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Isolation, identification and determination of sulfadiazine and its hydroxy metabolites and conjugates from man and Rhesus monkey by high-performance liquid chromatography

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Abstract

The following metabolites of sulfadiazine (S) were isolated from monkey urine by preparative HPLC: 5-hydroxysulfadiazine (5OH), 4-hydroxysulfadiazine (4OH) and the glucuronide (5OHgluc) and sulfate conjugate of 5OH (5OHsulf). The compounds were identified by NMR, mass and infrared spectrometry and hydrolysis by β-glucuronidase. The analysis of S, the hydroxymetabolites (4OH, 5OH) and conjugates N_4-acetylsulfadiazine (N_4), 5OHgluc and 5OHsulf in human and monkey plasma and urine samples was performed using reversed-phase gradient HPLC with UV detection. In plasma, S and N_4 could be detected in high concentrations, whereas the other metabolites were present in only minute concentrations. In urine, S, the metabolites and conjugates were present. The limit of quantification of the compounds in plasma varies between 0.2 and 0.6 μg/ml (S 0.31, N_4 0.40, 4OH 0.20, 5OH 0.37, 5OHgluc 0.33 and 5OHsulf 0.57 μg/ml). In urine it varies between 0.6 and 1.1 μg/ml (S 0.75, N_4 0.80, 4OH 0.60, 5OH 0.80, 5OHgluc 0.80 and 5OHsulf 1.1 μg/ml). The method was applied to studies with healthy human subjects and Rhesus monkeys. The metabolites 5OH, 5OHgluc and 5OHsulf were present in Rhesus monkey and not in man. Preliminary results of studies of metabolism and pharmacokinetics in Rhesus monkey and man are presented.

1. Introduction

Sulfadiazine is used for the prophylaxis and treatment of toxoplasmosis gondii infection and bacterial infections, e.g., due to *Pneumocystis carinii* [1,2]. The known metabolism of sulfadiazine (benzenesulfonamide-4-amino-N-pyrimidine; CAS No. 68-35-9; S) involves acetylation and oxidation at the N_4-nitrogen atom, leading to N_4-acetylsulfadiazine (N_4) and N_4-hydroxysulfadiazine (N_4OH). The hydroxylamines of sulfonamides are thought to be responsible for the occurrence of side-effects of long-term prophylactic treatment of *Pneumocystis carinii*

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pneumonia in HIV-positive patients [1]. Hydroxylation also takes place at the C_4-carbon of the pyrimidine ring, leading to 4-hydroxysulfadiazine (4OH), and at the C_5-carbon, leading to 5-hydroxysulfadiazine (5OH). The hydroxy metabolites can be conjugated with glucuronic acid and sulfate [3–6]. This metabolic picture of sulfadiazine, as shown in Fig. 1, is obtained by means of TLC with detection reagents and isocratic HPLC analysis [3–5]. The metabolism of sulfonamides is species dependent; acetylation is the main pathway in man, but non-existent in dog, and oxidation mainly occurs in turtle, cow, horse, etc. [5,6].

Recently, we explored the pharmacokinetics of sulfadiazine in Rhesus monkeys (*Macaca mulatta*) and human volunteers by gradient HPLC and observed novel conjugates of the hydroxysulfadiazines. The aims of this investigation were (a) to isolate hydroxy metabolites with their conjugates of sulfadiazine from monkey urine, (b) to develop a gradient HPLC method for the determination of sulfadiazine, its hydroxy metabolites and novel conjugates and (c) to obtain preliminary results of studies of the metabolism and pharmacokinetics of S in Rhesus monkey and humans.

2. Experimental

2.1. Chemicals

S and N_4 were obtained from Astra (Södertälje, Sweden). The metabolites 4OH and 5OH, were isolated from monkey urine and were pure

