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High-performance liquid chromatography of sulphadimethoxine and its N_1-glucuronide, N_4-acetyl and N_4-acetyl-N_1-glucuronide metabolites in human plasma and urine

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SUMMARY

Sulphadimethoxine is metabolized in humans by N_1-glucuronidation and by N_4-acetylation. Sulphadimethoxine-N_1-glucuronide can be measured by the direct high-performance liquid chromatographic analysis and without enzymic deglucuronidation. The N_1-glucuronide can be measured by an isocratic as well as by a gradient mobile phase. The group contribution of the N_1-glucuronide moiety to the capacity factor is a reduction of 0.24 in the isocratic system and 0.55 in the gradient system. N_4-Acetylation increases the capacity factor by a factor 1.4 in the isocratic system and by 1.06 in the gradient system.

INTRODUCTION

Sulphonamides are metabolized by phase I oxidation, and by phase II N_4-acetylation and N_1-glucuronidation as shown in Fig. 1 [1–4]. The N_4-acetyl

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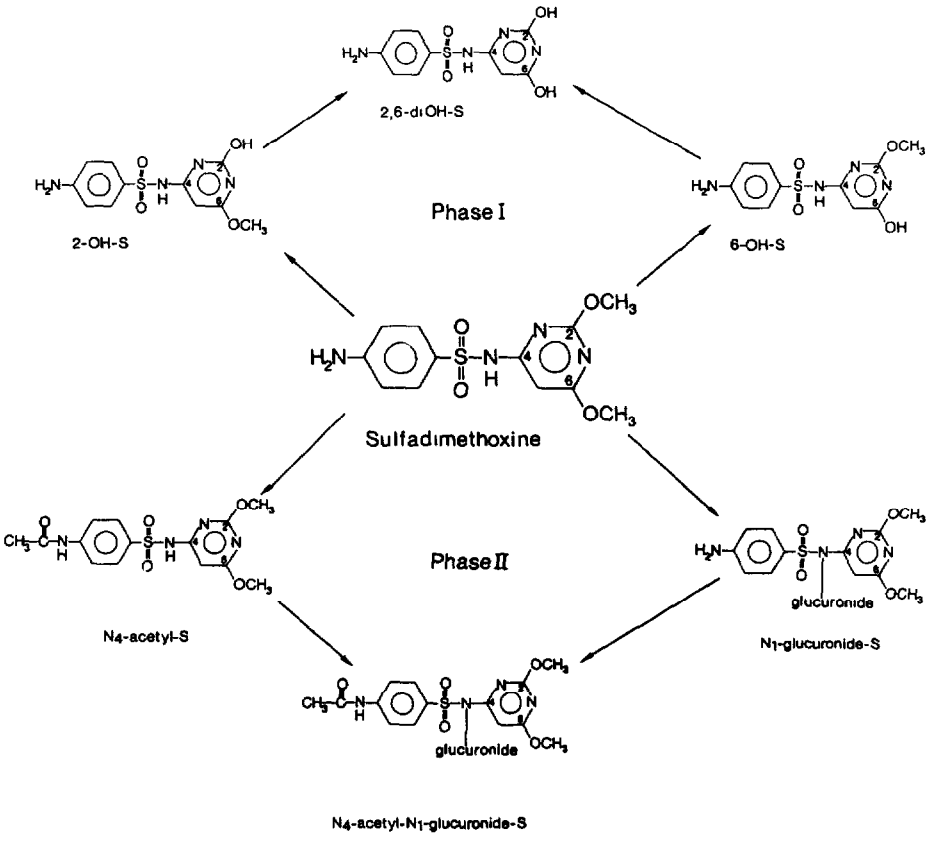

Fig. 1. Structures of sulphadimethoxine and its metabolites.

conjugates can be analysed by high-performance liquid chromatography (HPLC) [5,6]; in a reversed-phase system they have a slightly higher capacity factor than the parent compound. Hydroxysulphonamides can also be analysed by HPLC in the reversed-phase mode; the capacity factors of these metabolites are in general slightly smaller than those of the parent drugs [7,8].

