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Isolation, identification and determination of sulfamethoxazole and its known metabolites in human plasma and urine by high-performance liquid chromatography

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Abstract

From human urine the following metabolites of sulfamethoxazole (S) were isolated by preparative HPLC: 5-methylhydroxysulfamethoxazole (SOH), N_4-acetyl-5-methylhydroxysulfamethoxazole (N_4SOH) and sulfamethoxazole-N_1-glucuronide (Sgluc). The compounds were identified by NMR, mass spectrometry, infrared spectrometry, hydrolysis by ß-glucuronidase and ratio of capacity factors. The analysis of S and the metabolites N_4-acetylsulfamethoxazole (N_4), SOH, N_4-hydroxysulfamethoxazole (N_4OH), N_4SOH, and Sgluc in human plasma and urine samples was performed with reversed-phase gradient HPLC with UV detection. In plasma, S and N_4 could be detected in high concentrations, while the other metabolites were present in only minute concentrations. In urine, S and the metabolites and conjugates were present. The quantitation limit of the compounds in plasma are respectively: S and N_4 0.10 μg/ml; N_4SOH 0.13 μg/ml; N_4OH 0.18 μg/ml; SOH 0.20 μg/ml; and Sgluc 0.39 μg/ml. In urine the quantitation limits are: N_4 and N_4OH 1.4 μg/ml; S 1.5 μg/ml; N_4SOH 1.9 μg/ml; SOH 3.5 μg/ml; and Sgluc 4.1 μg/ml. The method was applied to studies with healthy subjects and HIV positive patients.

1. Introduction

The known metabolism of sulfamethoxazole [4-amino-N-(5-methyl-3-isoxazolyl-benzenesulfonamide; CAS number 723-46-6; S] involves acetylation and oxidation at the N_4 nitrogen atom leading to N_4-acetylsulfamethoxazole (N_4) and N-hydroxysulfamethoxazole (N_4OH). The

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latter compound is thought to be responsible for the occurrence of side effects during long-term prophylactic treatment of *Pneumocystis carinii* pneumonia in HIV positive patients [1]. Hydroxylation takes also place at the C_5 methyl group, leading to 5-methylhydroxysulfamethoxazole (SOH) and N_4-acetyl-5-methylhydroxysulfamethoxazole (N_4SOH) [2–10]. Moreover sulfamethoxazole is glucuronidated at the N_1 atom, leading to sulfamethoxazole-N_1-glucuronide [2,6–9]. This metabolic profile of S, shown in Fig. 1, has been elucidated by paper chromatography with detection reagents [2,6–9] and isocratic HPLC analysis [4,5,11]. Analysis of the sulfonamides in blood and urine was performed with the well known Bratton and Marshall method [12] until in 1972 the first HPLC method for separation of sulfonamides was reported [13].

Recently we investigated the pharmacokinetics of the N_4-hydroxylamine of sulfamethoxazole in human volunteers by isocratic as well as gradient HPLC and discovered a glucuronide conjugate of sulfamethoxazole (Sgluc) which was not detected before by HPLC analysis [14].

HPLC analysis of N-glucuronides of sulfonamides had already been described for sulfadimethoxine [15,16], sulfa-6-monomethoxine [17], sulfamethomidine [18] and sulfaphenazole [19], but not yet for S. For a full pharmacokinetic analysis of sulfamethoxazole, all metabolites should be available for the validation of the analytical method.

The aims of this investigation were (a) the isolation and identification of three metabolites of sulfamethoxazole: 5-methylhydroxysulfamethoxazole, N_4-acetyl-5-methylhydroxysulfamethoxazole and sulfamethoxazole-N_1-glucuronide by preparative HPLC and standard spectrometric techniques, and (b) to develop a simple and direct gradient HPLC analysis of S with its metabolites and glucuronide conjugate in human plasma and urine.

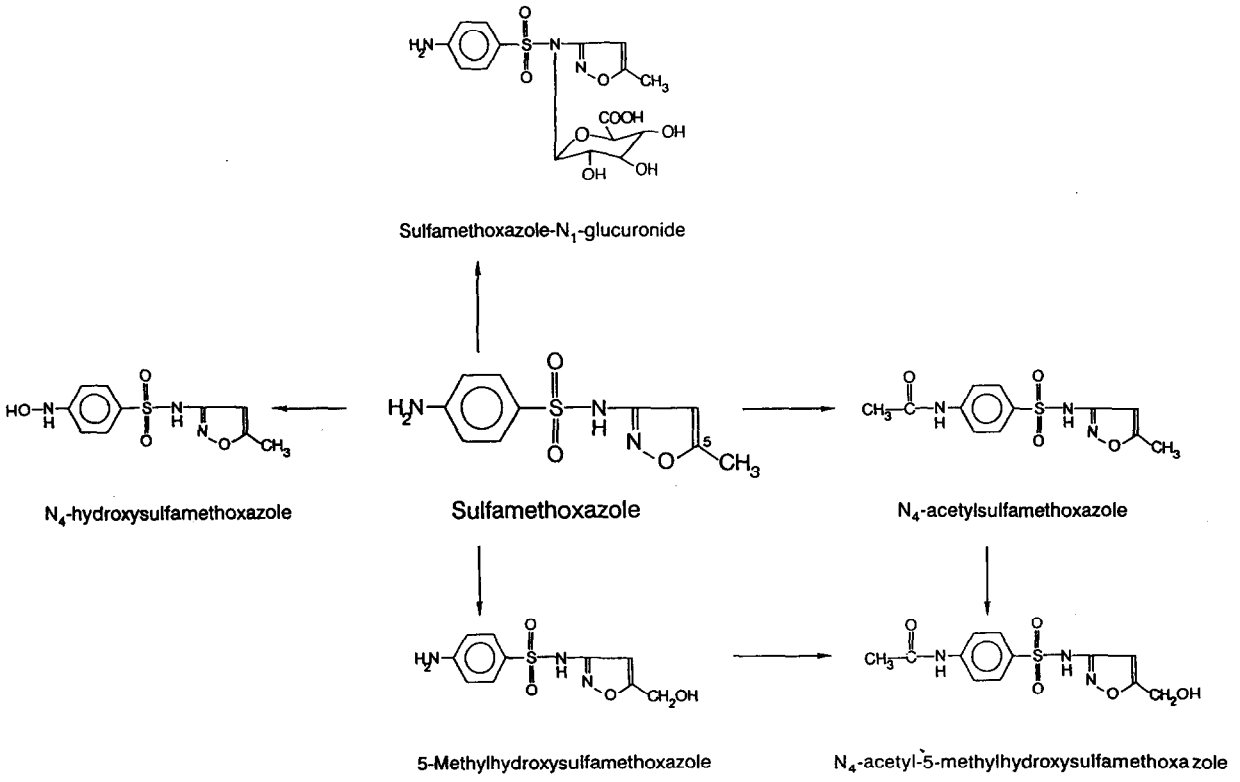

Fig. 1. Structures of sulfamethoxazole and its metabolites.