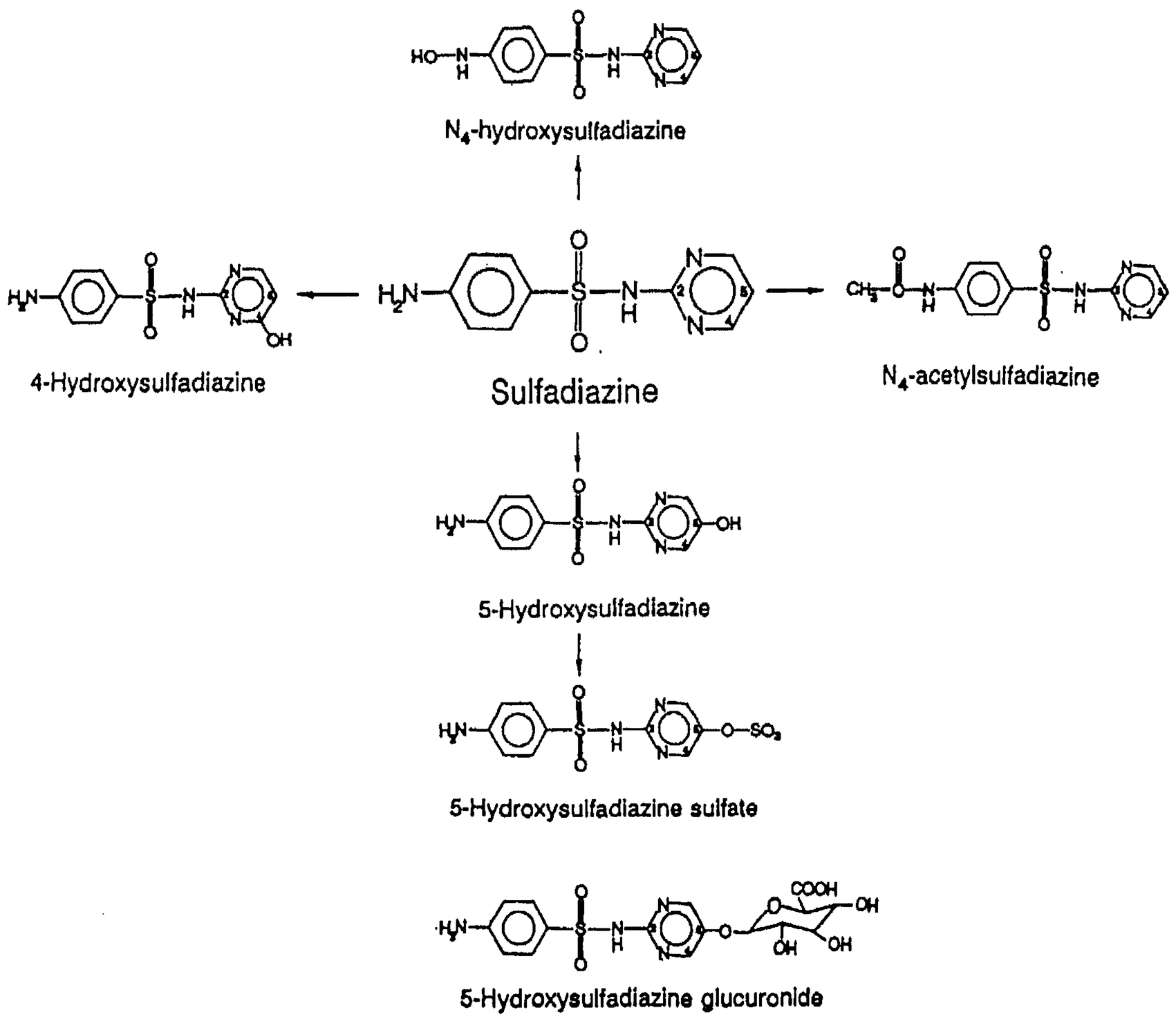

Fig. 1. Structures of sulfadiazine and its metabolites.

by HPLC. All reagents were of analytical-reagent grade from Merck (Darmstadt, Germany). Amberlite XAD-2 absorption resin and Celite 545 were obtained from Fluka (Perstorp Analytical, Oud Beyerland, Netherlands).

2.2. Subject and monkeys

A single oral dose of 500 mg S was administered to one healthy human subject (male, 35 years old, 85 kg, slow acetylator of sulfonamides) with 100 ml of tap water after an overnight fast. The study had the approval of the hospital ethics committee and informed consent was obtained from the volunteer.

Rhesus monkeys were obtained from the Central Animal Laboratory, University of Nijmegen and the Laboratory Animals Breeding Experimental Farm of Shunde Guangdong (Beijing, China). One Rhesus monkey (female, 5 years old and weighing about 5 kg) was housed in a metabolic cage and fed orally with 50 mg/kg of sulfadiazine and serial plasma and urine samples were collected for 2 days. Three Rhesus monkeys (female, 4–5 years old and weighing about 5 kg) obtained from the Central Animal Laboratory, University of Nijmegen, were housed in a metabolic cage and fed orally for 5 days with 50 mg/kg of sulfadiazine and all urine was collected for 6 days. The study had the approval of the ethics committee of the Animal Laboratory.

2.3. Sampling

Blood samples of 2 ml were drawn from the volunteer 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 from 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.

Blood samples of 2 ml were drawn from the monkey by puncture of the vena mediana and collected in heparinized Eppendorf vials at 0.1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 and 48 h after administration. Urine was collected at the same time intervals. 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 (200 μl) were deproteinized with acetonitrile (1:1, v/v), centrifuged at 3000 *g* for 5 min and 50 μl of the supernatant were injected on to the column.

Urine samples were centrifuged at 3000 *g*, the supernatant was diluted 1:5 with 0.6% acetic acid (AcOH) and 50 μl were injected on to the column.

2.5. Gradient HPLC determination of S and metabolites

The HPLC system consisted of a Marathon autosampler (Separations, Hendrik Ido Ambacht, Netherlands), a Spectra System P 4000 quaternary gradient pump, a Spectroflow 757 UV detector (Separations) and a Hitachi D2500 integrator (Merck, Amsterdam, Netherlands).

The column was a Dynamax 60 Å, 8 μm, 150 mm × 4.6 mm I.D. (Meyvis, Bergen op Zoom, Netherlands) with a guard column (15 mm × 4.6 mm I.D.), packed with 8-μm C_8 reversed-phase material (Meyvis).

The mobile phase was a mixture of acetonitrile and a mixture of 0.5% AcOH plus 0.5 g/l of $AcONH_4$ (pH 3.3). At time $t = 0$, the mobile phase consisted of 0% acetonitrile and 100% (v/v) AcOH–$AcONH_4$. During the following 5 min, the mobile phase was changed linearly until it attained a composition of 10% acetonitrile and 90% AcOH–$AcONH_4$. From 5 to 18 min it was changed to 18% acetonitrile and 82% AcOH–$AcONH_4$. At 18 min, the mobile phase was changed within 1 min to the initial composition, followed by equilibration for 4 min. The flow-rate was 1.5 ml/min. The effluent was monitored at 273 nm.

2.6. Isolation of the metabolites from urine

Column chromatography

Two litres of monkey urine (48 h, pH 9.3) was adjusted to pH 5.0 and allowed to stand overnight for precipitation of endogenous urates. Celite 545 (40 g) was added to the urine and the suspension was filtered. The pH of the filtrate was adjusted to 5.0.

A preparative column (40 cm × 6 cm I.D., packed with 1 kg of XAD-2) was rinsed with 2 l of methanol, 2 l of water and 1 l of 0.2 *M* KH_2PO_4 buffer (pH 5.0). Thereafter, 1 l of urine was passed through the column, followed by 2 l of the 100-fold diluted phosphate buffer (pH 5.0). The column was dried by air suction for 10 min.

