Journal of Chromatography, 305 (1984) 470—476
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1913

Note

Determination of sodium azodisalicylate, salazosulphapyridine and their metabolites in serum, urine and faeces by high-performance liquid chromatography

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(First received April 22nd, 1983; revised manuscript received August 31st, 1983)

Salazosulphapyridine (SASP) is extensively used in the treatment of inflammatory bowel disease. The mode of action of SASP is unknown. After oral ingestion the larger part of SASP reaches the colon intact where it is split

0378-4347/84/$03.00

by colonic bacteria at the azo bond into sulphapyridine (SP) and 5-amino-salicylic acid (5-ASA). Virtually all SP is absorbed and subsequently metabolized and excreted in the urine. Some of the 5-ASA is absorbed and excreted in the urine in the acetylated form. The larger part of 5-ASA is excreted in the faeces [1—3].

Recent studies have demonstrated that 5-ASA is the active moiety of SASP [4, 5]. SP functions as a carrier to transport 5-ASA to the distal part of the bowel. Most side-effects of SASP are attributable to SP [6]. Therefore, the treatment of inflammatory bowel disease has been focused on the use of 5-ASA [7, 8]. A promising compound in which 5-ASA is linked to another 5-ASA molecule is sodium azodisalicylate (di-5-ASA) [9, 10]. In pharmacokinetic studies of this new compound a reliable method for the determination of di-5-ASA is necessary. No satisfactory analytical method for measuring di-5-ASA and 5-ASA is available. This paper presents a suitable method for the determination of di-5-ASA, 5-ASA, acetyl-5-ASA (ac-5-ASA) and also for SASP in serum, urine and faeces.

EXPERIMENTAL

Reagents and material

5-Amino-2-hydroxybenzoic acid (5-ASA) and 4-amino-2-hydroxybenzoic acid (PAS) were purchased from Merck (Darmstadt, F.R.G.). 5-ASA was purified prior to use. Salazosulphapyridine, sulphapyridine and acetylsulphapyridine were supplied by Pharmacia (Uppsala, Sweden).

Acetyl-5-ASA (ac-5-ASA) and di-5-ASA were synthesized by the Department of Organic Chemistry of the University of Nijmegen. Propionyl-PAS and propionyl-5-ASA were synthesized by the reaction of PAS and 5-ASA with propionic anhydride and purified by recrystallization from water. All other reagents were of analytical grade (Merck).

For the clean-up of serum samples, Baker chemical extraction columns (3 ml) filled with quaternary amines (No. 7091) were used. They could be regenerated about ten times.

Apparatus

High-performance liquid chromatographic (HPLC) analyses were performed on a Spectra Physics instrument (Model SP 740) equipped with a Chrompack 3 mm × 150 mm analytical column packed with LiChrosorb RP-18 (5 μm) coupled to a Chrompack guard-column (12 mm × 75 mm).

Detection was performed with a Schoeffel fluorescence monitor Model FS 970, with excitation at 305 nm and a cut-off filter at 396 nm. For injection of the samples an automatic sampler was used.

Chromatographic conditions

The mobile phase consisted of deionized water adjusted to pH 3.3 (for SASP pH 2.85) with 0.01 *M* citric acid and 20% methanol. A flow-rate of 1.4 ml/min was established. The run-time was 16 min (for SASP 25 min).

Standards

Standards of 5-ASA, ac-5-ASA, di-5-ASA and SASP were prepared in serum (range 0.1—20 μmol/l), urine (range 20—1000 μmol/l) and faeces (range 20—1000 μmol/l).

Sample preparation

Urine. To 1 ml of urine were added propionyl-PAS as internal standard (0.1 ml of a solution of 10 mmol/l; for SASP 0.5 ml) and 20 μl of propionic anhydride. After standing for a few minutes at room temperature, 9 ml of methanol were added (for SASP 8.5 ml of methanol). After Vortex mixing, the mixture was left for at least 30 min and centrifuged. To 0.5 ml of the supernatant 4.5 ml of distilled water were added and 20 μl of this mixture were injected into the HPLC apparatus, assaying ac-5-ASA and free 5-ASA, the latter as propionyl-5-ASA.

For the determination of di-5-ASA or SASP an extra step was inserted, viz. cleavage of the azo bond. Before addition of propionic anhydridie 300 μl of freshly prepared 10% sodium dithionite ($Na_2S_2O_4$) were added to 1 ml of urine. After warming the mixture at 30°C for 5 min, 20 μl of propionic anhydride were added. The mixture was then treated as described above.

Faeces. Faeces were sampled directly into about 300 ml of mercury chloride (0.2%) and mixed thoroughly to prevent cleaving of the azo bond by colonic bacterial enzymes, then stored at −20°C. With 1 g of this suspension the same procedure as mentioned above for urine was followed.