2. Experimental

2.1. Chemicals

S and N_4 were obtained from Hoffmann-LaRoche (Mijdrecht, Netherlands). The metabolites SOH, N_4SOH and Sgluc were isolated from human urine and were HPLC pure. N_4OH was synthesized by Synthon (Nijmegen, Netherlands). All other reagents were of p.a. quality and obtained from Merck (Darmstadt, Germany). Amberlite XAD-2 absorption resin and Celite 545 were obtained from Fluka (Perstorp Analytical, Oud Beyerland, Netherlands).

Four ß-glucuronidase enzymes were obtained from Sigma (St. Louis, MO, USA): ß-glucuronidase type B1 (Bovine liver, Cat. No. G-0251), type H2 (*Helix pomatia*, Cat. No. G-0876), type LII (lyophilized powder from limpets *Patella vulgata*, Cat. No. G-8132), type VIIA (*Escherichia coli*, Cat. No. G-7646)

2.2. Subject

To one healthy human subject (male, 35 years of age, 85 kg) a single oral dose of 800 mg S in a gelatin capsule was administered with 100 ml tap water after an overnight fast. The study had the approval of the hospital ethics committee and informed consent was obtained from the volunteer.

2.3. Sampling

Blood samples were drawn and collected in heparinized Eppendorf vials (2 ml) at regular time intervals during 2 days after administration by means of fingertip puncture with Monolet lancets (Monoject, St. Louis, MO, USA). After centrifuging at 3000 *g* for 5 min, plasma samples were stored at −20°C pending analysis. Urine was collected upon untimed voiding. The total time of sample collection was 96 h. Three samples of 7 ml of each void were stored at −20°C pending analysis. The remainder of the urine was collected for 48 h in a tank and stored at −20°C, pending isolation.

2.4. Sample treatment

Plasma samples (100 μl) were deproteinized with 100 μl of acetonitrile, centrifuged at 3000 *g* for 5 min, and 20 μl of the supernatant were injected onto the column.

For the measurement of N_4OH, urine was centrifuged at 3000 *g* for 5 min, the supernatant was diluted 1:9 with 0.2 *M* KH_2PO_4 buffer pH 5.0 and 20 μl were injected onto the column.

For the measurement of S and the other metabolites, 50 μl diethylamine was added to the urine sample of 7 ml (pH 10) to increase the solubility of all sulfonamides. The urine was then centrifuged at 3000 *g* for 5 min, and subsequently the supernatant was diluted 1:9 with 0.2 *M* K_2PO_4 buffer pH 5.0 and 20 μl were injected onto the column.

2.5. Gradient HPLC analysis of S and metabolites

The HPLC system consisted of a Spectra Physics SP 8775 autosampler (Spectra Physics, Eindhoven, Netherlands), a Spectra Physics SP 8800 ternary HPLC pump, a Spectroflow 783 UV detector (Separations, H.I. Ambacht, Netherlands) and a Spectra Physics SP 4290 integrator. The column was a Spherisorb ODS 5 μm, 250 × 4.6 mm I.D. (Cat. no 28812, Chrompack, Bergen op Zoom, Netherlands) with a guard column (75x2.1 mm), packed with pellicular reversed phase (Chrompack Cat. No. 28653). The mobile phase was a mixture of acetonitrile, dimethylformamide (DMF) and a mixture of 0.9 g H_3PO_4 (85% w/w) and 0.225 g tetramethylammonium chloride (TMACl) in 1 l H_2O (pH 2.1).

At $t = 0$, the mobile phase consisted of 1% acetonitrile, 3% DMF and 96% of the H_3PO_4/TMACl mixture (v/v). During the following 30 min, the mobile phase changed linearly until it attained a composition of 20% acetonitrile, 10% DMF and 70% of the H_3PO_4/TMACl mixture (v/v). At 30 min, the mobile phase was changed within 3 min to the initial composition, followed by equilibration for 2 min. The flow-rate was 1.2 ml/min. The effluent was monitored at 271 nm.

2.6. Isolation of the metabolites from urine

Column chromatography

Two liters of urine (48 h) were brought dropwise to pH 2.5 with H_3PO_4 (25%, w/w) and allowed to stand for 1 h to precipitate endogenous urates. Celite 545 (40 g) was added to the urine, and the suspension was filtered. The pH of the filtrate was adjusted to 2.5.

A preparative column (40 × 6 cm, packed with 1 kg XAD-2) was rinsed with 2 l of methanol, 2 l of water and with 1 l of 0.06 *M* KH_2PO_4 buffer (pH 2.5). Thereafter, 1 l of urine was passed through the column, followed by 2 l of the 50-fold diluted phosphate buffer (pH 2.5). The column was dried by air suction for 10 min. Elution of the column was carried out with a mixture of methanol–1% acetic acid according to the following scheme: First, 500 ml CH_3OH–AcOH (20:80, v/v) was passed through the column, followed by 8 fractions of 250 ml with the methanol concentration increased by steps of 10% to 100% CH_3OH. The effluent fractions 5 to 8 (60:40–90:10, v/v) contained the sulfamethoxazole metabolites.

This procedure was repeated with the second liter of urine on a regenerated column, and fractions 5–8 from both procedures were combined. Methanol was evaporated under reduced pressure. The combined effluent was brought onto the column, and the gradient elution was repeated. Most of the yellow–brown urine color had now been removed, together with the parent compound and its N_4-acetyl conjugate.

Preparative high-performance liquid chromatography

The volume of the isolated fraction from the preparative column was concentrated to 130 ml by evaporation under reduced pressure (Rotavapor).

