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Determination of salicylazosulphapyridine, sulphapyridine and its metabolites in serum and urine*

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Abstract: Methods for the determination of salicylazosulphapyridine (Salazopyrin®), sulphapyridine, *N*-acetylsulphapyridine, sulphapyridine-*O*-glucuronide and *N*-acetylsulphapyridine-*O*-glucuronide in serum and urine have been developed. In one method samples were diluted with methanol to precipitate proteins present and thereafter injected directly onto the liquid chromatographic column. The sulphapyridine-*O*-glucuronide could not be determined by this method. For the determination of all the main sulphapyridine metabolites the sulphapyridine aglycones were formed after treatment with β-glucuronidase. These sulphapyridine compounds were then extracted with isobutylmethyl ketone at pH 5, and re-extracted to a phosphate buffer at pH 13 prior to injection. Separation and retention of all compounds was affected both by pH and concentration of methanol in the mobile phase. The proposed method made determinations down to 1 μg ml^{-1} possible, which was found to be sufficient. Comparisons were made with spectrophotometric methods.

Keywords: *Salicylazosulphapyridine; sulphapyridine and metabolites; liquid chromatography; method comparison.*

Introduction

When salicylazosulphapyridine (SASP) reaches the colon it is metabolized by bacterial azoreductase to sulphapyridine (SP) and 5-aminosalicylic acid (5-ASA) (Fig. 1). SP is almost completely absorbed from the colon and might be metabolized by both acetylation and/or hydroxylation to *N*-acetylsulphapyridine (AcSP), 5-hydroxysulphapyridine (5-OHSP) and *N*-acetyl-5-hydroxysulphapyridine (Ac-5-OHSP). The hydroxysulphapyridines are mainly excreted in the urine after conjugation with glucuronic acid to the corresponding sulphapyridine-*O*-glucuronide (SPGluc) or *N*-acetylsulphapyridine-*O*-glucuronide (AcSPGluc) [1]. 5-ASA is only partly absorbed from the colon and is mainly excreted as *N*-acetyl-5-aminosalicylic acid (Ac-5-ASA).

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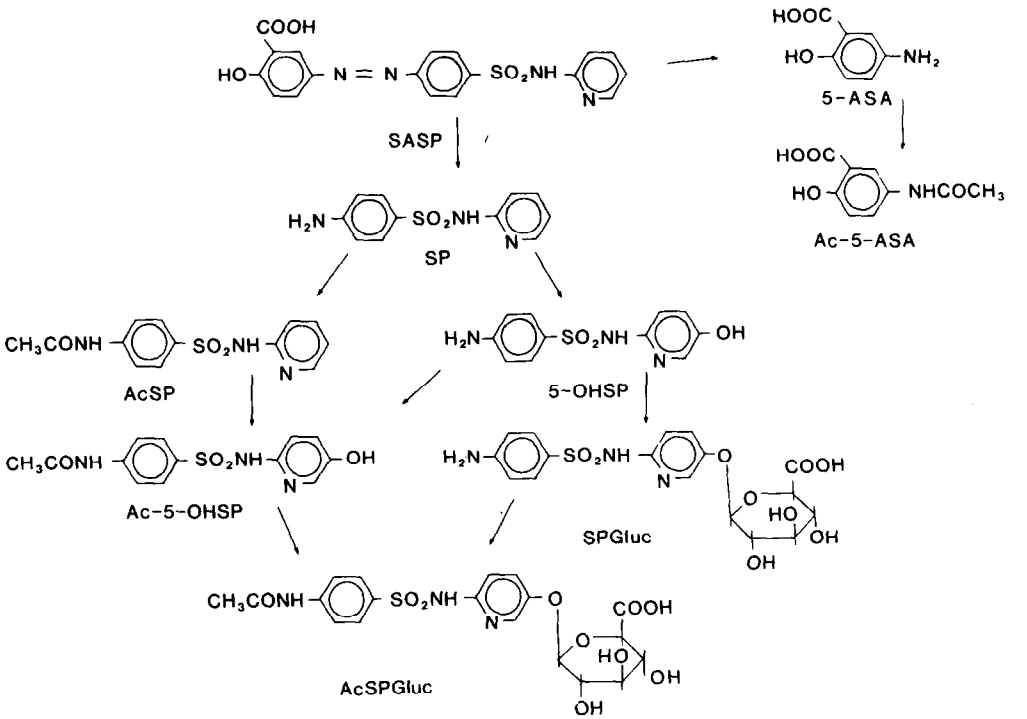

Figure 1
Metabolism of salicylazosulphapyridine.

