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Note

High-performance liquid chromatography of sulfapyridine and its acetyl and glucuronide metabolites in rat and human urine

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Sulfapyridine (2-sulfanilamidopyridine) coupled with *p*-aminosalicylic acid (salazopyrine, sulfasalazine) is widely used for the treatment of Crohn's disease, rheumatoid arthritis and ulcerative colitis [1–3]. The daily dose of the salazopyrine is 2–4 g, resulting in a certain percentage of the patient population in serious side-effects, such as male infertility [4,5]. The incidence of the side-effects is mainly related to sulfapyridine and the acetylator phenotype, though acetylation seems to be a minor metabolic pathway, since only 15–20% of the dose is excreted in the urine as N_4-acetylsulfapyridine. Oxidation of sulfapyridine at the 5-position takes place [6–8], and the 5-hydroxy metabolites become conjugated with glucuronic acid (Fig. 1). Glucuronide conjugates are in general measured after treatment of the biological matrix with β-glucuronidase. Recently we reported the direct high-performance liquid chromatography (HPLC) measurement of the N-glucuronides of sulfadimethoxine and sulfamonomethoxine [9–11] with gradient elution and UV detection. Hansen [12] reported a direct HPLC method for sulfapyridine and its glucuronide conjugates with UV absorption, fluorescence and electrochemical detection, based on the ion-pairing technique.

The aim of this investigation was to develop a simple HPLC method for the separation and analysis of sulfapyridine, its hydroxy metabolites, and N_4-acetyl and O-glucuronide conjugates.

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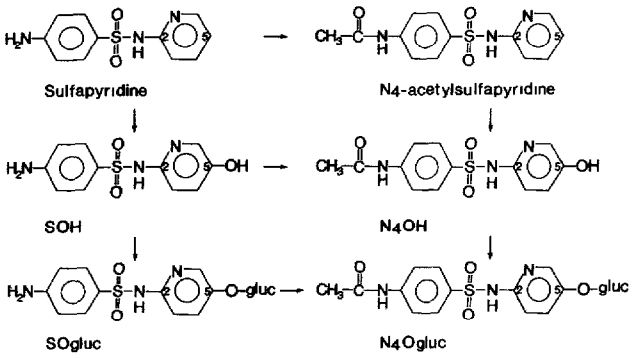

Fig 1 Structures of sulfapyridine and its metabolites.

EXPERIMENTAL

Drugs

Sulfapyridine (S), (OPG, Utrecht, The Netherlands), N_4-acetylsulfapyridine (N_4), 5-hydroxysulfapyridine (SOH) and N_4-acetyl-5-hydroxysulfapyridine (N_4OH) were synthesized and isolated as described elsewhere [8,13].

Gradient HPLC analysis

The HPLC system consisted of a Spectra Physics SP 8775 autosampler, an SP 8800 ternary HPLC pump, a Kratos Spectroflow 757 UV detector and SP 4290 integrator. The column was a Cp Spher C_8 (5 μm particle size, 250 mm × 4.6 mm I.D., from Chrompack, Middelburg, The Netherlands) with a guard column (75 mm × 2.1 mm I.D.) packed with pellicular reversed-phase material 10 μm particle size, Chrompack, Cat. No. 028 653.

The mobile phase was a mixture of acetonitrile and buffer (6.75 ml of 89% orthophosphoric acid and 2 ml of diethylamine adjusted with water to 1 l). At $T = 0$ the eluent consisted of 3% acetonitrile and 97% buffer. In 30 min ($T = 30$) the eluent was changed stepwise to 20% acetonitrile and 80% buffer. At 31 min ($T = 31$) the system was changed within 1 min to the initial composition. The flow-rate was 2 ml/min. UV detection was performed at 272 nm.

The capacity factors of the compounds are shown in Table I.

Sample preparation

Human plasma samples (100 μl) were deproteinized with 0.4 ml of 0.33 *M* trichloroacetic acid and centrifuged at 3000 *g*, and 20 μl of the supernatant were injected onto the column.

Urine samples were diluted ten times with distilled water, and 20 μl were injected onto the column.