N_1-Glucuronides were reported for sulphonamides bearing a methoxy group on their N_1-substituent. They were identified by thin-layer chromatography in a portion of 24-h human urine after oral administration of the methoxysulphonamide (sulphadimethoxine) [1,2]. No HPLC analysis of the N_1-glucuronide metabolites of sulphadimethoxine and its N_4-acetyl metabolite have been described in the literature. A common approach to the identification and quantification of glucuronide metabolites is to assay for the free aglycone following enzymic or chemical hydrolysis of the sample, and then subtract the amount of free aglycone that was originally in the sample.

The polar glucuronides are difficult to extract from plasma and urine. At-

tempts to analyse intact glucuronides are made with gas chromatography following derivatization of the carboxylic group with diazomethane and the hydroxyl groups with N,O-bis(trimethylsilyl)acetamide [9].

HPLC analysis and retention index measurements of some drugs with their glucuronide metabolites were described earlier [10,11]. The glucuronide group decreases strongly the capacity factor of the parent compound. The N_1-glucuronide conjugate of sulphadimethoxine and its N_4-acetyl metabolite would be expected to elute from a reversed-phase column very early and together with the endogenous waste compounds in human urine. The objectives of this study were to determine if the glucuronides of sulphadimethoxine could be chromatographed using reversed-phase columns, and if this analysis could be used satisfactorily for pharmacokinetic studies of the drug in humans.

EXPERIMENTAL

Chemicals and reagents

Sulphadimethoxine was obtained from Sigma (St. Louis, MO, U.S.A.) and N_4-acetylsulphadimethoxine was synthesized [8].

Alkaline hydrolysis of sulphadimethoxine was used at Daiichi Seiyaku (Tokyo, Japan) to synthesize 2- and 6-hydroxysulphadimethoxine (i.e. 2- and 6-demethylsulphadimethoxine, respectively). The N_1-glucuronides of sulphadimethoxine and N_4-acetylsulphadimethoxine were present in the urine of human volunteers.

All reagents were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

Isocratic HPLC analysis

The HPLC system consisted of a Spectra Physics SP 870 XR autosampler, an SP 8810 isocratic pump, a Kratos Spectroflow 783 UV detector and an SP 4290 integrator. The column was Cp Spher C_8 (5 μm, particle size, 250 mm$\times$4.6 mm I.D.) from Chrompack (Middelburg, The Netherlands), with a guard column (75 mm$\times$2.1 mm I.D.) packed with 10-μm pellicular reversed phase (Chrompack, Cat. No. 028 653). The mobile phase was a mixture of 840 ml of buffer (2 ml of diethylamine plus 6.75 ml of 89% orthophosphoric acid adjusted to 1000 ml with water; pH 2.1), 200 ml of acetonitrile and 20 ml of methanol (v/v). The flow-rate was 2 ml/min. UV detection was performed at 271 nm.

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 an SP 4290 integrator. The column was Cp Spher C_8 (5 μm particle size, 250 mm$\times$4.6 mm I.D.) from Chrompack, with a guard column (75 mm$\times$2.1 mm I.D.) packed with 10-μm pellicular reversed phase (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 1000 ml). Initially the eluent consisted of 10% acetonitrile and 90% buffer. After 35 min the eluent was changed stepwise to 25% acetonitrile and 75% buffer, and 1 min later it was changed over a period of 1 min back to the initial composition. The flow-rate was 2 ml/min. UV detection was performed at 271 nm.

The capacity factors and retention times of the compounds are shown in Table I for the isocratic and for the gradient system.

Sample preparation

Human plasma samples (200 μl) were deproteinized with 0.4 ml of 0.33 *M* trichloroacetic acid and centrifuged at 3000 *g*; 20 μl of the supernatant were injected onto the column.

Urine samples were diluted ten-fold with distilled water, and 100 μl were injected onto the column.