A number of papers on the determination of SASP and its metabolites have been published [2–12]. The spectrophotometric determination of SASP after extraction from an acidified sample into isoamylacetate and re-extraction into strong alkali is a convenient method [2], although liquid chromatography might improve the selectivity [3, 4]. SP and its metabolites have been determined by spectrophotometry after a Bratton–Marshall diazotation reaction [5]. Determination of the metabolites is made using a combination of enzymatic hydrolysis with β-glucuronidase and deacetylation with hydrochloric acid to obtain different sums of SP, AcSP, SPGluc and AcSPGluc [5]. The main advantage with these spectrophotometric methods has so far been their simplicity, making automation possible, and the opportunity to determine glucuronide metabolites. The aim of the present study was to develop a reliable method for the individual determination of SASP, SP and its metabolites AcSP, SPGluc and AcSPGluc, since none of the published chromatographic assays [7–10] discriminated between conjugated and nonconjugated sulphapyridine compounds.

Experimental

Apparatus

The liquid chromatographic system used for the determination of salicylazosulphapyridine comprised the following: a Waters model 6000A solvent delivery system, a Waters U6K injector, a Waters model 440 UV-detector employed at 365 nm and a Spectra Physics SP 4270 integrator for peak area measurements.

For the determination of sulphapyridine and its metabolites the following apparatus was used: a Beckman model 112 solvent delivery system with a Beckman 210 injector, a Waters model 440 UV-detector employed at 254 nm and a Spectra Physics SP 4270 integrator. The flow rate was 2.0 ml min^{-1} in all separation systems.

Photometric measurements were made on a Zeiss Spektralphotometer II. Spectra were recorded on a Varian Techtron model 635 or a Varian 2300 Spectrophotometer. A Beckman model 3560 digital pH meter was used for pH measurements. A Technicon AutoAnalyzer® equipment was used for the sulphapyridine determinations according to ref. [5].

Columns

To protect the analytical column, a precolumn (30 × 4 mm i.d.), dry-packed with 40-μm RP-18 Perisorb (Merck, Darmstadt, FRG) was used. The analytical columns (250 × 4 mm i.d.) were packed with 10-μm C_{18}-Nucleosil (Macherey-Nagel & Co, Düren, FRG). More than 100 ml of mobile phase was pumped through the columns before use.

Chemicals

Salicylazosulphapyridine (Salazopyrin®) (SASP), sulphapyridine (SP), *N*-acetylsulphapyridine (AcSP), 5-hydroxysulphapyridine (5-OHSP), *N*-acetyl-5-hydroxysulphapyridine (Ac-5-OHSP) and 6-methyl sulphapyridine (MSP) were all from Chemical Research, Pharmacia AB, Uppsala, Sweden. Sodium hydroxide was from EKA, Bohus, Sweden. Trisodium phosphate, disodium hydrogen phosphate, sodium dihydrogen phosphate and *ortho*-phosphoric acid were from Merck, Darmstadt, FRG. Hydrochloric acid and glacial acetic acid were from M & B Ltd, Dagenham, UK. Methanol, HPLC-grade, was from Rathburn Chemicals Ltd, Walkerburn, UK. Isobutylmethyl ketone was from Merck-Schuchardt, Munich, FRG. Dichloromethane was from Kebo-Grave AB, Solna, Sweden. β-Glucuronidase/arylsulphatase was from Boehringer Mannheim GmbH, FRG. Other chemicals used in the spectrophotometric determinations of SASP, SP and its metabolites were as described in refs [2] and [5]. All mobile phase solutions were stirred and filtered through a 0.45-μm Millipore filter before use.

Determination of SASP, SP and AcSP (Method A)

To 400 μl of thawed serum or urine sample, 800 μl of methanol was added to precipitate proteins. The sample was mixed, kept on an ice-bath for 10 min and after centrifugation at 8240 ***g*** for 2 min, 50 μl was injected onto the SASP separation system and 20 μl was injected onto the SP separation system.