Elution of the column was carried out with methanol–water according to the following scheme: first, 500 ml of CH_3OH–H_2O (20:80, v/v) was passed through the column, followed by eight fractions of 250 ml with the methanol concentration increased in steps of 10% to 100% CH_3OH.

Effluent fractions 1–2 (20:80–30:70, v/v) contained 5-hydroxysulfadiazine glucuronide. Effluent fractions 2–4 (30:70–50:50, v/v) contained 5-hydroxysulfadiazine sulfate and fractions 5–7 (60:40–80:20, v/v) contained the hydroxy metabolites 4- and 5-hydroxysulfadiazine.

This procedure was repeated with the second litre of urine on a regenerated column, and fractions 2–4 and 5–7 from both procedures were combined. Methanol was evaporated under reduced pressure. The combined fraction 5–7 was placed on the column and the gradient elution was repeated. Fractions 40:60 and 50:50 contained the sulfate conjugate and fractions 70:30 and 80:20 both contained the unconjugated hydroxysulfadiazines. Most of the yellow-brown urine colour 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 containing the hydroxysulfadiazines from the preparative column was concentrated to 100 ml by evaporation under reduced pressure (Rotavapor), and acidifed with 5 *M* HCl to pH 2.0 in order to prevent precipitation of the hydroxysulfadiazines.

The preparative HPLC system consisted of a Model 302 sample pump (Gilson, Meyvis, Bergen op Zoom, Netherlands), two Gilson Model 305 gradient pumps, a Model 811 B dynamic mixer, a Kratos Model 757 UV detector (Separations), a Model 2211 Superrac fraction collector (LKB, Woerden, Netherlands) and a BD7 recorder (Kipp & Zonen, Delft, Netherlands). The column was a C_8, 8-μm particle size, 250 mm × 10 mm I.D., Rainin Dynamax 60 Å column (Meyvis). The mobile phase consisted of 1% acetic acid in water–acetonitrile (85:15, v/v). The flow-rate was 4.7 ml/min and absorbance 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). Two crude samples containing 4OH- and 5OHsulfadiazine, respectively, were collected.

2.7. Final purification by preparative HPLC

4-Hydroxysulfadiazine (4OH)

For isolation, the mobile phase was acetonitrile–1% AcOH (18:82, v/v). The collected sample was concentrated to 20 ml under reduced pressure with the Rotavapor, then further reduced in volume to 0.5 ml in a smaller flask. The final volume of 0.5 ml was transferred to a small tube for freeze-drying.

5-Hydroxysulfadiazine (5OH)

For isolation, the mobile phase was tetrahydrofuran (THF)–1% AcOH (12:88, v/v). The collected sample was concentrated to 20 ml under reduced pressure with the Rotavapor, then further reduced in volume to 0.5 ml in a smaller flask. The final volume of 0.5 ml was transferred to a small tube for freeze-drying.

5-Hydroxysulfadiazine sulfate (5OHsulf)

For the first isolation, the mobile phase was acetonitrile–1% AcOH, starting at 8:92 (v/v) and changed linearly in 18 min to 26:74.

For the second isolation, the mobile phase was methanol–water (4:6, v/v).

For the third isolation, the mobile phase was acetonitrile–1% AcOH, starting at 12:88 (v/v) for 10.5 min, then changed in 1 min to 26:74.

For the final isolation, the mobile phase was acetonitrile–water (4:6, v/v).

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

5-Hydroxysulfadiazine glucuronide (5OHgluc)

For the first isolation, the mobile phase was acetonitrile–1% AcOH, starting at 10:90 (v/v) and changed linearly in 16 min to 26:74.

For the second isolation, the mobile phase was THF–1% AcOH (12:88, v/v).

For the third isolation, the mobile phase was methanol–1% AcOH starting at 10:90 (v/v) and changed linearly in 15 min to 25:75.

The collected sample was concentrated to dryness under reduced pressure with the Rotavapor (43 mg).

2.8. Identification of the metabolites

Mass spectrometry

A VG 7070E double-focusing mass spectrometer was used (Fisons Instruments, Weesp, Netherlands). Electron impact (EI) ionization (70 eV) and fast atom bombardment (FAB) (matrix nitrobenzyl alcohol, xenon 8 keV, acceleration voltage 6–7 kV) were performed.

Nuclear magnetic resonance spectrometry

^{1}H NMR spectra were recorded on a Bruker AM 400 spectrometer (400 MHz, Fourier transform (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 and deuterochloroform were used as solvents.

Infrared spectrometry

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

Melting point

Melting points were recorded with a melting point apparatus (Büchi, Flawil, Switzerland).

2.9. Deconjugation

Deconjugation reactions with β-glucuronidase [urine–β glucuronidase–buffer (1:1:8, v/v/v), 9 days, 37°C] were carried out. Four different β-glucuronidase enzymes were tested (all from Sigma, St. Louis, MO, USA): (A) 20 000 U/ml β-glucuronidase type B1 (bovine liver, Cat. No. G-0251) in phosphate buffer at pH 5.0; (B) 120 600 U/ml β-glucuronidase type H2 (*Helix pomatia*, Cat. No. G-0876) in phosphate buffer at pH 5.0; (C) 100 000 U/ml β-glucuronidase type LII (lyophilized powder from limpets *Patella vulgata*, Cat. No. G-8132) in phosphate buffer at pH 3.8; and (D) 20 000 U/ml β-glucuronidase type VIIA (*Escherichia coli*, Cat. No. G-7646) in phosphate buffer at pH 6.8.

Deconjugation with arylsulfatase was carried out with 19 U/ml sulfatase VI (*Aerobacter aerogenes*; Sigma, Cat. No. S-1629) in Tris solution of pH 7.1.

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

2.10. Limits of quantification

The limits of detection in water and determination of sulfadiazine 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 metabolites were prepared as follows: S 10.9 mg/ml in dimethylformamide (DMF), N_4 3.55 mg/ml in DMF, 4OH 1.16 mg/ml in DMF–0.1 *M* HCl (1:1, v/v), 5OH 1.78 mg/ml in 0.1 *M* HCl, 5OHgluc 1.78 mg/ml in water and 5OHsulf 2.0 mg/ml in water.