The preparative Gilson HPLC consisted of a Gilson 302 sample pump (Gilson, Meyvis, Bergen op Zoom, Netherlands), two 305 Gilson gradient pumps, a 811 B Dynamic mixer, a Kratos 757 UV detector (Separations, Hendrik Ido Ambacht, Netherlands), an LKB 2211 superrac fraction collector (LKB, Woerden, Netherlands), and a BD7 recorder (Kipp and Zonen, Delft, Netherlands). The column was a C_8, 8 μm particle size, 250 × 10 mm I.D., Rainin Dynamax 60 Å column (Meyvis).

The mobile phase consisted of 1% acetic acid in water and acetonitrile (85:15%, v/v) for 1 min at the start, thereafter changing linearly in 20 min to 65:35 (v/v). The flow-rate was 4.7 ml/min and peaks were detected at 271 nm.

Concentration of the separated metabolites was carried out on an IKA rotavapor (Janke and Kunkel, Staufen, Germany) equipped with a Trivac vacuum pump (Leybold-Heraeus, Woerden, Netherlands). Three crude samples each containing a metabolite were collected.

2.7. Final purification by preparative HPLC

Sulfamethoxazole-N_1-glucuronide (Sgluc)

First isolation: The mobile phase was methanol–1% AcOH (27:73, v/v), at a flow-rate of 4.7 ml/min.

Second isolation: The mobile phase started with a composition of acetonitrile–1% AcOH (5:95, v/v) and this was changed linearly in 20 min to acetonitrile–1% AcOH (40:60, v/v).

The collected sample was concentrated to 20 ml under reduced pressure with the rotavapor, and thereafter reduced in volume in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred to a small tube for freeze drying.

5-Hydroxysulfamethoxazole (SOH)

First isolation: Starting with a mobile phase of methanol–1% AcOH (20:80, v/v), the mobile phase changed linearly in 12 min to methanol–1% AcOH (40:60, v/v).

Second isolation: Starting with a mobile phase of acetonitrile–water (10:90, v/v), the mobile phase was changed linearly in 15 min to acetonitrile–water (35:65, v/v).

The collected sample was concentrated to 20 ml under reduced pressure with the rotavapor, thereafter further reduced in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred to a small tube for freeze drying.

N_4-Acetyl-5-hydroxysulfamethoxazole (N_4SOH)

First isolation: Starting with a mobile phase of acetonitrile–1% AcOH (20:80, v/v), the mobile

phase was changed linearly in 12 min to acetonitrile–1% AcOH (32:68, v/v).

Second isolation: Starting with methanol–water (40:60, v/v), the mobile phase changed linearly in 8 min to methanol–water (60:40, v/v).

The collected sample was concentrated to 20 ml under reduced pressure with the rotavapor, thereafter reduced in volume in a smaller flask to 0.5 ml. The final volume of 0.5 ml was transferred to a small tube for freeze drying.

2.8. Identification of the metabolites

Mass spectrometry

A double focussing VG 7070E mass spectrometer with direct inlet was used (Fisons Instruments, Weesp, Netherlands). EI (70 eV), positive CI methane (100 eV), and FAB (matrix nitrobenzylalcohol, Argon, acceleration voltage 6–7 kV) were performed.

Nuclear magnetic resonance

^{1}H-NMR spectra were recorded on a Bruker AM 400 spectrometer (400 MHz, FT; Bruker, Wormer, Netherlands) on solutions in CD_3OD/$CDCl_3$ (internal standard Me_4Si). ^{13}C-NMR spectra with ^{1}H-decoupling were recorded with a Bruker AM 400 spectrometer operating at 100.6 MHz on solutions in $CD_3OD/CDCl_3$ (internal standard Me_4Si). Chemical shift values are reported as δ-values relative to Me_4Si as internal standard; deuteromethanol/deuterochloroform were used as solvents.

Elemental analysis

A Carlo Erba EA 1108 element analyzer (Interscience, Breda, Netherlands) was used for CHNS elemental analysis.

Infrared spectrometry

Infrared spectra in KBr were recorded on a Perkin Elmer 881 infrared spectrophotometer (Perkin Elmer, Gouda, Netherlands).

2.9. Deconjugation

Deconjugation reactions with ß-glucuronidase (urine–ß-glucuronidase–buffer 1:1:8, v/v, 9 days, 37°C) were carried out. Four different ß-

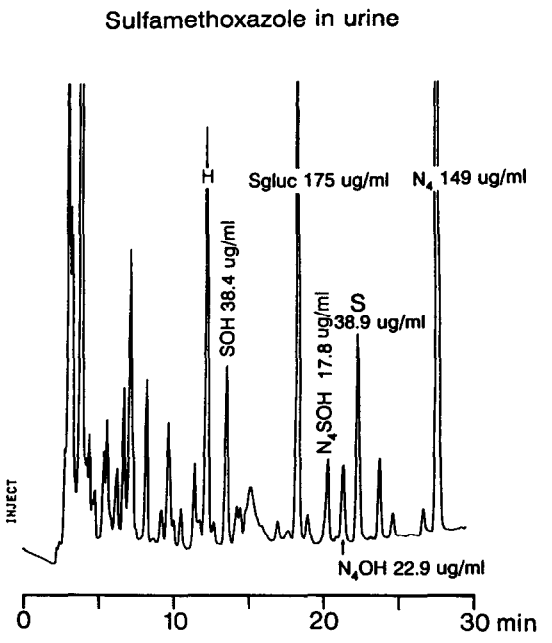

Fig. 2. Chromatogram of human urine sample from a healthy human subject obtained 1 h after administration of a single oral dose of 800 mg S, containing sulfamethoxazole (S) and the metabolites N_4-acetylsulfamethoxazole (N_4), 5-methylhydroxysulfamethoxazole (SOH), N_4-hydroxysulfamethoxazole (N_4OH), N_4-acetyl-5-methylhydroxysulfamethoxazole (N_4SOH), and sulfamethoxazole-N_1-glucuronide (Sgluc). H is hippuric acid.