The mobile phase for the elution of SASP was methanol–0.025 M phosphate buffer (60:40, v/v). The mobile phase for the separation of SP, AcSPGluc and AcSP was methanol–0.167 M acetic acid (15:85, v/v).

Determination of SP and its metabolites (Method B)

To 200 μl of thawed serum or urine sample was added 200 μl of 1 M acetate buffer (pH = 5 saturated with sodium chloride and containing MSP as internal standard) and 5 μl of β-glucuronidase/arylsulphatase. After mixing the solutions in the Eppendorftube on a Whirlimixer for 5 s, the samples were kept at 37°C for 30 min.

The sulphapyridine compounds were then extracted into 800 μl of isobutylmethyl ketone for 10 min. After centrifugation 600 μl of the organic phase was transferred to another tube containing 800 μl of phosphate buffer (pH = 12.8). The sample was

extracted for 10 min, centrifuged and the organic phase discarded. The aqueous phase was extracted with 600 μl of dichloromethane to remove traces of isobutylmethyl ketone. After centrifugation 500 μl of the aqueous phase was transferred to another tube. Finally the pH was adjusted with 100 μl of 0.65 M hydrochloric acid and 20 μl was injected.

The mobile phase for the separation of SP and unconjugated SP metabolites was methanol–0.02 M phosphate buffer (pH = 7) (20:80, v/v).

Results and Discussion

Assay for SASP, SP and AcSP (Method A)

Since the pKa values of SASP are 0.6, 2.4, 8.3 and 11.0 [11], the retention is strongly affected by pH and the concentration of the organic modifier, as showed by Jansson and Modin (unpublished results). An increase in retention was observed when the pH was decreased to 2.5. Symmetrical peaks were obtained at this pH and two chromatograms from the determination of SASP in serum and urine are presented in Fig. 2. Sensitive determination of SASP is possible due to its high molar absorptivity [11].

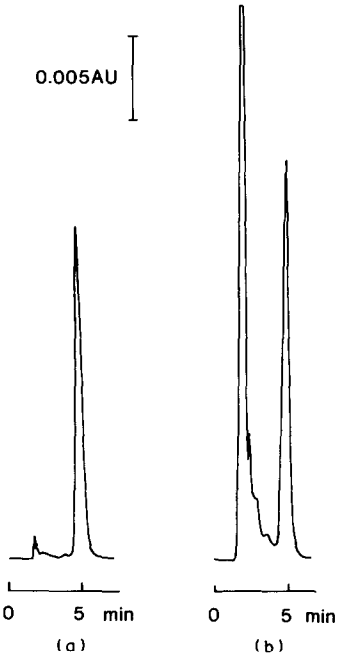

Figure 2
(a) Chromatogram of salicylazosulphapyridine from serum. Serum spiked with salicylazosulphapyridine 14.0 μg ml^{-1}, and processed as described for Method A. Injection volume 50 μl.
(b) Chromatogram of salicylazosulphapyridine from urine. Urine spiked with salicylazosulphapyridine 14.8 μg ml^{-1}, and processed as described for Method A. Injection volume 50 μl. Mobile phase: methanol–0.025 M phosphate buffer (pH = 2.5) (60:40, v/v). Detection: UV at 365 nm.

The precipitation of proteins with methanol is not a very efficient method [12] since at least two volumes of methanol are needed for quantitative precipitation. Several hundred injections of serum and urine samples have, however, been injected onto the Nucleosil C_{18} column with only a change of the precolumn after about 200 injections. The simple sample treatment used for the determination of SASP could also be used for the direct determination of SP and AcSP. The liquid chromatographic method for the determination of SASP was found to be linear in the range 0.5–110 μg ml^{-1} (r = 0.9998 in serum and r = 0.9999 in urine), with a recovery from both spiked serum and urine of

98%. The relative standard deviation (RSD) for urine SASP was somewhat higher at low concentrations due to matrix effects. At 1.1 μg ml^{-1} the RSD (n = 10) was 6.6% in urine and 2.9% in serum. A method comparison according to Eksborg [13] was made between the liquid chromatographic method ("Method A") and the spectrophotometric method [2] using spiked serum samples. The results show that both methods gave comparable results at concentrations higher than 10 μg ml^{-1}. At 4.1 μg ml^{-1} the RSD was 3.0% (n = 10) for the spectrophotometric method and 2.9% (n = 10) at 1.1 μg ml^{-1} for the liquid chromatographic method.