2.12. Recovery

A calibration graph for four concentrations (0.5–60 μg/ml) of S and its metabolites in 0.9% NaCl solution was compared with a calibration graph for 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 [7].

3. Results

3.1. Identification

Metabolites of S were detected (Fig. 1 and Table 1) in a urine sample from a human volunteer after oral intake of 500 mg of S and in urine from a Rhesus monkey treated with 50 mg/kg of S orally. Four metabolites of S were isolated from the monkey urine sample: 4OH, 5OH, 5OHgluc and 5OHsulf. The isolated compounds were identified as follows.

4-Hydroxysulfadiazine

The EI mass spectrum of 4-hydroxysulfadiazine ($C_{10}H_{10}N_4O_3S$, M_r 266) shows *m/z*, relative intensity, as follows: 267 [$M^+ + 1$], 12.1%; 266 [M^+], 100%; 201, 17.9%; 202, 23.2%; 109, 12.3%; 108, 44.3%, 93, 11.6%; 92, 79.5%; 28, 70.5%.

The EI mass spectrum of sulfadiazine ($C_{10}H_{10}N_4O_2S$, M_r 250) shows *m/z*, relative intensity, as follows: 251 [$M^+ + 1$], 0.14%; 250 [M^+], 0.06%; 186, 100%; 109, 11.9%; 108, 19.6%; 93, 37.9%; 92, 51.6%.

Table 1
Retention times and capacity factors of sulfadiazine and its metabolites and conjugates

Compound	t_R (min)	k'
t_0	1.80	
5OHgluc	6.99	2.88
4OH	9.85	4.47
Hippuric acid	11.35	5.31
5OH	12.00	5.67
5OHsulf	12.70	6.06
S	12.70	6.06
N_4	15.56	7.64

Introduction of a hydroxyl group in the sulfadiazine structure at the 4-position reduces the capacity factor by a factor of 0.74, whereas at the 5-position the reduction factor is 0.94. Introduction of an acetyl group in the sulfadiazine structure increases the capacity factor by a factor of 1.26. Introduction of a glucuronide group in 5OH reduces the capacity factor by a factor of 0.51, while introduction of a sulphate group has a smaller effect, with a reduction factor of 0.89. Hippuric acid is an endogenous compound present in urine.

The NMR spectrum in CD_3OD–$CDCl_3$ (3:1, v/v) is $\delta = 7.48$ ppm (2H, d, $H_a + H_{a'}$, $J_{ab} = 8.4$ Hz); 7.48 ppm (1H, d, H_c, $J_{cc'} = 7.6$ Hz); 6.57 ppm (2H, d, $H_b + H_{b'}$, $J_{ab} = 8.8$ Hz); 5.90 ppm (2H, s, NH_2); 5.70 ppm (1H, d, $H_{c'}$, $J_{cc'} = 7.6$ Hz); 3.16 ppm (1H, d, OH).

The melting point is 269–270°C with decomposition. The IR spectrum shows the sulfone moiety at 1600 and 1630 nm.

5-Hydroxysulfadiazine

The EI mass spectrum of 5-hydroxysulfadiazine ($C_{10}H_{10}N_4O_3S$, M_r 266) shows *m/z*, relative intensity, as follows: 267 [$M^+ + 1$], 0.65%; 266 [M^+], 0.62%; 203, 13.7%; 202, 100%; 201, 80.9%; 156, 14.3%, 109, 3.5%; 108, 45.2%; 93, 11.6%; 92, 94.2%; 65, 65.3%.

The positive-ion FAB mass spectrum shows *m/z*, relative intensity, as follows: 267 [$M + H^+$], 100%; 289 [$M^+ + Na$], 25%; 533 [$2M + H^+$], 4%; 555 [$2M + Na^+$], 4%.

The NMR spectrum in CD_3OD–$CDCl_3$ (3:1, v/v) is $\delta = 8.02$ ppm (2H, s, $H_c + H_d$); 7.69 ppm (2H, d, $H_a + H_{a'}$, $J_{ab} = 8.8$ Hz); 6.62 ppm (2H, d, $H_b + H_{b'}$, $J_{ab} = 8.8$ Hz).

The melting point is 182–183°C with decompo-

sition. The IR spectrum shows the sulfone moiety at 1600 and 1630 nm.

The NMR spectrum of sulfadiazine in CD_3OD–$CDCl_3$ (3:1, v/v) is $\delta = 8.39$ ppm (2H, d, $H_c + H_{c'}$, $J_{cd} = 4.8$ Hz); 7.76 ppm (2H, d, $H_a + H_{a'}$, $J_{ab} = 7.0$ Hz); 6.88 ppm (1H, t, H_d, $J_{cd} = J_{c'd} = 4.8$ Hz); 6.62 ppm (2H, d, $H_b + H_{b'}$, $J_{ab} = 7.0$ Hz). The melting point is 252–256°C.

5-Hydroxysulfadiazine sulfate

The positive-ion FAB mass spectrum of 5-hydroxysulfadiazine sulfate ($C_{10}H_{10}N_4O_6S_2$, M_r 345) shows m/z, relative intensity, as follows: 347, 5%; 345, 4%; 369, 7% [$M^+ + Na$].

The NMR spectrum in CD_3OD is $\delta = 8.28$ ppm (2H, s, $H_c + H_{c'}$); 7.63 ppm (2H, d, $H_a + H_{a'}$, $J_{ab} = 8.7$ Hz); 6.65 ppm (2H, d, $H_b + H_{b'}$, $J_{ab} = 8.7$ Hz).

Acid hydrolysis in 5 *M* HCl at 50°C for 2 h resulted in 5-hydroxysulfadiazine. Treatment of the isolated compound with arylsulfatase VI resulted in 5-hydroxysulfadiazine.

