
Inter-day			
5-ASA	0.20	0.21	2.8
Ac-5-ASA	0.20	0.21	5.2

phasalazine, which had a retention time of 6.5 min and was well resolved from Ac-5-ASA. The identity of this peak could not be established but is probably a metabolite of sulphasalazine.

The calibration graphs for 5-ASA and Ac-5-ASA were linear over the concentration range prepared. The within-day and between-day variabilities of the assay were consistently less than 6% (Table I).

Of the thirteen patients studied, eight had rheumatoid arthritis, three ulcerative colitis and two Crohn's disease. The concentrations of 5-ASA and Ac-5-ASA in the sera of patient 6 were below the limits of detection (Table II). The sulphapyridine concentrations in this patient had also been confirmed to be very low (unpublished data).

TABLE II

SERUM CONCENTRATIONS OF 5-ASA AND Ac-5-ASA IN PATIENTS TREATED WITH SULPHASALAZINE

N.D. = not detectable.

Patient No.	Sulphasalazine dose (g/day)	5-ASA (μg/ml)	Ac-5-ASA (μg/ml)
1	1.5	0.14	0.49
2	3.0	0.64	1.18
3	3.0	0.87	2.40
4	3.0	0.55	1.23
5	3.0	1.16	1.50
6	1.5	N.D.	N.D.
7	3.0	0.30	0.95
8	3.0	0.18	0.64
9	3.0	0.14	0.28
10	3.0	0.57	1.23
11	4.0	0.81	1.62
12	3.0	0.16	1.12
13	3.0	0.27	1.00

DISCUSSION

The assay reported here is simple and efficient. It is also sensitive, reproducible and may be easily automated to measure a large number of samples. Hence it is suitable for routine clinical use. With meticulous attention to volumes, no internal standard is required. We have operated the method for several months without an apparent deterioration in column efficiency. We have found, however, that the successful resolution of Ac-5-ASA from the preceding peak and from sulphapyridine is critically dependent on the pH of the buffer. An unidentified peak was consistently present in all sera of patients treated with sulphasalazine. Its fluorescence and chromatographic characteristics suggest that it might be a metabolite of 5-ASA.

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