Deconjugation

Deglucuronidation was carried out with 100 μl of plasma or urine, 100 μl of deglucuronidase and 800 μl of buffer. The urine samples, containing the glucuronides of sulphadimethoxine and N_4-acetylsulphadimethoxine, were taken from a volunteer after oral intake of sulphadimethoxine and from a volunteer after oral intake of N_4-acetylsulphadimethoxine. Four different glucuronidase and sulphatase enzymes (A–D) were used:

TABLE I

CAPACITY FACTORS AND RETENTION TIMES OF SULPHADIMETHOXINE AND ITS METABOLITES

Compound[a]	Isocratic mobile phase		Gradient mobile phase	
	Retention time (min)	Capacity factor (k')	Retention time (min)	Capacity factor (k')
Unretained	1 0		1 2	
2-OH-S	2.15	1.15	3.4	1.83
6-OH-S	3 33	2.33	6.4	4.33
S-Glucuronide	6.01	5.01	17 13	13.28
N_4-Glucuronide	8.16	7.16	21 87	17.23
S	21.64	20.64	31.38	25.15
N_4	24.61	23.61	33 03	26.53

[a]S = sulphadimethoxine; N_4 = N_4-acetylsulphadimethoxine, 2-OH-S = 2-hydroxysulphadimethoxine; 6-OH-S = 6-hydroxysulphadimethoxine

(A) Acetate buffer (pH 5.0) and 5600 U/ml β-glucuronidase plus 100 U/ml arylsulphatase (type IV, limpets *Patella vulgata*, Sigma, Cat. No. S8504).

(B) 0.2 *M* Phosphate buffer (pH 5.0) and 5600 U/ml β-glucuronidase plus 100 U/ml arylsulphatase (type IV, limpets *P. vulgata*, Sigma, Cat. No. S8504). The phosphate buffer eliminates the arylsulphatase action.

(C) Phosphate buffer (pH 6.8) and 20.000 U/ml β-glucuronidase (*Escherichia coli*, type VII, Sigma, Cat. No. G1758).

(D) Phosphate buffer (pH 3.8) and 50.000 U/ml β-glucuronidase plus 893 U/ml arylsulphatase type LII (lyophilized powder from limpets *P. vulgata*; Sigma, Cat. No. G8132).

The acetate buffer consisted of 0.2 *M* sodium acetate (pH 5.0) and the phosphate buffer consisted of 0.2 *M* KH_2PO_4 (pH 3.8) and 0.2 *M* KH_2PO_4 plus 0.2 *M* Na_2HPO_4 (pH 6.8).

Deacetylation was carried out by heating the samples with 1 *M* hydrochloric acid at 50°C for 1 h.

Calibration curves

Urine samples from one subject containing different concentrations of the glucuronides of sulphadimethoxine (peak height) were deconjugated by system D. The increase in the concentrations of sulphadimethoxine and N_4-acetylsulphadimethoxine represented the concentration of the conjugate. A calibration curve was constructed with the help of the following formula:

$$[\text{S-gluc}] = d[\text{S}] \times M_{\text{S-gluc}}/M_{\text{S}}$$

where $d[\text{S}]$ is the difference in concentration of sulphadimethoxine before and after deconjugation and M is relative molecular mass ($r = 0.995$).

Calibration curves for sulphadimethoxine and N_4-acetylsulphadimethoxine were constructed by spiking plasma and urine with known concentrations of the compounds ($r = 0.999$).

Volunteer study

A human volunteer (female, 20 years, 70 kg) was given 687 mg of sulphadimethoxine. Blood samples were collected at predetermined times for one week. Urine samples were collected on spontaneous sampling for one week. Plasma and urine samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Figs. 2 and 3 show the HPLC profiles of a human urine sample containing sulphadimethoxine and its N_4-acetylated and N_1-glucuronidated metabolites in the isocratic and gradient system, respectively. In both systems the parent drug and metabolites are well separated. Depending on the endogenous waste compounds present in the urine sample, one can decide whether the gradient