TABLE I

CAPACITY FACTORS OF SULFAPYRIDINE AND ITS METABOLITES IN A LINEAR GRADIENT MOBILE PHASE

Compound	Capacity factor (k')	k' ratio		
		SOH/S	SOgluc/S	N_4/S
SOgluc	8.85		0.79	
S	11 22			
SOH	13.33	1.19		
N_4Ogluc	16 97		0.82	
N_4	20 55			1 83
N_4OH	22 89	1 11		1 72

Deconjugation

Deglucuronidation was carried out with 100 μl of plasma or urine, 100 μl of β-glucuronidase (50 000 U/ml glucuronidase + 893 U/ml arylsulfatase type LII lyophilized powder from limpets *Patella vulgata*; Sigma, Cat. No. G 8132, St. Louis, MO, U.S.A.) and 800 μl of 0.2 *M* KH_2PO_4 phosphate buffer (pH 3.8).

Deacetylation was carried out by heating the samples with 5 *M* HCl (1:1) at 75°C for 1 h.

Human urine samples, containing the glucuronides of 5-hydroxysulfapyridine and N_4-acetyl-5-hydroxysulfapyridine, were taken from a volunteer after oral intake of sulfapyridine. The 24-h rat urine samples were taken from rats on a sulfapyridine-containing food regimen.

Calibration curves

Urine samples of one subject containing different concentrations of the glucuronides of 5-hydroxysulfapyridine and N_4-acetyl-5-hydroxysulfapyridine (represented by different peak heights) were deconjugated. The increase in the concentration of 5-hydroxysulfapyridine and N_4-acetyl-5-hydroxysulfapyridine represented the concentration of the conjugate. A calibration curve was constructed with the help of the following formulas:

$$[\mathrm{SOH}] = H_{\mathrm{SOH}} \times F_{\mathrm{S}} \times \mathrm{MW}_{\mathrm{SOH}}/\mathrm{MW}_{\mathrm{S}}$$

$$[\mathrm{SOgluc}] = (\mathrm{d}H_{\mathrm{SOH}} \times F_{\mathrm{S}} \times \mathrm{MW}_{\mathrm{SOH}}/\mathrm{MW}_{\mathrm{S}}) \times \mathrm{MW}_{\mathrm{SOgluc}}/\mathrm{MW}_{\mathrm{SOH}}$$

$\mathrm{dH}_{\mathrm{SOH}}$ represents the difference in peak height (concentration) of 5-hydroxysulfapyridine before and after deconjugation, MW is the molecular mass, F is the slope of the regression line between concentration and peak height ($r = 0.995$) and H is the peak height.

Calibration curves of sulfapyridine and N_4-acetylsulfapyridine were constructed by spiking plasma and urine with known concentrations of the compounds (r = 0.999). Since the molar extinctions of 5-hydroxysulfapyridines are similar to those of the (acetyl)sulfapyrine, the calibration curves of sulfapyridine and 5-hydroxysulfapyridine are similar [13].

Volunteer study

A human volunteer (male, 45 years, 90 kg) was given 497 mg of sulfapyridine in gelatine capsules. Blood samples were collected at predetermined times for four