Assay for SP, AcSP, SPGluc and AcSPGluc (Methods A and B)

Two reversed-phase liquid chromatographic separation systems have been tested for the determination of SP and its metabolites. Both systems employed the same Nucleosil C_{18} column as employed for the determination of SASP, but with different mobile phases. Since the sulphonamide group of SP is a relatively weak acid with a pKa of 8.6 [14] and the glucuronic acid moiety of the conjugated SP compounds might be expected to have a pKa of about 3 [15], retention can be regulated by adjustment of mobile phase pH. A mobile phase composed of methanol–0.167 M acetic acid (15:85, v/v) retarded AcSPGluc as shown in Table 1. An increase in pH of the mobile phase made this compound less retarded. Since only the hydroxylated SP compounds were available as reference material the existence of AcSPGluc could only be proved indirectly by treatment of patient urine with β-glucuronidase. This produced 5-OHSP and Ac-5-OHSP from SPGluc and AcSPGluc respectively, and the disappearance of the AcSPGluc peak. No 5-OHSP or Ac-5-OHSP was found in untreated patient serum or urine.

The recovery of SP from spiked serum samples using precipitation with methanol gave a recovery of 97% and the precision (RSD, n = 10) was 3.1% for SP and 3.4% for AcSP at 2.0 μg ml^{-1}. Calibration curves were linear in the range 2–25 μg ml^{-1} (r = 0.9998, n = 19). For serum samples the concentration of methanol in the mobile phase can be increased in order to decrease the retention times.

Since SPGluc could not be quantitated using this method, other possibilities were evaluated. Enzymatic hydrolysis with β-glucuronidase, as described previously, produced 5-OHSP and Ac-5-OHSP from the conjugated SP and AcSP compounds respectively. These aglycones formed were poorly separated from the nonhydroxylated SP and AcSP at pH = 3 (Table 1). An increase in pH to 7 produced a change in elution order of the hydroxylated and nonhydroxylated sulphapyridines. A further increase to pH = 7.5 lead to poor resolution for SP and Ac-5-OHSP. The reason for the change in elution order might be due to lower pKa values of 5-OHSP and Ac-5-OHSP, compared to SP and AcSP. Blank urine contains several compounds which interfere with 5-OHSP and SP and some purification step was found necessary to make determinations of SP and its metabolites possible. The compounds were extracted into isobutylmethyl ketone at pH = 5 and re-extracted at pH = 13. To remove dissolved isobutylmethyl ketone a final extraction with dichloromethane was made.

All SP compounds show high molar absorptivity at 254 nm (ϵ = 1.5–2.0 × 10^4) making determination down to 2 μg ml^{-1} in urine and 1 μg ml^{-1} in serum possible. The higher limit for urine samples is due to a small interfering peak with a similar retention time as SP. Calibration curves were linear ($r > 0.999$, n = 12) up to 100 μg ml^{-1} and precision (RSD, n = 10) was between 1.5 and 3.1% at 100 μg ml^{-1} and at 2 μg ml^{-1} RSD was in the range 4.7–14% for the different SP compounds. This method was also used for serum analysis, although the non-enzymatic pre-treatment method ("Method

Table 1
Capacity ratios for sulphapyridine (SP), 5-hydroxysulphapyridine (5-OHSP), *N*-acetylsulphapyridine (AcSP), *N*-acetyl-5-hydroxysulphapyridine (Ac-5-OHSP) and *N*-acetylsulphapyridine-*O*-glucuronide (AcSPGluc) on 10-μm C_{18} Nucleosil

Mobile phase	SP	5-OHSP	AcSP	Ac-5-OHSP	AcSPGluc
Methanol–0.167 M acetic acid (pH = 3) (15:85, v/v)	6.7	7.9	19.2	23.4	15.0
Methanol–0.02 M phosphate buffer (pH = 7) (20:80, v/v)	5.9	4.4	12.2	7.5	<2*

*Co-eluted with endogenous peaks.