Table 1
Retention times (t_R) and capacity factors (k') of sulfamethoxazole, its metabolites and conjugates

Compound	t_R (min)	k'	k' Ratio	
			+OH	+acetyl
t_0	2.20			
Hippuric acid	12.29	4.59		
SOH	13.67	5.21	0.56	
S-glucuronide	18.50	7.41		
N_4-SOH	20.61	8.36	0.71	1.60
N_4-OH	21.61	8.82		
S	22.60	9.27		
N_4	28.07	11.76	1.27	

HPLC conditions see under gradient HPLC analysis.
Abbreviations: sulfamethoxazole (S), N_4-acetylsulfamethoxazole (N_4), 5-methylhydroxysulfamethoxazole (SOH), N_4-hydroxysulfamethoxazole (N_4OH), N_4-methylhydroxysulfamethoxazole (N_4SOH), and sulfamethoxazole-N_1-glucuronide (Sgluc), H = hippuric acid.

glucuronidase enzymes were tested (all from Sigma, St. Louis, MO, USA):
A. 20 000 U/ml ß-glucuronidase type B1 (Bovine liver, Cat. No. G-0251) in phosphate buffer at pH 5.0.
B. 120 600 U/ml ß-glucuronidase type H2 (*Helix pomatia*, Cat. No. G-0876) in phosphate buffer at pH 5.0.
C. 100 000 U/ml ß-glucuronidase type LII (lyophilized powder from limpets *Patella vulgata*, Cat. No. G-8132) in phosphate buffer at pH 3.8.
D. 20 000 U/ml ß-glucuronidase type VIIA (*Escherichia coli*, Cat. No. G-7646) in phosphate buffer at pH 6.8.

Deconjugation reactions with 5 *M* HCl (urine–HCl, 1:1, v/v, 75°C, 1 h) were carried out.

2.10. Limits of quantitation

The limits of detection in water and quantitation of sulfamethoxazole and its metabolites in plasma and urine were determined at a signal-to-noise ratio of 3.

2.11. Standard solutions

Standard solutions of S and the metabolites were prepared as follows: S 9.58 mg/ml in DMF, N_4 9.59 mg/ml in DMF, N_4OH 2.62 mg/ml in methanol with 1 mg Vitamin C, N_4SOH 1.16 mg/ml in ethanol, Sgluc 3.0 mg/ml in water, SOH 0.43 mg/ml in water.

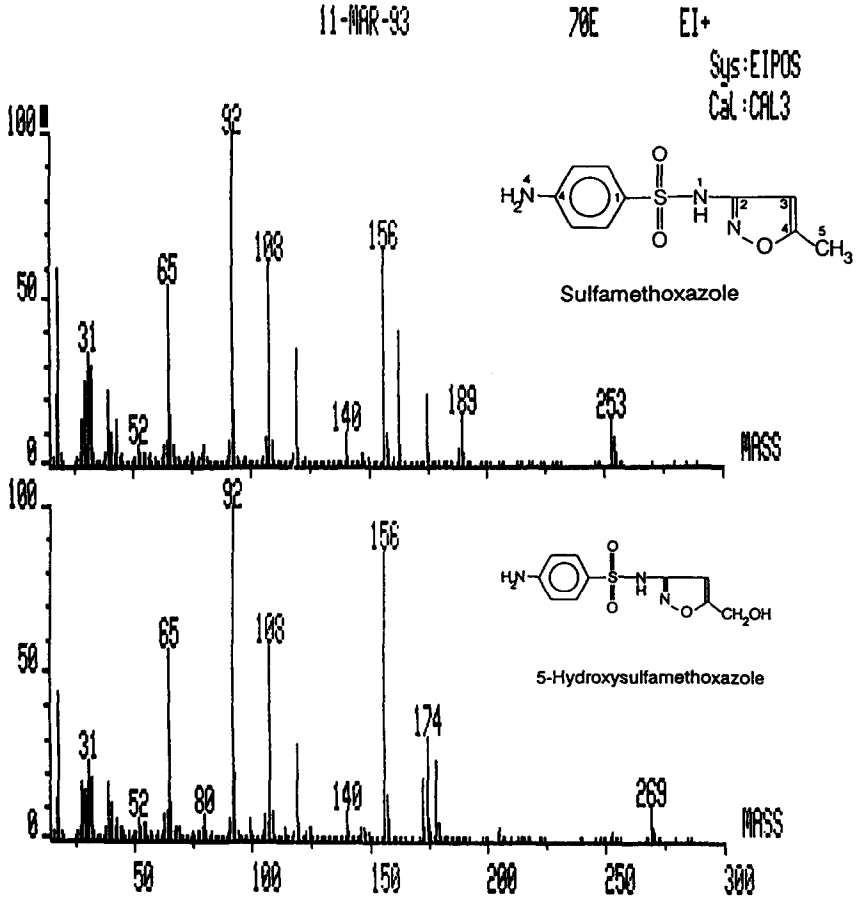

Fig. 3. Electron-impact mass spectra of sulfamethoxazole and its metabolite 5-methylhydroxysulfamethoxazole (Abscissa *m/z*; ordinate: Intensity %).

2.12. Recovery

A calibration curve of 4 concentrations (0.1–60 μg/ml) of S and its metabolites in 0.9% NaCl solution was compared with a calibration curve of the same concentrations in plasma. All samples were treated with acetonitrile 1:1 (v/v).

2.13. Pharmacokinetics

The pharmacokinetic parameters were calculated using the MediWare computer package [20].

3. Results

Identification: Metabolites of S were detected in a urine sample of a volunteer after oral intake of 800 mg of S (Fig. 2 and Table 1).

Three metabolites of S were isolated from the human urine sample: SOH, N_4SOH and Sgluc). The isolated compounds were identified as described below.

5-Hydroxysulfamethoxazole (SOH). Fig. 3 shows the EI mass spectra of S and SOH. The mass spectrum of SOH ($C_{10}H_{11}N_3O_4S$, M_r 269) reads: m/z, relative intensity; 269 [M^+] 9.5% ; 174, 30.1%; 156, 86.3%; 108, 60%; 92, 100%.

The mass spectrum of S ($C_{10}H_{11}N_3O_3S$, M_r 253) reads: m/z, relative intensity; 253 [M^+] 9.5% ; 156, 70.5%; 108, 65%; 92, 100%.