Serum. To 0.5 ml of serum were added propionyl-PAS as internal standard (0.1 ml of a solution of 500 μmol/l) and 20 μl of propionic anhydride. After standing for a few minutes at room temperature 4 ml of a phosphate buffer (0.01 *M*, pH 8) were then added and after centrifugation transferred to a quaternary amine extraction column (pre-equilibrated with 10 ml of phosphate buffer, 0.01 *M*, pH 8). The column was washed with 3 ml of the same buffer. The compounds were extracted with 2 ml of phosphate buffer (0.2 *M*, pH 5.7) into a test tube; 20 μl of this mixture were then injected into the HPLC apparatus.

For the determination of di-5-ASA or SASP the azo bond was cleaved as described above for urine and faeces. Before addition of propionic anhydride, 200 μl of freshly prepared 10% sodium dithionite were added to 0.5 ml of serum. After warming the mixture at 30°C for 5 min, 20 μl of propionic anhydride were added. The same procedure as described above was then followed.

RESULTS

The chromatograms of the samples, prepared as described in the preceding section, showed three well separated peaks. Some typical chromatograms are shown in Fig. 1. The retention time (t_R) for ac-5-ASA was 4.5 min. Free 5-ASA was propionylated to give propionyl-5-ASA with a t_R of 6.5 min. The internal standard (propionyl-PAS) had a t_R of 12.5 min. Under these conditions the acetylated sulphapyridine, sulphapyridine and propionylsulpha-

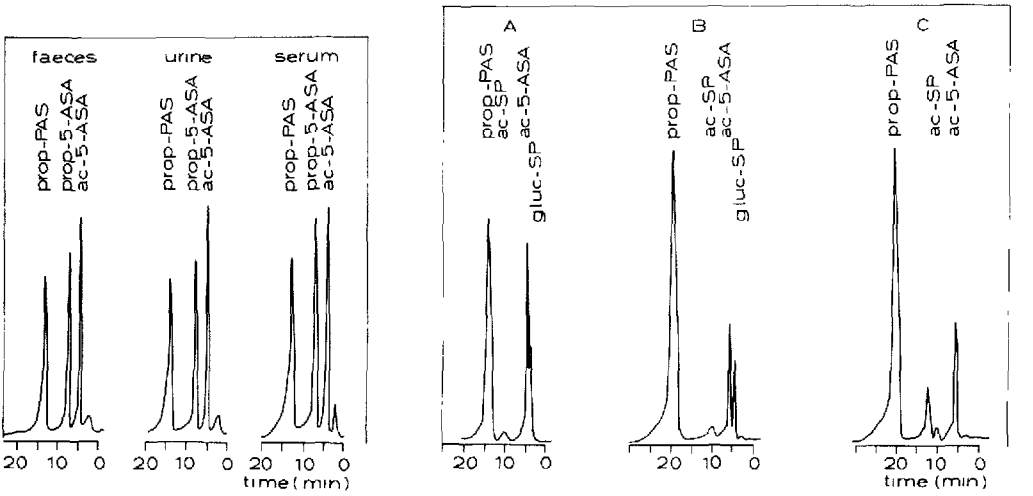

Fig. 1. Typical chromatograms of faeces, urine and serum samples. Concentrations measured: 5-ASA: faeces 502 μmol/l, urine 480 μmol/l and serum 20.7 μmol/l. Ac-5-ASA: faeces 464 μmol/l, urine 499 μmol/l and serum 19.6 μmol/l.

Fig. 2 Typical chromatograms of a urine sample of a patient who ingested a single dose of 2.3 g of salazosulphapyridine; (A) with mobile phase pH 3.3, (B) with mobile phase pH 2.85, and (C) with mobile phase pH 3.3, after deglucuronidation of gluc-SP. Ac-5-ASA concentrations measured: 587 μmol/l (B) and 561 μmol/l (C).

pyridine could hardly be detected and there was no interference with the peaks we wished to measure.

The only interfering metabolite peak we found was glucuronyl-SP (gluc-SP) resulting from SASP (Fig. 2A). This peak interfered with that of ac-5-ASA and could be separated when the pH of the mobile phase was reduced to 2.85 (Fig. 2B). The run-time was then 25 min, resulting in a low internal standard peak, which could be raised by adding more propionyl-PAS (0.5 ml). An alternative way (Fig. 2C) was deglucuronidation of gluc-SP by which 20 μl of β-glucuronidase (Boehringer Mannheim, 100,000 Fishman units /ml) were added to the sample and incubated for 3 h, before adding propionic anhydride or before cleavage with sodium dithionite. By the latter method the gluc-SP peak disappeared, but another peak of unknown origin appeared. In the chromatograms no interfering peaks were observed arising from other compounds in urine, serum and faeces.

Calibration graphs were constructed by plotting the peak height ratios (ac-5-ASA/internal standard and prop-5-ASA/internal standard) against the concentrations of ac-5-ASA and prop-5-ASA (Fig. 3). Straight lines were obtained. The detection limit of ac-5-ASA and prop-5-ASA amounted to 0.1 μmol/l.

Table I demonstrates the intra-assay variation for different concentrations of ac-5-ASA, 5-ASA, di-5-ASA and SASP in urine, faeces and serum. The coefficients of variance are quite low.

