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## Note

# Determination of sulfapyridine and its major metabolites in plasma by high-pressure liquid chromatography

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Sulfapyridine (SP) is one biologically active moiety of salicylazosulfapyridine (trade name Azulfidine®; Pharmacia, Uppsala, Sweden) [1, 2]. This drug consists of 5-aminosalicylic acid and sulfapyridine which are linked by an azo bond. It is split in the colon by gut bacteria [1]. The sulfonamide is widely used in the treatment of Crohn's disease and ulcerative colitis [3, 4]. Similar to other sulfonamides, the metabolism of SP includes acetylation of the free amino group and hydroxylation of the benzene ring followed by conjugation with glucuronic acid to their corresponding glucuronides (Fig. 1) [4, 5]. Since the rate of acetylation of SP is genetically controlled [6, 7] and the observed side effects are found primarily in patients of the slow acetylator phenotype, it

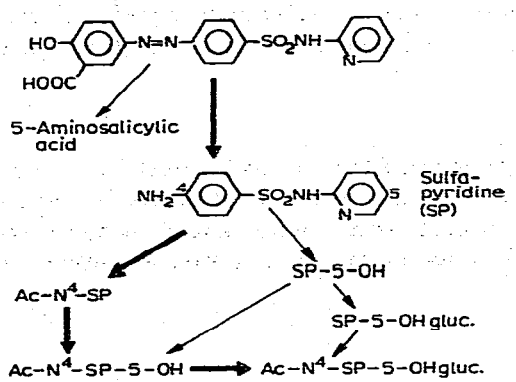


**Fig. 1. Structure and metabolism of salicylazosulfapyridine (Azulfidine) in man as described by Das and Dubin [4].**

seems advisable to determine the phenotype of the treated patients. In addition, a therapeutic range of 20—50 μg/ml has been postulated for the total SP [4]. This necessitates a specific method for the determination of SP and its acetylated metabolite. In the past plasma level monitoring was performed by a time-consuming and rather unspecific photometric method [10]. Therefore we have developed a fast and specific high-pressure liquid chromatography (HPLC) method for the simultaneous measurement of SP and its major metabolites in human plasma.

## EXPERIMENTAL

### *Apparatus*

The chromatographic separations were performed with an HPLC apparatus (Spectra-Physics, Darmstadt, G.F.R.) equipped with a 250×4.6 mm I.D. column packed with Nucleosil RP-18, 10 μm (Macherey & Nagel, Düren, G.F.R.) and a UV detector (254 nm; Spectra-Physics; cell volume 8 μl). Flow-rate was 1.4 ml/min at ambient temperature. The same results have been obtained with a self-packed 250×3 mm I.D. column filled with Nucleosil RP-18, 5 μm.

### *Reagents*

All solvents were of analytical reagent grade (Merck, Darmstadt, G.F.R.). The mobile phase consisted of methanol—1% acetic acid (20:80, v/v). SP and the internal standard sulfadimidine (SD) were purchased from Serva (Heidelberg, G.F.R.). $N^4$-acetylsulfapyridine (AcSP) and 5-hydroxysulfapyridine (SPOH) were gifts from Pharmacia. A stock solution of 0.1 mg per ml of twice-distilled water was prepared for each compound. To achieve complete dissolution 50 μl of 0.1 *N* NaOH in 10 ml of twice-distilled water were added. The solutions were stable when stored at 4°. Plasma samples were kept frozen at —20° until analysis.

### *Calculations*

Quantification of the plasma concentrations of SP, AcSP and SPOH was performed by calculating the peak height ratios of the drug/metabolites to the added internal standard SD and relating these to previously constructed calibration curves. These curves were computed by a linear regression program. All samples were run in duplicate.

### *Extraction procedure*

Plasma samples and the added internal standard SD were extracted from acetate buffer by chloroform. The separated organic phase was evaporated to complete dryness. The residue was redissolved in methanol—water and injected into the HPLC system. The details of the complete extraction procedure are given in Fig. 2.

## RESULTS

All the various compounds (SP, AcSP, SPOH and SD; see Fig. 1) were extracted with chloroform in a single step from 100 μl human plasma. The evapo-