NMR spectrum of SOH in $CD_3OD/CDCl_3$ (1:8, v/v) reads: $\delta = 7.61$ ppm (2H,d,$H_a + H_{a'}$, $J_{ab} = 8.8$ Hz); 6.64 ppm (2H,d,$H_b + H_{b'}$, $J_{ab} = 8.8$ Hz); 6.33 ppm (1H,s,H_d); 4.56 ppm (2H,s,H_c).

The NMR spectrum of S in $CD_3OD/CDCl_3$ (1:1, v/v) reads: $\delta = 7.59$ ppm (2H,d,$H_a + H_{a'}$, $J_{ab} = 7.0$ Hz); 6.64 ppm (2H,d,$H_b + H_{b'}$, $J_{ab} = 7.0$ Hz); 6.08 ppm (1H,s,H_d); 2.31 ppm (3H,s,H_c).

N_4-Acetyl-5-methylhydroxysulfamethoxazole (N_4SOH). The methane chemical ionization mass spectrum of N_4SOH ($C_{12}H_{13}N_3O_5S$, M_r 311) showed m/z values of 296 [$MH^+ - CH_4$] 4.5%, 312 [$M + H^+$] 3.5% and 328 [$MH^+ + CH_4$] 0.12%; base peak 43, 100%.

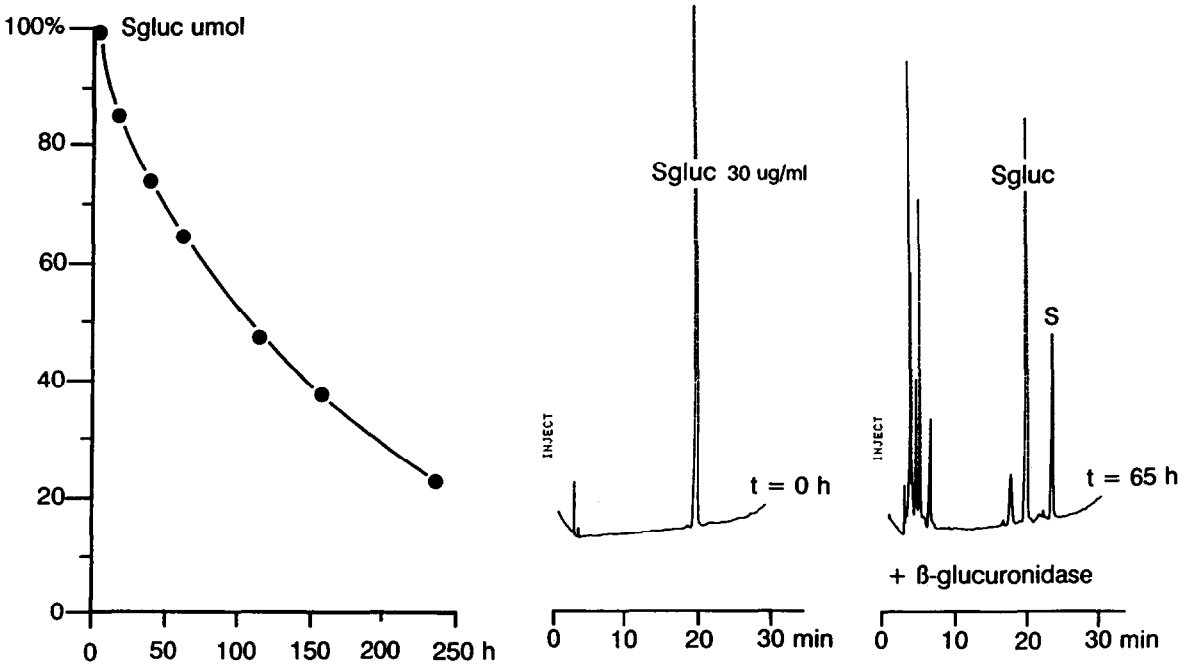

Fig. 4. Percent of sulfamethoxazole-N_1-glucuronide remaining with time of hydrolysis by ß-glucuronidase type LII (left panel) and chromatograms obtained at the start of the reaction ($t = 0$) (central panel) and at $t = 65$ h (right panel). Ordinate UV absorption at 271 nm (mV).

For N_4 ($C_{12}H_{13}N_3O_4S$, M_r 295), the mass spectrum showed the m/z values of 296 [M + H^+] 100% and 311 [$MH^+ + CH_4$] 4.2%.

NMR spectrum of N_4SOH in CD_3OD reads: $\delta = 7.82$ ppm (2H,d,$H_a + H_{a'}$, $J_{ab} = 8.8$ Hz); 7.71 ppm (2H,d,$H_b + H_{b'}$, $J_{ab} = 8.8$ Hz); 6.28 ppm (1H,s,H_d); 4.52 ppm (2H,s,H_c); 2.14 ppm (3H,s,H_j) (Fig. 4.).

The NMR spectrum of N_4 in $CD_3OD/CDCl_3$ (1:1, v/v) reads: $\delta = 7.80$ ppm (2H,d,$H_a + H_{a'}$, $J_{ab} = 8.8$ Hz); 7.69 ppm (2H,d,$H_b + H_{b'}$, $J_{ab} = 8.8$ Hz); 6.06 ppm (1H,s,H_d); 2.29 ppm (3H,s,H_c); 2.13 ppm (3H,s,H_j).

Hydrolysis: Acid hydrolysis of N_4SOH in 5 *M* HCl (1 h, 70°C) resulted in SOH according to HPLC analysis.

Sulfamethoxazole-N_1-glucuronide (Sgluc). The FAB mass spectrum of Sgluc ($C_{16}H_{19}N_3O_9S$, M_r 429) reads: m/z values 430 [$M^+ + H$] 5%, 308, 22%; 273, 30%; 176, 15%; 155, 100%. A mass fragment m/z 452 [$M^+ + 23$] 14% shows the presence of Na.