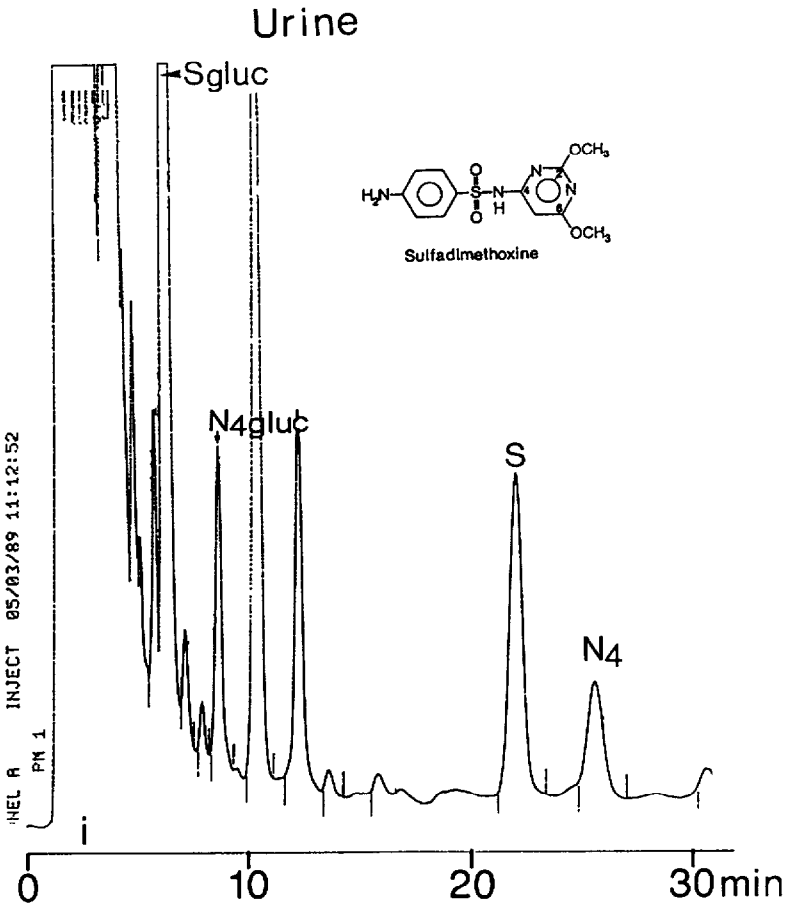

Fig 2. Chromatogram of a human urine sample containing sulphadimethoxine (S) and its metabolites N_4-acetylsulphadimethoxine (N_4), sulphadimethoxine-N_1-glucuronide (Sgluc), and N_4-acetylsulphadimethoxine-N_1-glucuronide (N_4gluc) in the isocratic mobile phase.

or the isocratic system should be used. Table I shows the retention times and the capacity factors of the parent drug and its N_4-acetyl and N_1-glucuronide metabolites. The assay sensitivity in urine is 1 μg/ml for sulphadimethoxine and its three metabolites. The assay sensitivity in plasma is 0.5 μg/ml for the parent drug and its N_4-acetyl metabolite (signal-to-noise ratio of 3).

The group contribution of the N_1-glucuronide moiety to the capacity factor is a reduction of 0.24 in the isocratic system and 0.55 in the gradient system. N_4-Acetylation increases the capacity factor by a factor 1.14 in the isocratic system and by a factor 1.06 in the gradient system. When there is a double conjugation, i.e. N_4-acetylsulphadimethoxine-N_1-glucuronide, the N_4-acetyl