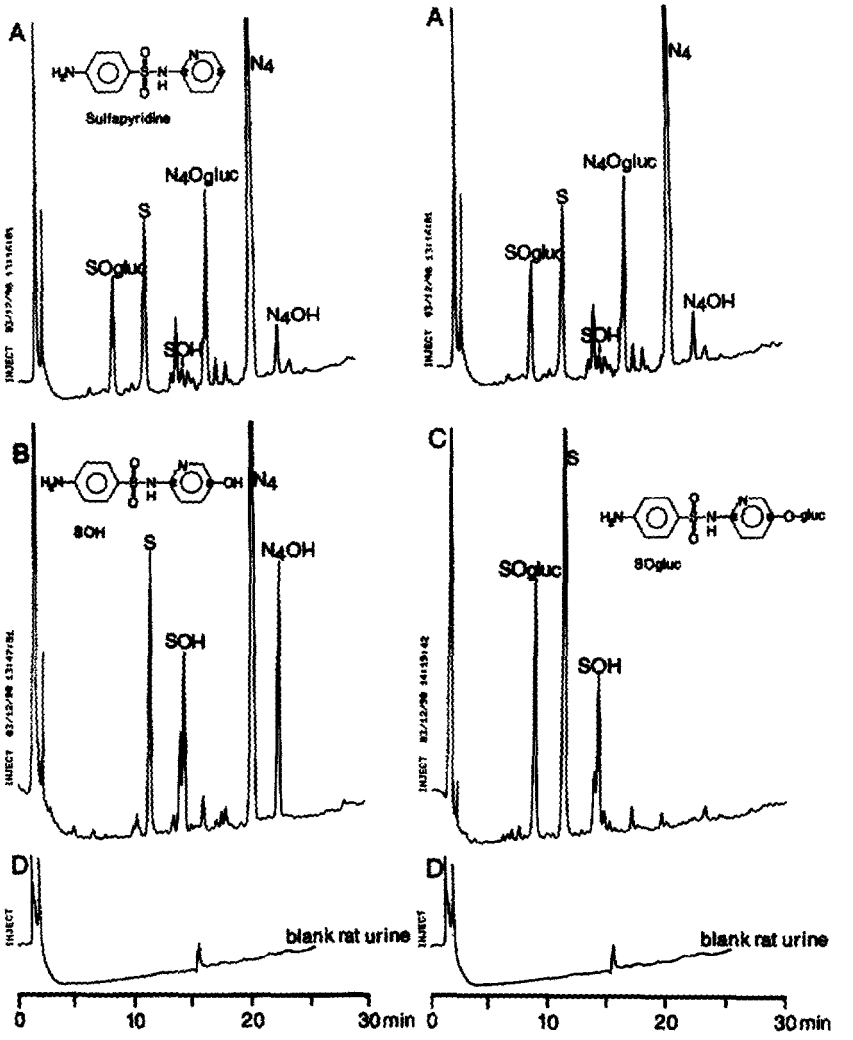

Fig. 2. Chromatograms of a 24-h rat urine sample (A), after glucuronidase action (B), after acid hydrolysis (C) and blank rat urine (D).

days. Urine samples were collected on spontaneous voiding for four days. Plasma and urine samples were stored at −20°C until analysis.

RESULTS

Fig. 2 shows chromatograms of rat urine containing sulfapyridine and its hydroxy and glucuronide metabolites. The peaks are well separated from each other. Table I lists the capacity factors. Deglucuronidation reduces the number of peaks from six to four (S, SOH, N_4 and N_4OH), and acid hydrolysis reduces the number from six to three (S, SOH, SOgluc). The contribution of the chemical

TABLE II

INTER-DAY AND INTRA-DAY COEFFICIENTS OF VARIATION

Compound ($n=6$)	Concentration ($n=6$) (μg/ml)	Inter-day C.V. (%)	Intra-day C.V (%)	Sample[a]
S	46.8±1 1	2.3	2 2	5
	34.1±0 8	2 4	2.4	9
	31.4±1 3	4 1	3 9	10
	9.4±0 3	3.5	3 2	12
	5.8±0 3	4.5	4 1	18
SOgluc	80.3±2 9	3.5	3.2	5
	40 2±2.1	4 9	4.5	9
	24 5±0.9	3 7	3.3	10
	13 3±0.7	5.2	5 0	12
	7 8±0 4	5 2	5 1	18
N_4	69.0±0 8	1 1	1 0	5
	68.8±0 3	0 4	0 4	9
	40.3±0 5	1.4	1 2	10
	19 4±0 4	1.9	1 3	12
	10 2±0 2	1.6	1 2	18
N_4OH	9 1±0 2	1.8	1 5	5
	12 9±0.3	2 0	1 6	9
	8 9±0.2	2 0	1.5	10
	5 2±0.2	3 2	3 1	12
	8 4±0.4	4 5	4 4	18
N_4Ogluc	168.3±3 7	3.4	3 3	5
	223 5±3 7	1.7	1 5	9
	125 3±4 0	3.2	3 3	10
	70-3±1.5	2.2	2 2	12
	35 1±2.3	8 3	8 0	18

[a] Urine sample number taken from the human volunteer

TABLE III

CONCENTRATIONS OF SULFAPYRIDINE AND ITS METABOLITES IN RAT URINE

Values are in μg/ml; samples taken over 24 h.