The NMR spectrum of Sgluc in $CD_3OD/CDCl_3$ (1:1, v/v) reads: $\delta = 7.52$ ppm (2H,d,$H_a + H_{a'}$, $J_{ab} = 8.8$ Hz); 6.61 ppm (2H,d,$H_b + H_{b'}$, $J_{ab} = 8.8$ Hz); 6.08 ppm (1H,s,H_d); 5.32 (1H,d,H_i, $J_{hi} = 9.2$ Hz); 3.91 ppm (1H,d,H_e, $J_{ef} = 9.4$ Hz), 3.54 ppm (1H,t,H_g, $J_{fg} = 9.0$ Hz, $J_{gh} = 9.0$ Hz), 3.47 ppm (1H,t,H_f, $J_{fg} = 9.0$ Hz, $J_{ef} = 9.4$ Hz), 3.21 ppm (1H,t,H_h, $J_{gh} = 9.0$ Hz, $J_{hi} = 9.2$ Hz), 2.39 ppm (3H,s,H_c).

The ^{13}C NMR spectrum of Sgluc, shown in Table 2, elucidates the presence of a ß-glucuropyranoyl group and an intact sulfamethoxazole moiety.

Elemental analysis of Sgluc. Proposed structure: $C_{16}H_{19}N_3SO_9$, M_r 429. Calculated percentages: C 44.75%, H 4.46%, N 9.79%, S 7.47%. Found percentages C 42.62%, H 4.29%, N 8.61% and S 6.51%.

The IR spectrum of Sgluc shows a carbonyl moiety at 1724 nm and sulfone absorption at 1594 and 1620 nm.

Table 2
Summary of the ^{13}C chemical shifts and assignments of sulfamethoxazole (S), sulfamethoxazole-N_1-glucuronide (Sgluc) and ß-D-glucuronopyranoic acid [24]

C	M	*n*	*m/z* (ppm)		
			S	Sgluc	ß-D-glucurono pyranoic acid
Aromatic ring					
C_1	s	1	157.5	156.8	
C_4	s	1	169.8	170.3	
$C_2 + C_6$	d	2	128.5	129.9	
$C_3 + C_5$	d	2	112.9	112.7	
Isoxazoyl ring					
$C_{2'}$	s	1	124.9	123.7	
$C_{3'}$	d	1	94.7	86.9	
$C_{4'}$	s	1	152.2	152.6	
$C_{5'}$	q	1	11.3	11.8	
Glucuronide ring					
$C_{1''}$	d	1		102.3	96.9
$C_{2''}$	d	1		76.4	76.3
$C_{3''}$	d	1		76.3	75.4
$C_{4''}$	d	1		70.6	74.7
$C_{5''}$	d	1		69.4	72.2
$C_{6''}$	s	1		170.9	173.8

TMS: tetramethylsilane (0 ppm).
M: multiplicity in ^{13}C (s = singlet, d = doublet, q = quartet; DEPT distortionless enhanced polarization transfer).
n: number of C atoms.

Hydrolysis of Sgluc with ß-glucuronidase (type LII) proceeded very slowly. After 2 weeks 50% was hydrolyzed (Fig. 4).

Table 1 shows the retention times, the capacity factors of parent drug and its metabolites and group contributions to the retention behaviour. Introduction of the hydroxyl group in the sulfamethoxazole isoxazoyl structure reduces the capacity factor by a factor of 0.56. Introduction of the acetyl group in the sulfamethoxazole structure increases the capacity factor by a factor of 1.27.

Fig. 2 and Fig. 5 show the chromatograms of a human plasma and urine sample respectively after oral administration of 800 mg S. The chromatograms show the presence of S and its metabolites. Table 3 shows the equations of the calibration curves of sulfamethoxazole and its metabolites in plasma and urine. Table 4 shows

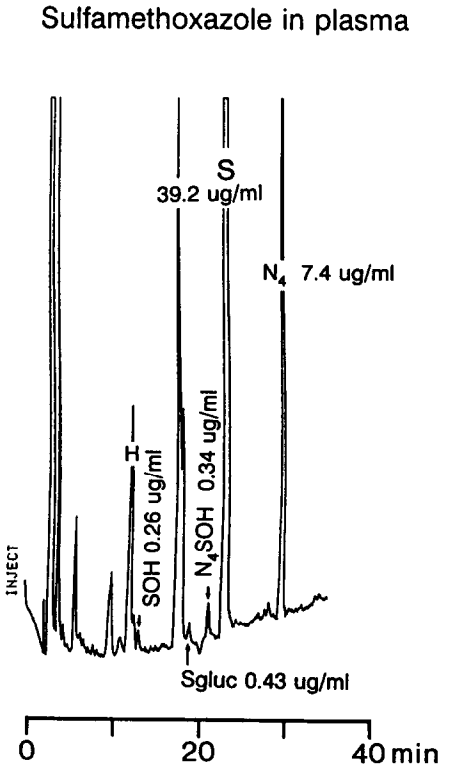

Fig. 5. Chromatogram of a plasma sample, from a healthy human subject obtained 1 h after administration of a single oral dose of 800 mg S, containing sulfamethoxazole (S) and the metabolites N_4-acetylsulfamethoxazole (N_4), 5-methylhydroxysulfamethoxazole (SOH), N_4-hydroxysulfamethoxazole (N_4OH), N_4-acetyl-5-methylhydroxysulfamethoxazole (N_4SOH), and sulfamethoxazole-N_1-glucuronide (Sgluc). H is hippuric acid.

Table 3
Calibration curves of sulfamethoxazole and its metabolites

	Concentration (μg/ml)	Curve	Correlation (r)
Plasma			
SOH	0.20–1.31	$y = 288x - 4.10$	0.9997
Sgluc	0.40–3.0	$y = 151x - 9.96$	0.9984
N_4SOH	0.20–1.16	$y = 342x + 4.71$	0.9996
N_4OH	n.d		
S	0.20–66.3	$y = 392x - 43.6$	0.9999
N_4	0.20–15.9	$y = 376x - 12.6$	0.9999
Urine			
SOH	3.5–39	$y = 87.9x - 19.0$	0.9993
Sgluc	4.1–75	$y = 46.1x - 17.8$	0.9992
N_4SOH	1.9–35	$y = 86.1x + 28.8$	0.9994
N_4OH	1.4–34	$y = 61.0x + 46.5$	0.9993
S	1.5–200	$y = 97.1x - 300$	0.9989
N_4	1.4–476	$y = 91.2x - 444$	0.9996

Peak height y (integration units) and concentration x (μg/ml). n.d. = not detected.