A") could be used in compliance studies where only the concentrations of SP and AcSP are to be determined. Chromatograms from patient serum and urine using "Method B" are given in Fig. 3a and b respectively. Method comparison was made between "Method A" and the spectrophotometric method ("Method D") [5] using serum spiked with SP. Both methods gave comparable results down to about 10 μg ml^{-1}. The calibration curve for the spectrophotometric method was slightly curved (r = 0.9986) compared to the HPLC method (r = 0.9998). At concentrations below 10 μg ml^{-1} the direct HPLC method is to be preferred. Owerbach *et al.* [7] found somewhat lower values for a liquid chromatographic method when compared with the results from a modified Bratton–Marshall method [5]. Method comparison analyses were made between "Method B" and the Bratton–Marshall method ("Method D") [5] using some randomly selected urine samples from healthy volunteers taking a single dose (2 g) of Salazopyrin®.

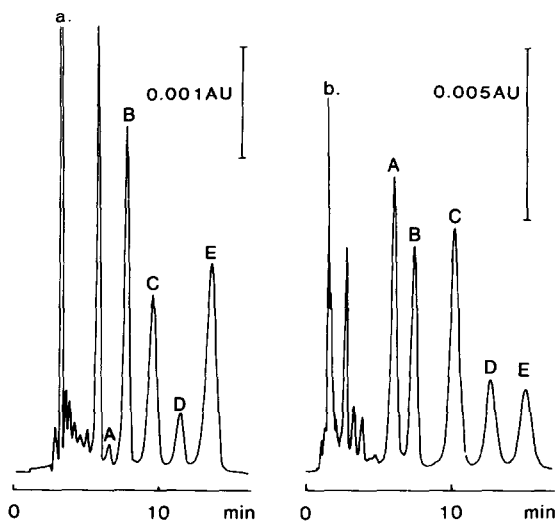

Figure 3
Chromatograms of sulphapyridine and its metabolites isolated from biofluids after enzymatic hydrolysis with β-glucuronidase. Key: sulphapyridine-*O*-glucuronide as 5-hydroxysulphapyridine (A), sulphapyridine (B), *N*-acetylsulphapyridine-*O*-glucuronide as *N*-acetyl-5-hydroxysulphapyridine (C), 6-methyl-sulphapyridine (D), as internal standard and *N*-acetylsulphapyridine (E). Mobile phase: methanol–0.02 M phosphate buffer (pH = 7) (20:80, v/v). Detection: UV at 254 nm. (a) Serum containing 3.2 μg ml^{-1} of A, 17.9 μg ml^{-1} of B, 21.8 μg ml^{-1} of C, 5.0 μg ml^{-1} of D and 21.6 μg ml^{-1} of E. (b) Urine containing 58.6 μg ml^{-1} of A, 29.9 μg ml^{-1} of B, 78.0 μg ml^{-1} of C, 20.0 μg ml^{-1} of D and 21.8 μg ml^{-1} of E.

The results are given both as total sulphapyridine (SP + AcSP + SPGluc + AcSPGluc) (Fig. 4), and as individual sulphapyridine and metabolite concentrations (Fig. 5). If the results are identical the observed quotient between the methods should be one. The total sulphapyridine values show reasonable agreement between the two methods (Fig. 4), although the HPLC method gives slightly higher concentrations. The sulphàpyridine and metabolite values differ to a great extent (Fig. 5a, b). This is due to hydrolysis of the acetyl group of AcSP and Ac-5-OHSP prior to the Bratton–Marshall diazotation [5]. The hydrolysis can be partly avoided by extraction at 0°C [5].

From Fig. 5c and d it is evident that this will lead to a constant underestimation of the acetylated sulphapyridine compounds. From Fig. 5a and b it is apparent that the

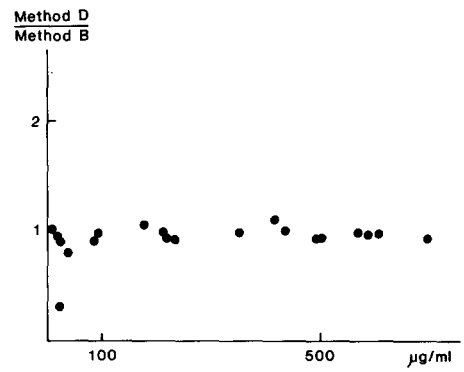

Figure 4
Method comparison analysis. Method B: total urine sulphapyridine concentration (SP + AcSP + SPGluc + AcSPGluc) after enzymatic hydrolysis using high-performance liquid chromatographic separation and spectrophotometric detection at 254 nm. Method D: data from spectrophotometric method [5].