The IR spectrum shows the sulfone moiety at 1640 nm. The melting point is 169°C with decomposition.

5-Hydroxysulfadiazine-O-glucuronide

The positive-ion FAB mass spectrum shows m/z, relative intensity, as follows: 443 [$M + H^+$], 20%; 465 [$M + Na^+$], 12%; base peak 273, 100%.

The NMR spectrum in CD_3OD at 40°C is $\delta = 8.30$ ppm (2H, s, $H_c + H_{c'}$); 7.71 ppm (2H, d, $H_a + H_{a'}$, $J_{ab} = 8.8$ Hz); 6.63 ppm (2H, d, $H_b + H_{b'}$, $J_{ab} = 8.8$ Hz); 4.86 ppm (1H, m, H_1); 3.94 ppm (1H, d, H_5, $J_{4,5} = 10$ Hz); 3.59 ppm (1H, m, H_4); 3.46 ppm (2H, m, $H_2 + H_3$).

Acid hydrolysis in 5 *M* HCl resulted in 5-hydroxysulfadiazine. Treatment of the isolated compound with β-glucuronidase (system C) for 1 h at 37°C resulted in 5-hydroxysulfadiazine. The melting point is 167°C with decomposition; there is a phase and colour transition at 104–105°C from yellow to rose-red.

The IR spectrum shows the sulfone moiety at 1600 and 1630 nm, the glucuronide carbonyl moiety at 1730 nm and a broad maximum 3200–3450 nm of the glucuronyl group.

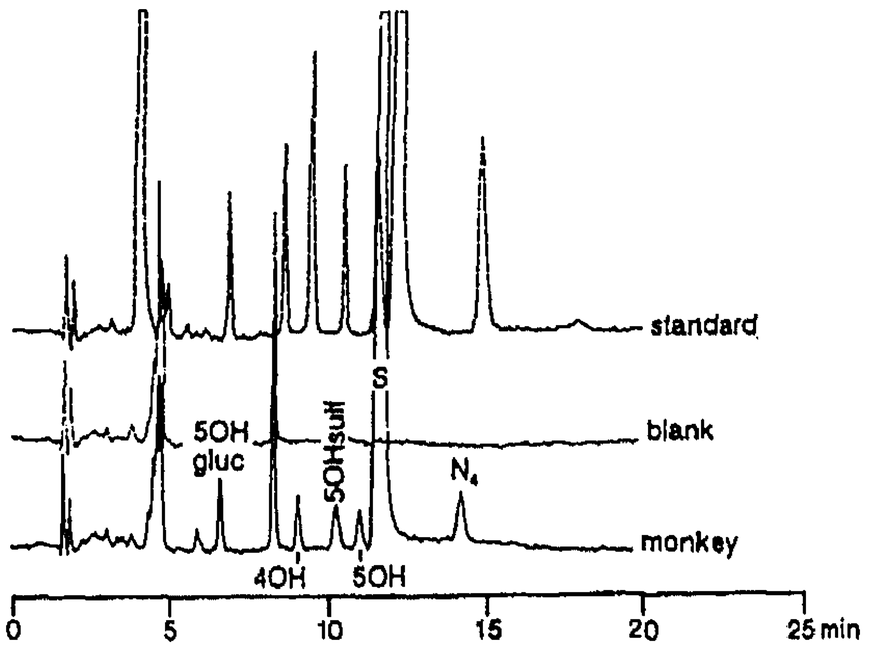

Fig. 2. Chromatograms of Rhesus monkey plasma containing sulfadiazine (S) and its metabolites, a blank monkey plasma and the isolated reference compounds. The concentrations of the compounds in the standard are S 52.6, 4OH 5.6, 5OH 8.6, 5OHgluc 5.7, 5OHsulf 7.1 and N_4 9.0 μg/ml.

3.2. Other data

Table 1 shows the retention times and the capacity factors of parent drug and its metabolites.

Fig. 2 shows the chromatograms for monkey plasma with blank and standards and Fig. 3

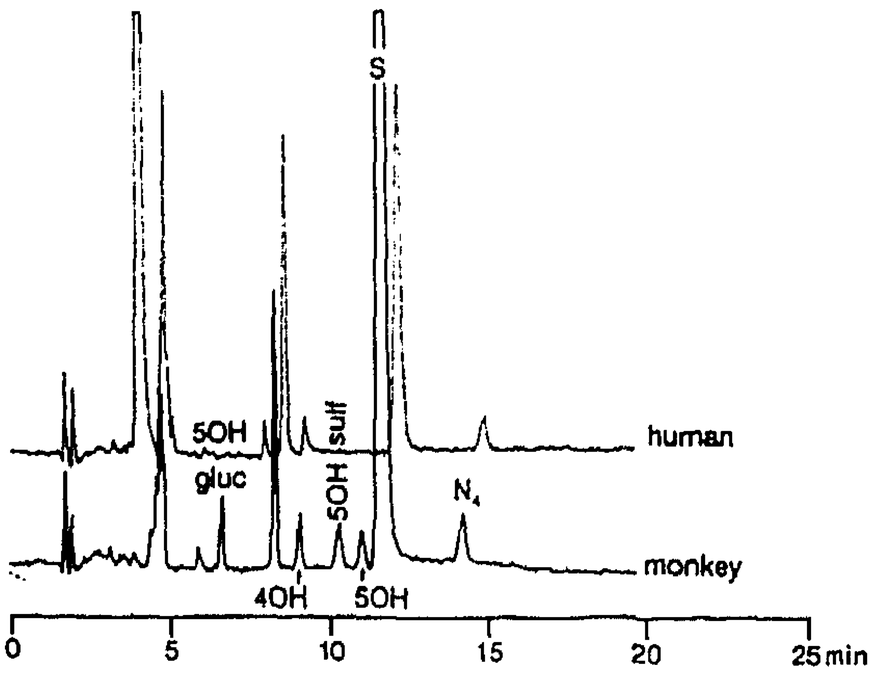

Fig. 3. Chromatograms of a Rhesus monkey and human plasma sample containing sulfadiazine (S) and its metabolites. 5-Hydroxysulfadiazine with its conjugates is not present in human plasma. The concentrations of the compounds in the human plasma sample are S 12.6, 4OH 0.6 and N_4 1.5 μg/ml. The concentrations of the compounds in the monkey plasma sample are S 57.7, 4OH 1.1, 5OH 1.6, 5OHgluc 2.3, 5OHsulf 2.1 and N_4 2.2 μg/ml.