Table 4
Recovery and limits of detection and quantitation of sulfamethoxazole and its metabolites

Compound	Detection limit in water (μg/ml)	Quantitation limit (μg/ml)	
		Urine	Plasma
SOH	0.064	3.5	0.20
Sgluc	0.150	4.1	0.39
N_4SOH	0.060	1.9	0.13
N_4OH	0.120	1.4	0.18
S	0.035	1.5	0.10
N_4	0.035	1.4	0.10

Recovery of deproteinization in plasma ($n = 4$)

	Recovery (%)	C.V. (%)
S	99.6	5.3
SOH	96.4	6.6
N_4	100.6	3.9
N_4SOH	92.8	5.9
Sgluc	100.6	5.9

Detection limit in water; quantitation limit in the biological matrix.

the limits of detection in water and limits of quantitation in plasma and urine of S and its metabolites.

Samples of pH 5 and kept in the dark were stable in the autosampler of the HLPC during 24 h. Sgluc was stable for 9 days when kept at pH

Table 5
Intra-day coefficient of variation (%C.V.) of spiked sulfamethoxazole and its metabolites in human urine ($n = 5$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V.%)	Accuracy (%)
SOH	19.58	19.84	3.0	5.0
	39.15	40.23	2.0	2.8
Sgluc	37.38	37.53	5.1	0.4
	74.75	78.39	3.7	4.9
N_4SOH	17.33	17.04	3.4	1.7
	34.65	35.50	2.4	2.5
N_4OH	17.05	16.43	2.3	3.6
	34.10	35.46	2.0	4.0
S	1.99	2.06	8.9	3.5
	99.45	100.6	3.3	1.1
	198.9	200.2	3.2	0.66
N_4	4.76	5.17	4.0	8.6
	237.8	244.2	3.1	2.7
	475.5	475.8	5.2	0.063

Table 6
Inter-day coefficient of variation (%C.V.) of spiked sulfamethoxazole and its metabolites in human urine ($n = 7$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V.%)	Accuracy (%)
SOH	19.58	18.33	8.2	6.4
	39.15	38.51	3.4	1.6
Sgluc	37.38	37.06	3.1	0.9
	74.75	75.78	3.8	1.4
N_4SOH	17.33	18.54	4.2	7.0
	34.65	33.01	3.4	4.7
N_4OH	17.05	17.70	1.8	3.8
	34.10	35.14	1.7	3.1
S	1.99	1.97	6.5	1.0
	99.45	100.2	3.5	0.7
	198.9	196.6	3.2	1.2
N_4	4.76	4.79	5.6	0.7
	237.8	237.6	2.3	0.1
	475.5	465.3	2.8	2.2

3.8, pH 5.0 and pH 6.8. Sgluc could be hydrolyzed slowly by ß-glucuronidase LII as shown in Fig. 4.

Table 5 and 6 show respectively the intra- and inter-day variations of S and its metabolites in urine.

Table 7 and 8 show respectively the intra- and inter-day variations of S and its metabolites in plasma.

Fig. 6 shows the plasma concentration–time curves of S and the metabolites and Fig. 7 the renal excretion rate–time profiles of S and its metabolites after a single oral administration of 800 mg of S in one male volunteer.

Table 7
Intra-day coefficient of variation (%C.V.) of spiked sulfamethoxazole and its metabolites in human plasma ($n = 5$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V.%)	Accuracy (%)
SOH	0.52	0.58	2.5	10.5
	1.04	1.12	1.5	7.7
Sgluc	1.20	1.29	5.1	7.5
	2.39	2.67	2.0	11.7
N_4SOH	0.46	0.52	12.3	12.6
	0.92	0.94	9.9	1.7
S	5.30	5.46	1.3	2.9
	26.5	28.0	1.1	5.6
	53.0	54.2	1.4	2.2
N_4	1.27	1.31	2.0	3.3
	6.34	6.83	1.1	7.7
	12.68	13.4	1.3	5.7

Table 8
Inter-day coefficient of variation (%C.V.) of spiked sulfamethoxazole and its metabolites in human plasma ($n = 7$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V.%)	Accuracy (%)
SOH	0.52	0.56	3.0	7.3
	1.04	1.13	3.5	8.7
Sgluc	1.20	1.28	4.4	6.7
	2.39	2.54	1.9	6.3
N_4SOH	0.46	0.48	13.0	2.8
	0.92	0.92	9.5	0.9
S	5.30	5.42	2.5	2.2
	26.5	28.3	3.5	6.7
	53.0	54.9	2.1	3.5
N_4	1.27	1.28	1.9	1.0
	6.34	6.75	2.9	6.5
	12.68	13.2	2.0	4.1

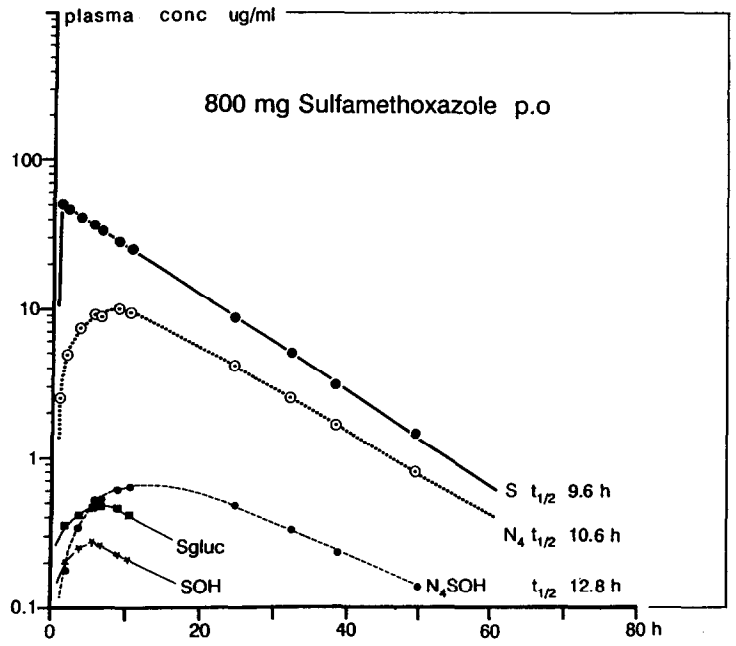

Fig. 6. Plasma concentration–time curves of sulfamethoxazole (S), sulfamethoxazole-N_1-glucuronide (Sgluc), 5-hydroxysulfamethoxazole (SOH), N_4-acetylsulfamethoxazole (N_4) and N_4-acetyl-5-methylhydroxysulfamethoxazole (N_4SOH) in a healthy human subject after a single oral dose of 800 mg of sulfamethoxazole.