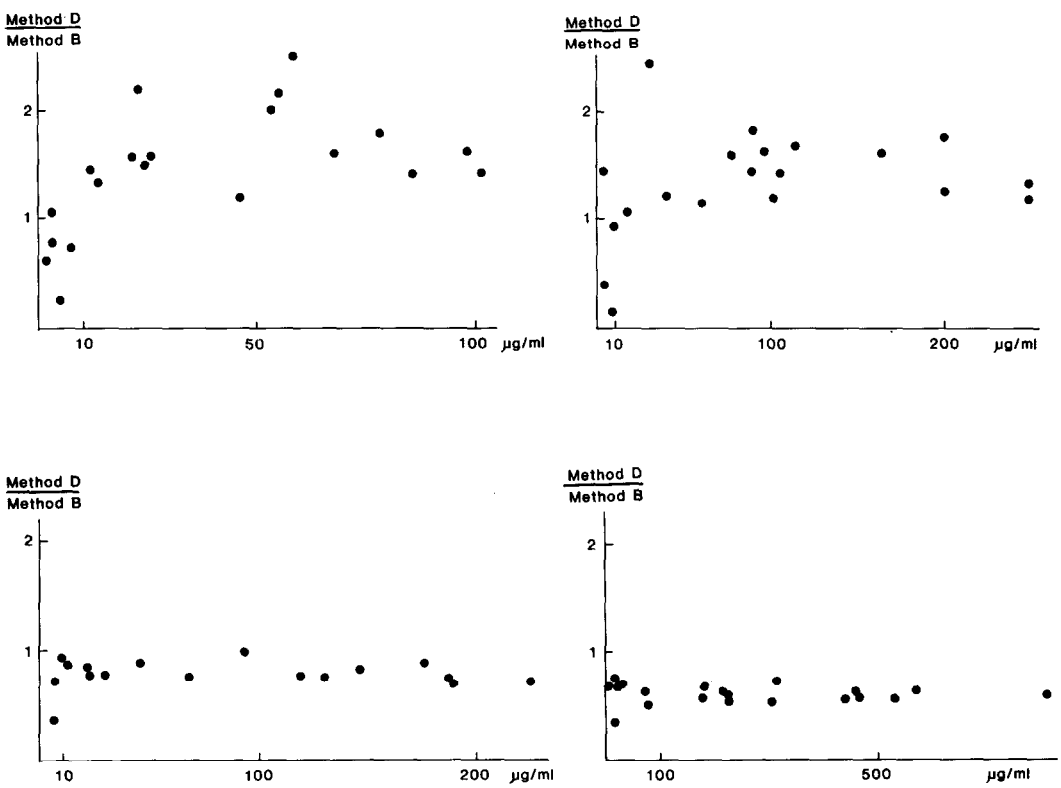

Figure 5
Method comparison analysis. As Fig. 4 but for (a) sulphapyridine (SP); (b) sulphapyridine-*O*-glucuronide (SPGluc); (c) *N*-acetylsulphapyridine (AcSP); (d) *N*-acetylsulphapyridine-*O*-glucuronide (AcSPGluc).

nonacetylated compounds will be overestimated with the Bratton–Marshall method. The extent of overestimation depends on the actual AcSP or AcSPGluc level in the sample. At lower concentrations there is a tendency that the Bratton–Marshall method underestimates the concentration of all SP compounds. Thus a combination of two effects is observed in Fig. 5a–d.

It may be noted that the graphical presentation of method comparison data according to Eksborg [13] has one major drawback. The magnitude of over- and under-estimation is not linear throughout the ordinates. A value of 2 and 0.5 indicates the same difference, although 0.5 seems closer to one. In order to reveal constant differences between methods one could instead take the logarithm of the quotient.

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