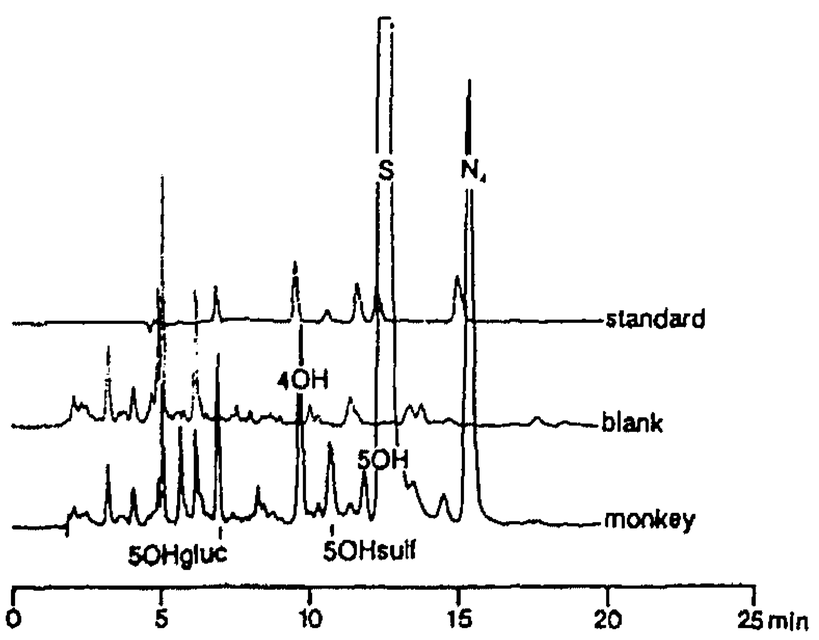

Fig. 4. Chromatograms of Rhesus monkey urine containing sulfadiazine (S) and its metabolites, a blank monkey plasma and the isolated reference compounds. The concentrations of the compounds in the urine are S 653, 4OH 32.4, 5OH 10.2, 5OHgluc 25.7, 5OHsulf 13.4 and N_4 85.5 μg/ml.

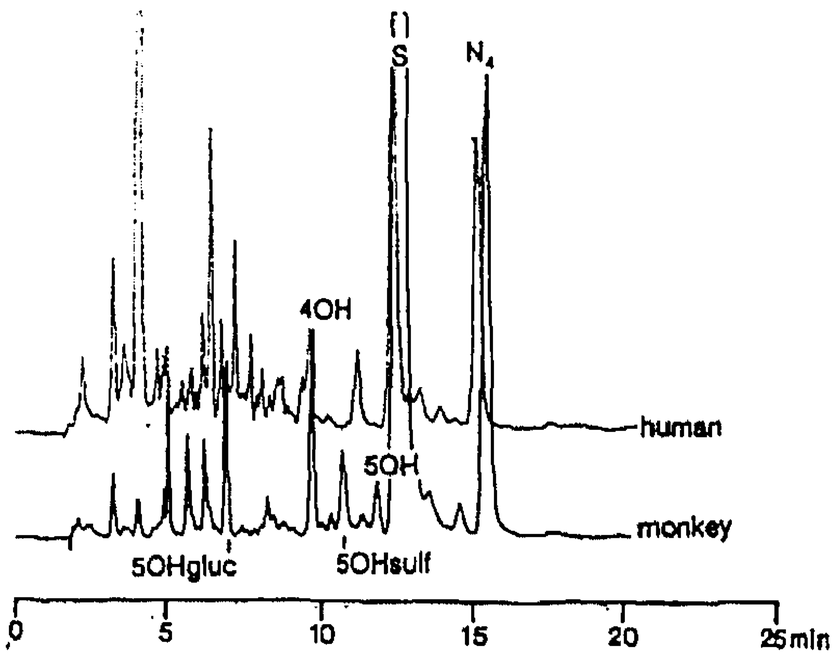

Fig. 5. Chromatograms of Rhesus monkey and human urine containing sulfadiazine (S) and its metabolites. 5-Hydroxysulfadiazine and its conjugates are not present in human urine. The concentrations of the compounds in the human urine sample are S 67.5, 4OH 12.3 and N_4 85.5 μg/ml. The concentrations of the compounds in the monkey urine sample are given in Fig. 4.

compares the chromatograms for monkey and human plasma after an oral dose of sulfadiazine. The chromatograms show the presence of S and its metabolites. Figs. 4 and 5 show chromatograms for monkey and human urine and a urine sample after oral administration of S.

Table 2 shows the equations for the calibration graphs for S and its metabolites in plasma and urine. Table 3 shows the limits of detection in water and limits of quantification in plasma and urine of S and its metabolites. Samples at pH 5 and kept in the dark were stable in the autosampler of the HPLC for 24 h.