Sample	SOgluc	S	SOH	N_4Ogluc	N_4	N_4OH
1	62.4	77.5	13 4	100 1	131 7	29.8
2	70 2	104 8	9.8	49.5	128 5	10.1
4	37 3	32 3	32.2	85.1	44 3	90 3
5	–[a]	13 0	21.5	–	50 4	57.4
6	28 2	21.7	19 0	58 9	66 0	38 9
7	50 1	73 9	20.1	53 2	147 5	12 4
13	49 6	244 9	12 1	4 9	143 4	1 4
Blank	–	–	–	–	–	–

[a] Not detected

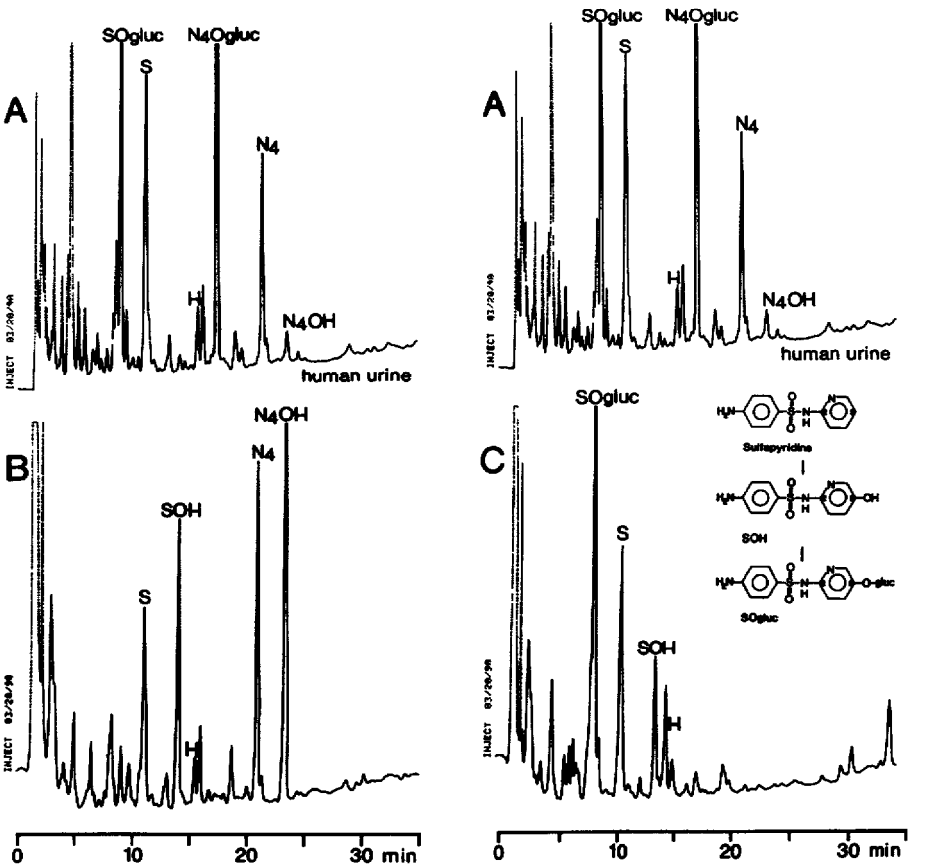

Fig. 3 Chromatograms of a human urine sample (A), after glucuronidase action (B), after acid hydrolysis (C) In blank human urine there were no compounds with the same capacity factors as the sulfonamides Peak H is hippuric acid

moiety of the sulfapyridine molecule (*i.e.* oxidation, acetylation, oxidation plus glucuronidation) to the capacity factor is constant.

Table II lists the inter-day and the intra-day variations, and Table III the urine concentrations of sulfapyridine and its metabolite in rat urine.

Fig. 3 shows chromatograms of human urine containing sulfapyridine and its metabolites As with the rat urine samples, deconjugation and acid hydrolysis result in fewer peaks and sulfonamide derivatives.

Fig. 4 shows the plasma concentration–time curves and renal excretion rate–time profiles of sulfapyridine and its metabolites in a human volunteer after an oral dose of 497 mg of sulfapyridine. The hydroxy and hydroxyglucuronide conjugates are detectable in urine but not in plasma. The main metabolite of this slow acetylator is N_4-acetyl-5-hydroxysulfapyridine-glucuronide (N_4-Ogluc). Of the administered dose, 54.1% is acetylated, 46.2% is oxidized and 42% is glucuronidated.

Table IV summarizes the preliminary pharmacokinetic parameters obtained from the same human volunteer.