The prepared samples, worked up in vials, ready to be injected into the HPLC apparatus, could be stored for at least 14 days at −20° C. There was no decrease in the content of the samples, nor did additional peaks appear.

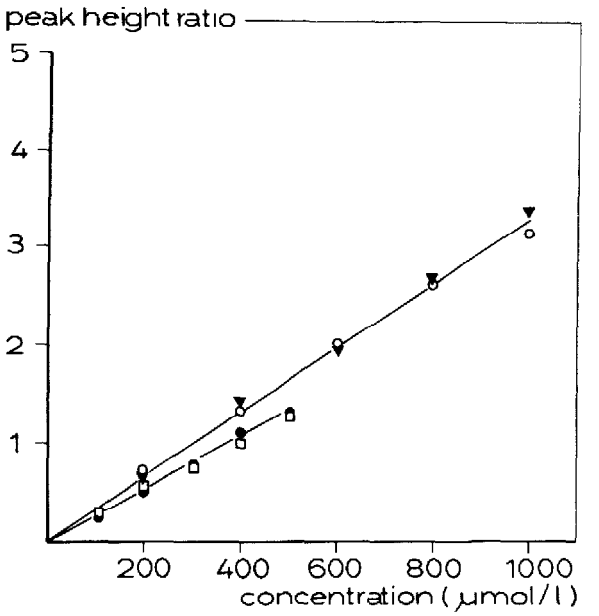

Fig. 3. Peak height ratios (ac-5-ASA/internal standard and prop-5-ASA/internal standard) against the concentrations of ac-5-ASA and prop-5-ASA. (▾), ac-5-ASA in urine; (○), ac-5-ASA in faeces; (•), prop-5-ASA in urine; (▫), prop-5-ASA in faeces.

TABLE I

INTRA-ASSAY VARIATION FOR DIFFERENT CONCENTRATIONS OF ac-5-ASA, 5-ASA, di-5-ASA AND SASP IN URINE, FAECES AND SERUM

	Concentration (μmol/l)	Mean amount detected (n = 6) (μmol/l)	S.D.	Coefficient of variation
Urine				
ac-5-ASA	1000	1010.3	22.9	2.3
ac-5-ASA	100	99.1	3.9	4.0
5-ASA	500	484.9	5.0	1.0
di-5-ASA	100	101.2	1.8	1.8
SASP	100	99.6	3.6	3.6
Faeces				
ac-5-ASA	1000	1017.4	16.3	1.6
ac-5-ASA	100	104.2	1.9	1.8
5-ASA	500	480.1	3.0	0.6
di-5-ASA	100	93.5	7.6	8.1
SASP	100	97.1	3.7	3.8
Serum				
ac-5-ASA	20	21.9	1.0	4.4
ac-5-ASA	10	9.8	0.3	3.5
5-ASA	20	20.6	0.7	3.3
di-5-ASA	20	21.5	1.8	8.6
SASP	20	20.1	0.6	3.0

DISCUSSION

In 1973 a colorimetric detection of SASP and its metabolites in serum, urine and faeces, and bile was described [11]. Currently, for the determination of these compounds the focus is on the use of an HPLC assay. Several investigators demonstrated good detection in serum and urine [12—17]. Sulphapyridine and ac-5-ASA could be analysed satisfactorily as well. With the method described in this article we demonstrate the determination of di-5-ASA, SASP, ac-5-ASA and 5-ASA in serum, urine and faeces. SASP and di-5-ASA have been measured indirectly by cleaving the azo bond with sodium dithionite into a molecule of 5-ASA and SP and two molecules of 5-ASA, respectively. In the earlier colorimetric assay of SASP [11] titanium trichloride was used for this cleavage. However, it has been suggested that Ti ions can form complexes between the carboxy and hydroxy functions of the aminosalicylic molecule and the reversed-phase material (unpublished observations). This problem of complex formations with Ti ions can be circumvented by using sodium dithionite for the azo bond cleavage.

In view of the pharmacokinetic and toxicological experiments with di-5-ASA we focused our attention on the metabolites ac-5-ASA and 5-ASA. By propionylating 5-ASA with propionic anhydride propionyl-5-ASA could be determined independently from ac-5-ASA. This method has an obvious advantage over the alternative procedure in which the amount of free 5-ASA is determined as the difference of total ac-5-ASA (obtained after acetylation of samples containing free 5-ASA and ac-5-ASA) and ac-5-ASA present prior to acetylation. Considerable errors will be introduced in this differential method because of the rather large ratios of ac-5-ASA and free 5-ASA.

The present method does not suffer from peaks that interfere with the detection. Characteristic peaks of serum, urine and faeces samples were found to appear in the front of the chromatograms. The use of fluorescence detection is to a large extent responsible for the high degree of selectivity.

In conclusion, the method described here can be used for monitoring SASP, di-5-ASA, ac-5-ASA and 5-ASA in serum, urine and faeces and therefore will be helpful in pharmacokinetic, toxicological and clinical studies with di-5-ASA.

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