Table 2
Calibration graphs for sulfadiazine and its metabolites

Compound	Concentration (μg/ml)	Curve[a]	Correlation coefficient (r)
Plasma			
4OH	0.20–2.32	$y = 702x + 41.2$	0.9981
5OH	0.37–3.56	$y = 420x - 30.4$	0.9989
5OHgluc	0.33–2.36	$y = 500x - 3.62$	0.9981
5OHsulf	0.57–4.00	$y = 243x + 20.5$	0.9960
S	0.31–218	$y = 484x - 141$	0.9999
N_4	0.40–7.1	$y = 379x + 10.7$	0.9998
Urine			
4OH	0.6–116	$y = 93.2x + 53.1$	0.9997
5OH	0.8–356	$y = 78.6x + 77.8$	0.9982
5OHgluc	0.8–118	$y = 89.3x + 73.0$	0.9997
5OHsulf	1.1–50	$y = 65.0x + 77.8$	0.9982
S	0.8–2180	$y = 95.7x + 466$	0.9997
N_4	0.8–187	$y = 75.5x + 2.4$	0.9997

[a] y = Peak height (integration units) and x = concentration (μg/ml).

Table 3
Recovery and limits of detection and quantification of sulfadiazine and its metabolites

Compound	Detection limit[a] in water (μg/ml)	Quantification limit[a] (μg/ml)[a]		Recovery in plasma ($n = 4$) (%)	
		Urine	Plasma	Mean	C.V.
4OH	0.07	0.6	0.20	95.6	4.1
5OH	0.12	0.8	0.37	92.8	8.3
5OHgluc	0.11	0.8	0.33	101.7	1.3
5OHsulf	0.11	1.1	0.57	96.6	4.9
S	0.08	0.8	0.31	98.4	3.5
N_4	0.12	0.8	0.40	84.3	6.1

[a] Detection limit in water; quanfitication limit in the biological matrix.

Tables 4 and 5 show the intra- and inter-day variations, respectively, for S and its metabolites in plasma, and Tables 6 and 7 show those for S and its metabolites in urine.

Fig. 6 shows an example of the plasma concentration–time curves for S and its metabolites in a Rhesus monkey and Fig. 7 shows the plasma concentration–time curve and renal excretion rate–time profiles for S and its metabolites after a single oral administration of 500 mg of S to one male volunteer.

Table 8 summarizes some pharmacokinetic parameters of S calculated from the plasma and urine concentrations of the parent drug and its

Table 4
Inter-day coefficients of variation (C.V.) for spiked sulfadiazine and its metabolites in human plasma ($n = 4$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V., %)	Accuracy (%)
4OH	2.32	2.64	11.6	13.8
5OH	3.56	3.56	3.0	0
5OHgluc	2.36	2.34	5.2	0.8
5OHsulf	4.00	3.76	15.1	6.0
S	218	222	2.4	1.7
N_4	7.10	6.93	1.0	2.4
4OH	1.16	1.26	14.6	8.6
5OH	1.78	1.76	3.3	1.1
5OHgluc	1.18	1.16	3.3	1.7
5OHsulf	2.00	1.80	13.2	10.0
S	109	111	3.4	1.4
N_4	3.55	3.45	2.7	2.8
4OH	0.56	0.61	16.4	7.1
5OH	0.86	0.78	9.1	9.3
5OHgluc	0.57	0.58	12.8	1.8
5OHsulf	0.32	n.d.	–	–
S	5.26	5.63	9.6	7.0
N_4	0.90	0.83	12.2	7.8

Table 5
Intra-day coefficients of variation (C.V.) for spiked sulfadiazine and its metabolites in human plasma ($n = 4$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V., %)	Accuracy (%)
4OH	2.32	2.27	4.8	2.2
5OH	3.56	3.55	1.7	0.3
5OHgluc	2.36	2.42	3.5	2.5
5OHsulf	4.00	4.01	4.4	0.0
S	218	216	1.1	1.0
N_4	7.10	6.89	0.6	3.0
4OH	1.16	1.11	3.6	4.4
5OH	1.78	1.82	0.8	2.2
5OHgluc	1.18	1.15	3.5	2.5
5OHsulf	2.00	1.98	7.6	1.0
S	109	106	1.1	2.6
N_4	3.55	3.41	1.3	4.0
4OH	0.56	0.60	10.0	7.1
5OH	0.86	0.83	9.0	3.5
5OHgluc	0.57	0.62	8.3	8.8
5OHsulf	0.32	n.d.	–	–
S	5.26	5.71	4.6	8.6
N_4	0.90	0.93	12.7	3.3