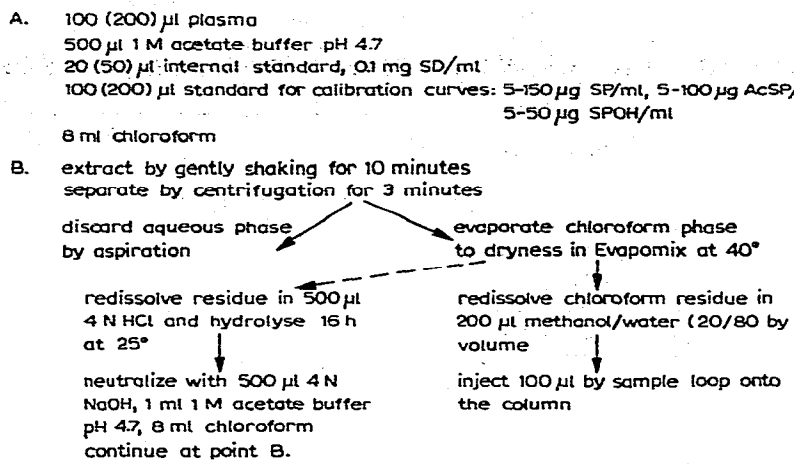


**Fig. 2. Scheme of the extraction procedure for the determination of SP, AcSP and SPOH. The specifications given in parentheses refer to the determination of total SP including hydrolysis of AcSP to SP.**

rated and redissolved chloroform extracts gave well-resolved, sharp and symmetrical peaks for SP and its metabolites after HPLC separation on an RP-18 column. Typical chromatograms are given in Fig. 3. To exclude any interference from other endogenous substances, a plasma sample drawn prior to the drug administration and without the addition of the internal standard served as blank.

No disturbing peaks were found during measurement of samples of more than 30 patients. Generally, elution was completed within 10—15 min depending on late peaks in some specimens. The calibration curves are linear from 5—150 µg/ml for SP, from 5—100 µg/ml for AcSP and from 5—50 µg/ml for SPOH. The lower limit of sensitivity was 5 µg/ml. The substances were stable if the plasma was stored for 7 days at room temperature. The accuracy and recoveries of the assay are summarised in Tables I and II, respectively.

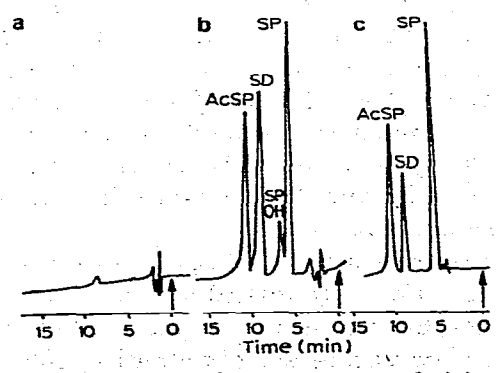


**Fig. 3. Typical chromatograms of: (a) patient's blank plasma; (b) blank plasma containing 20 µg SP per ml, 20 µg AcSP per ml, 20 µg SPOH per ml and 20 µg SD per ml; (c) sample of a patient treated chronically with 3 g salicylazosulfapyridine per day.**

TABLE I

DETERMINATION OF THE ACCURACY OF TWO DIFFERENT CONCENTRATIONS OF SP, AcSP AND SPOH

| Drug | Concentration (μg/ml) | n | Mean ± SD |
|---|---|---|---|
| SP | 10 | 10 | 9.88 ± 0.43 |
| SP | 50 | 4 | 51.29 ± 1.18 |
| AcSP | 10 | 4 | 9.58 ± 0.26 |
| AcSP | 50 | 7 | 49.34 ± 0.95 |
| SPOH | 10 | 10 | 9.41 ± 1.48 |
| SPOH | 20 | 10 | 18.65 ± 1.90 |

TABLE II

RECOVERY OF SP AND AcSP INCLUDING THE RESULTS AFTER HYDROLYSIS

| Drug | Concentration (μg/ml) | Recovery (%) | Recovery after hydrolysis (%) |
|---|---|---|---|
| SP | 20 | 96 (n=6) | 76 (n=2) |
| AcSP | 20 | 91 (n=4) | 83 (n=4) |

In contrast with our HPLC method (direct and simultaneous SP and AcSP measurement without hydrolysis) the photometric assay of Hannson and Sandberg [10] utilizes two measurements, one with boiling in 4 *N* hydrochloric acid to hydrolyse AcSP into SP. To compare our results with this assay, several samples were measured directly and split according to the above procedure. The conditions for this extraction and for the hydrolysis are included in Fig. 2.

Following the aggressive hydrolysis the resulting chromatograms exhibited (after extraction with ether—acetone (6 ml + 0.5 ml)) some additional peaks indicating decomposition of SP by the hydrochloric acid (Fig. 4a). If chloroform was used as extraction solvent these degradative products were not visible (Fig. 4b). If the hydrolysis was performed at 25° for ca. 16 h, easily measurable peaks were obtained when either ether or chloroform was used (see Fig. 4c). Under these conditions the total amount of SP (free SP and SP derived from AcSP) equalled the sum of the separately determined concentrations of SP and AcSP (Table III). Therefore this milder hydrolysis seems to be an alternative pathway for evaluation of the amounts of total SP and AcSP (=total SP — free SP) if no direct and specific method for AcSP is available.