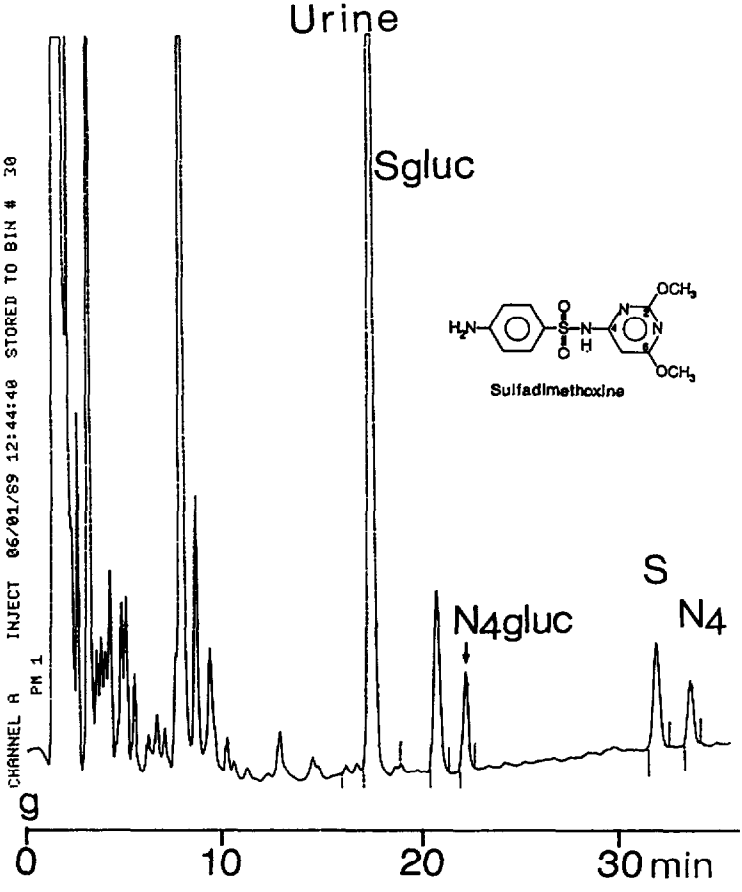

Fig. 3. Chromatogram of a human urine sample containing sulphadimethoxine (S) and its metabolites N_4-acetylsulphadimethoxine (N_4), sulphadimethoxine-N_1-glucuronide (Sgluc), and N_4-acetylsulphadimethoxine-N_1-glucuronide (N_4gluc) in the gradient mobile phase.

group slightly increases the capacity factor of the sulphadimethoxine-N_1-glucuronide by 43% in the isocratic system and by 29% in the gradient system.

Table II shows the inter- and intra-day variation of the analysis for sulphadimethoxine and its metabolites in the isocratic eluent, and Table III shows the equivalent values for the gradient eluent.

Fig. 4 shows the yield of the deglucuronidation reactions for system D. The deglucuronidation is complete after 24 h, whereas system A stopped at 96%, system B at 82% and system C at 54%.

Similar results were obtained for the deglucuronidation of N_4-acetylsulphadimethoxine-N_1-glucuronide. Systems A and D completed the reaction in 24 h, whereas system B stopped at 73% and system C at 43%.

Fig. 5 shows an example of the pharmacokinetic profile of sulphadimethox-

TABLE II

INTRA-DAY AND INTER-DAY VARIABILITY OF A URINE SAMPLE CONTAINING SULPHADIMETHOXINE AND ITS METABOLITES

Values obtained with the isocratic mobile phase; $n = 5$.

Compound[a]	High concentrations		Low concentrations	
	C.V. (%)	Concentration (μg/ml)	C.V. (%)	Concentration (μg/ml)
Intra-day variability				
S	2.9	18.77	4.4	6.97
S-Glucuronide	2.9	262.6	4.6	113.3
N_4	2.9	8.97	6.3	7.70
N_4-Glucuronide	3.1	6.39	5.7	3.61
Inter-day variability				
S	2.2	18.77	5.9	8.97
S-Glucuronide	0.1	262.6	2.5	113.3
N_4	1.2	8.97	5.6	7.70
N_4-Glucuronide	2 4	6.39	5.5	3 61

[a]For abbreviations see Table I.

TABLE III

INTRA-DAY AND INTER-DAY VARIABILITY OF A URINE SAMPLE CONTAINING SULPHADIMETHOXINE AND ITS METABOLITES

Values obtained with the gradient mobile phase; $n = 5$

Compound[a]	High concentrations		Low concentrations	
	C.V. (%)	Concentration (μg/ml)	C.V. (%)	Concentration (μg/ml)
Intra-day variability				
S	3.4	18.77	3.6	2.80
S-Glucuronide	1.7	262.6	8.0	29 7
N_4	2.1	8.97	5.9	3 05
N_4-Glucuronide	1 6	6.39	2.2	1.22
Inter-day variability				
S	2 5	17.9	3 9	2 56
S-Glucuronide	2.7	256.0	3 6	27 3
N_4	6.0	9.58	6.6	2 35
N_4-Glucuronide	1.6	7.43	6 8	1.18

[a]For abbreviations see Table I.