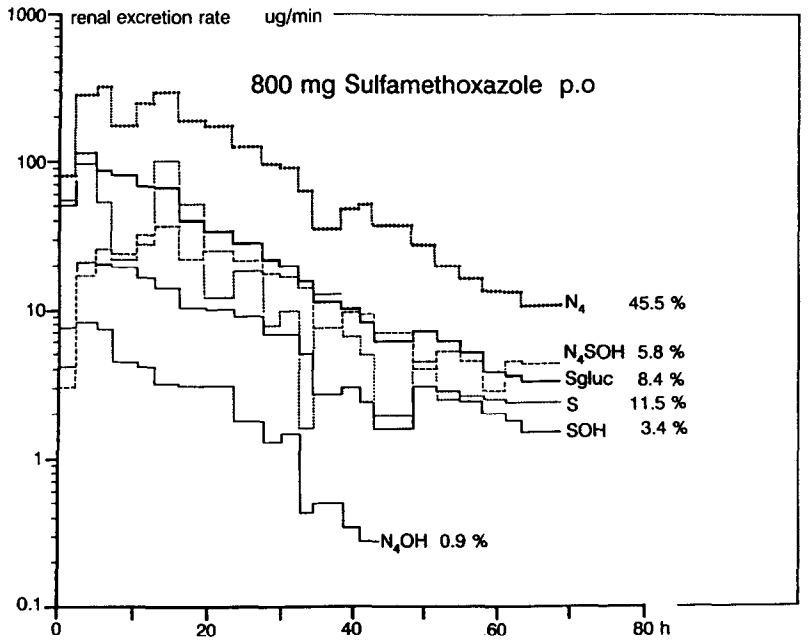

Fig. 7. Renal excretion rate–time profiles and cumulative excreted amounts (% mol dose) of sulfamethoxazole (S), sulfamethoxazole-N_1-glucuronide (Sgluc), 5-hydroxysulfamethoxazole (SOH), N_4-acetylsulfamethoxazole (N_4), N_4-acetyl-5-methylhydroxysulfamethoxazole (N_4SOH), and N_4-hydroxylsulfamethoxazole (N_4OH) in a healthy human subject after a single oral dose of 800 mg of sulfamethoxazole.

Table 9
Some pharmacokinetic parameters of sulfamethoxazole, its metabolites and conjugates from a healthy subject after a single oral administration of 800 mg sulfamethoxazole

Parameter	Value
Subject	A
Gender	male
Body weight (kg)	85
Dose (mg)	800
C_{max} (μg/ml)	51.4
t_{max} (h)	0.5
$t_{1/2absorption}$ (h)	0.05
$t_{1/2}$ (h)	
S	9.6
N_4	10.6
N_4SOH	12.8
MRT S (h)	14.0
Total oral body clearance (l/h)	0.87
Volume of distribution (l)	12.1
Percentage of the dose excreted (% mol)	
S	15.3
SOH	3.4
N_4	45.5
N_4OH	0.9
N_4SOH	5.8
Sgluc	8.4
Total	79.3

Urinary pH kept acidic (between pH 5.0 and 6.0).

Table 9 summarizes some pharmacokinetic parameters of S calculated from the plasma and urine concentrations of parent drug and metabolites in the pilot experiment after a single oral administration of 800 mg S to the human volunteer.

4. Discussion

The metabolite SOH was previously identified by Rieder [2,21], Ueda et al. [6–9] and Woolley et al. [10]. Vree et al. isolated this metabolite by preparative TLC from dog urine. Dog's urine is relatively easy to analyse, because they are unable to acetylate sulfonamides [4,5,22].

N_4SOH was detected in urine by Rieder [2,21], by Woolley et al. in renal calculi [10], and was observed in plasma of *pneumocystis carinii* patients by Vree et al. [23]. This compound could easily be recognized because acidic hydrolysis of the isolated product resulted in SOH. Other reported conjugates of S were the N_4-glycolylsulfamethoxazole, sulfamethoxazole-N_1-glucuronide and the N-glucuronide at the isoxazoyl ring [2,3,21]. In all studies, the presence of an N-glucuronide was reported. The N-glucuronides can be hydrolyzed by ß-glucuronidases, as reported for the methoxysulfonamides sulfadimethoxine [15,16], sulfa-6-monomethoxine [17], sulfamethomidine [18] and sulfaphenazole [19]. Of the four ß-glucuronidase systems tested, systems C and D were the most active ones for N-deglucuronidation of these methoxysulfonamides [15–19].

Sgluc was only partly vulnerable to enzymatic hydrolysis by the ß-glucuronidases tested. Hydrolysis proceeded very slowly and would have been completed after approximately one month of reaction time, but after 2 weeks, the most active of the tested ß-glucuronidases (LII, system C) had lost its activity (Fig. 4). This enzyme was the most active in hydrolysing the N_1-glucuronides of the methoxysulfonamides [15–19]. Enzyme H2 (B) produced a similar result.

The ^{13}C NMR spectrum showed a ring structure with 5 carbon atoms, resembling the spectrum of the reference compound glycuronopyranoic acid [24]. The glucuronide group was not attached at the N_4 or the N atom of the isoxazoyl group, as the two unsaturated bonds were still present (Fig. 1).

In conclusion, the hydroxy metabolites of sulfamethoxazole, 5-methylhydroxysulfamethoxazole and N_4-acetyl-5-methylhydroxysulfamethoxazole, were isolated and structure analysis could be compared with those reported in the literature. The N-glucuronide of sulfamethoxazole that was isolated was the N_1-glucuronide, according to the ^{13}C NMR spectrum obtained.

The analysis of S and metabolites in human plasma and urine samples can be easily performed with gradient HPLC with UV detection. In plasma only the parent drug and N_4-acetyl metabolite were detected at higher concentrations, while the metabolites were present as traces. In urine parent drug and the metabolites were quantitated. The method is in current use

in pharmacokinetic studies in healthy subjects and HIV positive patients on prophylactic treatment with sulfamethoxazole/trimethoprim.

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