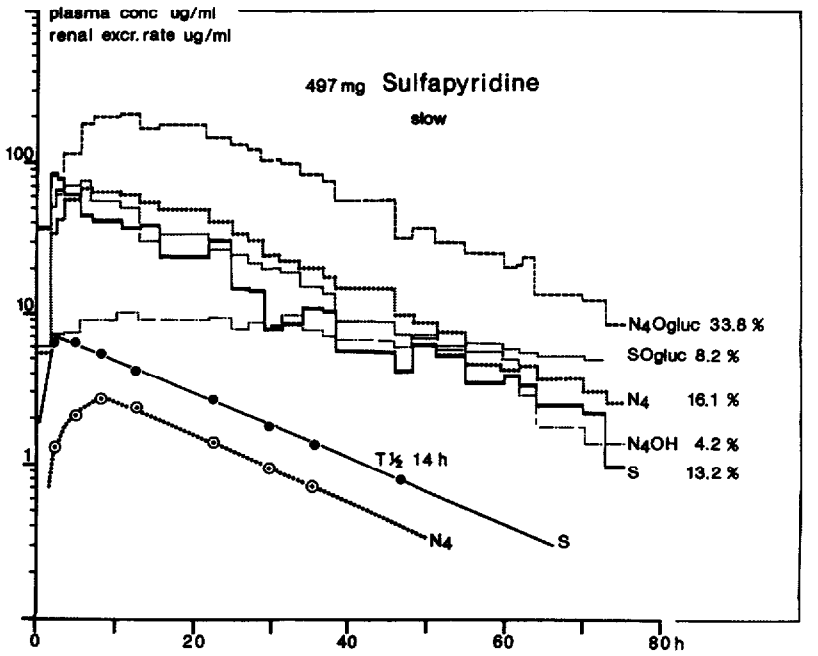

Fig 4 Plasma comcentration–time curves and renal excretion rate–time profiles of sulfapyridine (S) and its metabolites 5-hydroxysulfapyridine glucuronide (SOgluc), N_4-acetylsulfapyridine (N_4), N_4-acetyl-5-hydroxysulfapyridine (N_4OH) and its glucuronide (N_4Ogluc) in a human volunteer (slow acetylator) after administration of an oral dose of 497 mg of sulfapyridine

TABLE IV

SOME PHARMACOKINETIC PARAMETERS OF SULFAPYRIDINE AND ITS METABOLITES IN HUMANS

Parameter	Value
Subject slow acetylator	
Dose (mg)	497
Elimination half-life (h)	
Sulfapyridine (S)	14
SOH	14
SOgluc	14
N_4	14
N_4OH	>14
N_4Ogluc	14
Percentage of the dose excreted (%)	
Sulfapyridine (S)	13.2
SOH	0 0
SOgluc	8 2
N_4	16 1
N_4OH	4 2
N_4Ogluc	33.8
Total	75.5
Protein binding[a] (%)	
Sulfapyridine (S)	52.8 ± 4 4
SOH[b]	26.0 ± 1.0
SOgluc	18 3 ± 3.2
N_4	77 7 ± 3.6
N_4OH[b]	71 0 ± 1.0
N_4Ogluc	52 3 ± 4 5

[a] Measured with the Amicon Micropartition system

[b] Rat urine sample containing the SOH and N_4OH metabolites added to human plasma All other samples human urine containing the sulfa metabolites added to human plasma.

DISCUSSION

This gradient HPLC analysis of sulfapyridine and it metabolites is suitable for the metabolic phenotyping of patients, using urine samples, as only sulfapyridine and N_4-acetylsulfapyridine are present in plasma. With this method it is probable that the side-effects of sulfapyridine, released from salazopyridine, are correlated to the metabolic phenotype of the patient. Because sulfapyridine is no longer used, but is released after salazopyridine administration, the side-effects of the latter combination can be studied with sulfapyridine as a model compound. A first step must be an analytical method that enables the easy measurement of all the possible metabolites. Numerous HPLC methods have been described for sulfapyridine (salazopyridine), all of them dealing solely with parent drug and N_4-

acetyl metabolite. Hansen first described the direct measurement of hydroxy and hydroxyglucuronide metabolites of sulfapyridine; this method may be considered as more laborious than the method described in this paper.

With an isocratic mobile phase, the group contribution of each chemical moiety in the molecule is quite characteristic [14,15]. The same holds for a linear gradient mobile phase (Table I). The nearly constant ratio between the capacity factors of compounds differing by one functional group can be used as an additional tool in identifying the structural analogues.

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