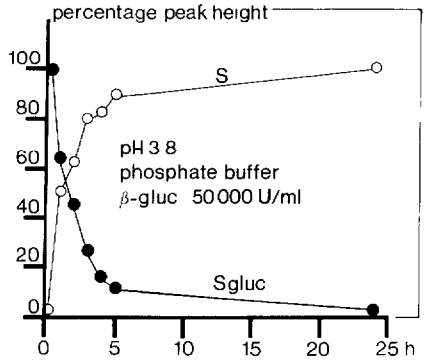

Fig. 4. Effect of the time of deglucuronidase treatment (system D) on the peak heights of sulphadimethoxine-N_1-glucuronide (Sgluc) and sulphadimethoxine (S).

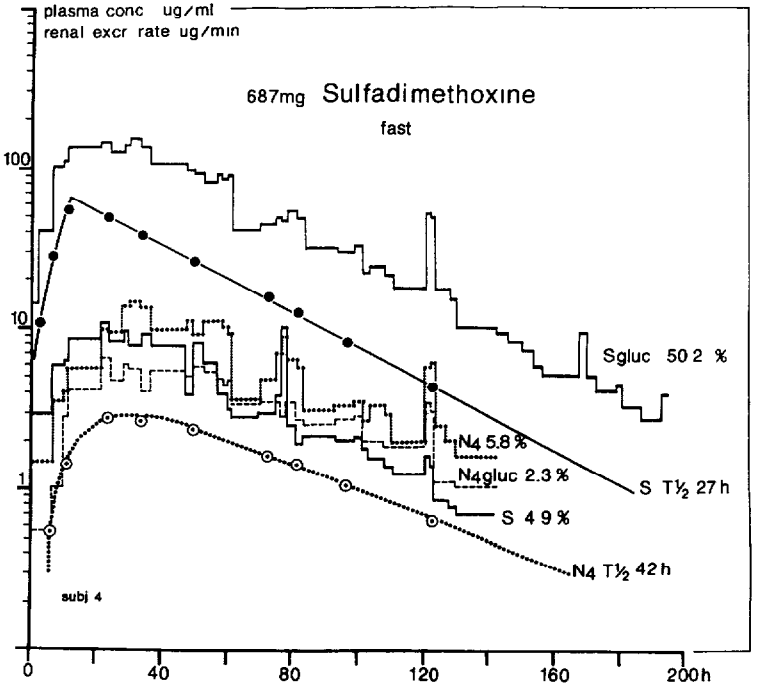

Fig 5. Plasma concentration-time curves and renal excretion rate-time profiles of sulphadimethoxine (S) and its N_4-acetyl- (N_4) and N_1-glucuronide (gluc) metabolites in a human volunteer (fast acetylator) after oral administration of 687 mg of sulphadimethoxine.

ine in a 'fast' acetylator. Sulphadimethoxine-N_1-glucuronide is not present in plasma but is, like the main metabolite, immediately excreted in the urine. Of the dose administered 50.2% is excreted as sulphadimethoxine-N_1-glucuro-

nide. The plasma protein binding of sulphadimethoxine-N_1-glucuronide is ca. 30%. A full pharmacokinetic analysis of sulphadimethoxine in fast and slow acetylators will be published elsewhere [12].

This direct analysis of the N_1-glucuronide allows the pharmacokinetics of this specific metabolite to be determined without deglucuronidation of numerous urine samples. Depending on the endogenous compounds or waste products in urine, and on the availability of the apparatus, the isocratic or the gradient mobile phase can be used. (Semi)preparative HPLC of the N_1-glucuronide can be performed with both systems for the isolation and purification of the N_1-glucuronides of a series of methoxysulphonamides, such as sulphadimethoxine, sulphamonomethoxine, and sulphadoxine, as drug conjugate standards.

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