The normal daily maintenance dose for the treatment of Crohn's disease or ulcerative colitis averages 3 g salicylazosulfapyridine (containing 1.83 g SP). With this dosage regimen we could not detect any free SPOH in plasma, but in the form of its glucuronide minor amounts were visible after incubating 100—200 μl plasma with β-glucuronidase—arylsulfatase for 3 h at 37°. However, only at the highest sensitivity setting of the UV detector could concentrations of less than 1 μg/ml be detected.

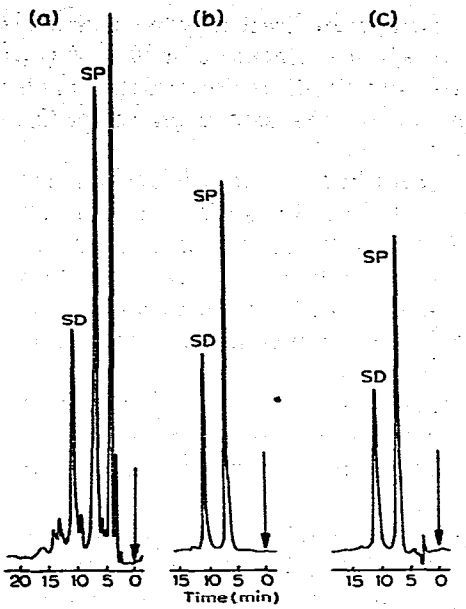


Fig. 4. Typical chromatograms of a patient's plasma sample for the determination of total SP. The extracts were hydrolyzed according to ref. 10 with 4 *N* HCl for 10 min at 100° and extracted with (a) ether—acetone or (b) chloroform: In (c) our milder conditions (see Fig. 2) for the hydrolysis of AcSP were used.

TABLE III

HPLC DETERMINATION OF TOTAL SP BY TWO DIFFERENT PROCEDURES

| Patients | Total SP measured after hydrolysis* (μg/ml) | Free SP** (μg/ml) | AcSP** (μg/ml) | Free SP + AcSP (μg/ml) |
|---|---|---|---|---|
| A.D. | 25.6 | 6.9 | 19.0 | 25.9 |
| N.D. | 32.3 | 7.1 | 29.2 | 36.3 |
| S.R. | 64.5 | 28.3 | 31.7 | 60.0 |
| H.Sch. | 46.0 | 29.9 | 12.0 | 41.9 |
| N.St. | 31.6 | 16.0 | 12.2 | 28.8 |

*Hydrolysed by 4 *N* HCl at 25° for 16 h.
**Measured directly; without hydrolysis.

## DISCUSSION

The photometric determination of SP as described by Hannson and Sandberg [10] represents a well-established method. However, they measured the major metabolite, AcSP, in the form of SP after hydrolysis in strong hydrochloric acid at 100°, which might also partly destroy SP or other metabolites. Our experiments under different conditions of hydrolysis support this assumption.

We have therefore developed a method which allows the simultaneous and direct detection of SP and its intact metabolites by a specific chromatographic

measurement without any derivatization. The detection limit of our method is adequate for measurement of SP and AcSP in 100-$\mu$l plasma samples within 10—12 min. The rate of acetylation of the sulfonamide SP is dependent on the phenotype [6]. This parameter can be determined by the measurement of free SP and AcSP in the same single plasma sample.

According to the ratio AcSP:total SP, the population can be divided into rapid and slow acetylators [4—6, 11]. Patients with Crohn's disease or ulcerative colitis are treated with this sulfonamide for long periods, sometimes even for their whole life. The incidence of side effects is much more pronounced and occurs almost exclusively among patients characterized as slow acetylators. About 80% of the side effects were observed in slow acetylators with plasma levels higher than 50 $\mu$g/ml [4, 7—9].

In addition to the toxic manifestations, therapeutic efficacy seems to be related to a therapeutic range of 20—50 $\mu$g/ml of total SP. Therefore determination of the acetylation phenotype and of the plasma levels seems to be of benefit to the patients.

The method described is a relatively simple and specific way of obtaining rapid information about the phenotype and effective plasma concentrations from one small plasma sample. From the 33 patients tested so far, 17 patients could be regarded as slow acetylators (acetylation range 11—35%) and the remaining 16 as fast acetylators (acetylation range 41—85%). In 11 patients plasma levels of total SP were in the toxic range (>50 $\mu$g/ml), while in 8 patients these concentrations were below the therapeutic range (<20 $\mu$g/ml). These routine measurements may help in improving therapy with salicylazosulfapyridine.

In summary, a specific and sensitive HPLC procedure for the simultaneous determination of SP, AcSP and SPOH in human plasma was developed. Following a single extraction step with chloroform the compounds and the added internal standard SD are eluated from a reversed-phase column by methanol—1% acetic acid (20:80).

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