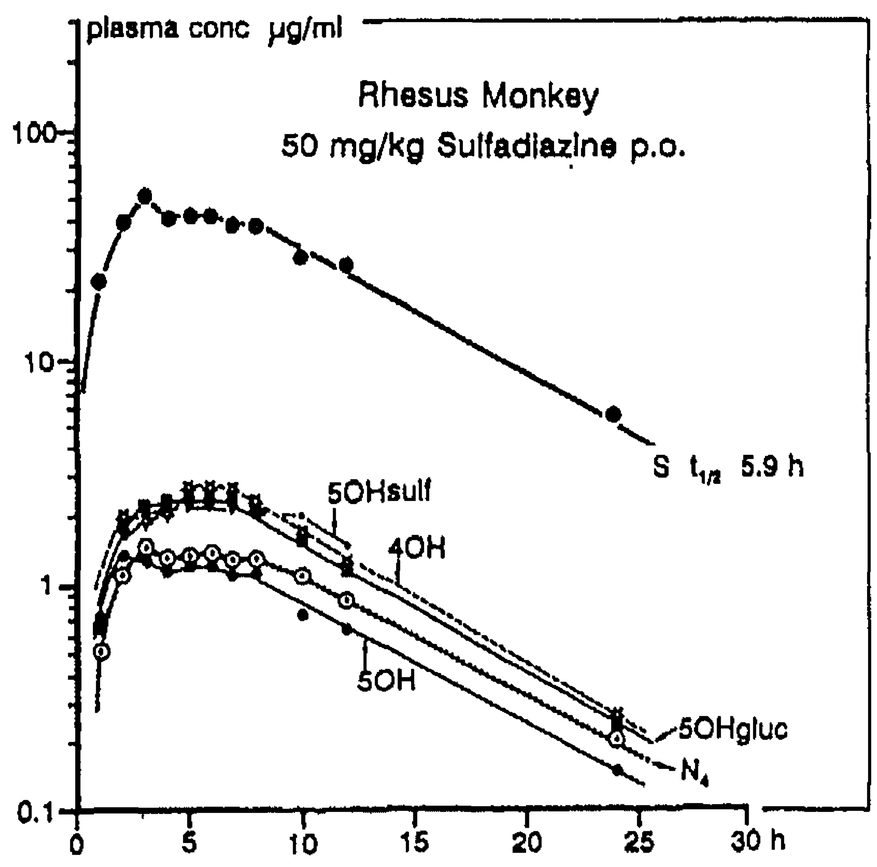

Fig. 6. Plasma concentration–time curve for sulfadiazine (S), N_4-acetylsulfadiazine (N_4), 4-hydroxysulfadiazine (4OH) and 5-hydroxysulfadiazine (5OH) and its glucuronide (5OHgluc) and sulfate (5OHsulf) conjugates in a Rhesus monkey after an oral dose of 50 mg/kg of sulfadiazine.

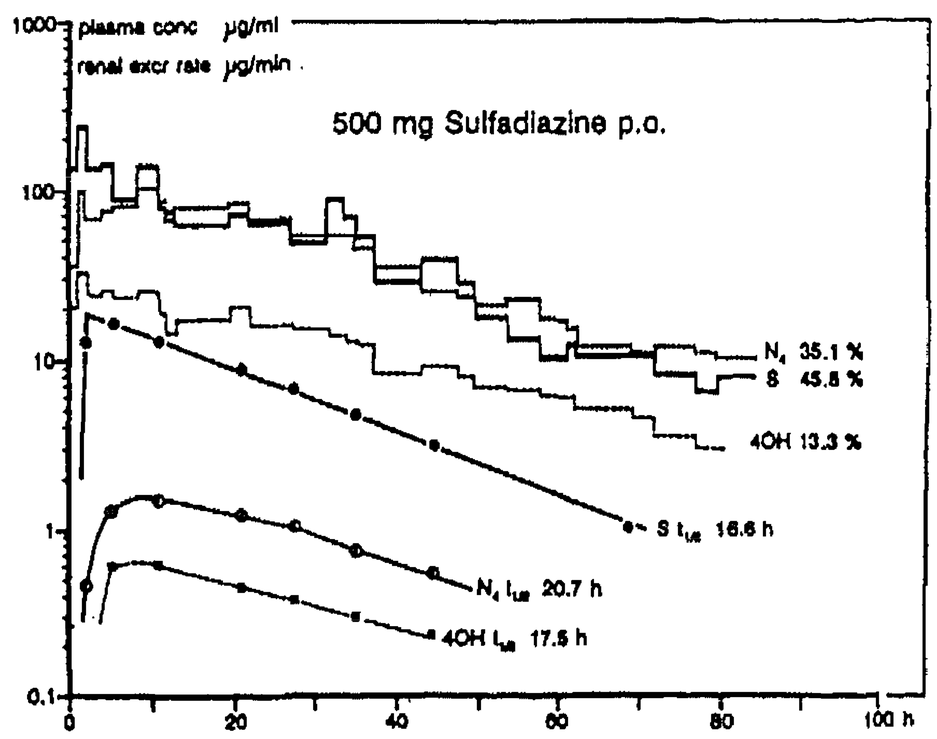

Fig. 7. Plasma concentration–time curve and renal excretion rate–time profiles for sulfadiazine (S), N_4-acetylsulfadiazine (N_4) and 4-hydroxysulfadiazine (4OH) in a human volunteer after an oral dose of 500 mg of sulfadiazine (6 mg/kg).

Table 6
Inter-day coefficients of variation (C.V.) for spiked sulfadiazine and its metabolites in human urine ($n = 4$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V., %)	Accuracy (%)
4OH	116.0	113.0	1.8	2.5
5OH	89.0	93.8	6.5	5.4
5OHgluc	118.0	116.0	1.4	1.4
5OHsulf	70.0	64.9	2.3	7.3
S	215.0	217.0	3.9	1.1
N_4	178.0	177.0	3.7	0.4
4OH	58.0	56.4	2.7	2.8
5OH	44.5	44.2	2.5	0.7
5OHgluc	59.0	58.4	2.1	1.0
5OHsulf	35.0	33.5	1.5	4.3
S	107.0	110.0	3.1	2.2
N_4	89.0	89.8	2.6	0.9
4OH	14.5	14.8	4.1	2.1
5OH	11.1	11.5	7.0	3.6
5OHgluc	14.8	14.2	11.3	4.2
5OHsulf	8.8	8.6	12.8	2.3
S	26.9	27.8	3.6	3.3
N_4	22.3	22.7	2.6	1.8
4OH	5.8	5.9	10.1	1.7
5OH	4.5	4.3	5.8	4.4
5OHgluc	5.9	6.0	12.5	1.7
5Osulf	3.5	2.9	12.4	17.1
S	10.8	10.5	4.4	2.8
N_4	8.9	8.7	5.3	2.2

metabolites in the pilot experiment after a single oral administration of 500 mg S to the human volunteer.

4. Discussion

The presence of metabolites of sulfadiazine and its availability for isolation strongly depends on the species. For instance, if only human urine had been investigated, 5-hydroxysulfadiazine with its conjugates would not have been detected and isolated.

The metabolites 4- and 5-hydroxysulfadiazine have already been described [4]. Vree and co-workers isolated the metabolite from the urine of dogs [5] and excreta of fresh water turtles *Pseudemys scripta elegans* by preparative TLC [8]. Dog's urine is relatively easy to analyse, because dogs are unable to acetylate sulfonamides [5]. Excreta of turtles diluted in tank water give clean chromatograms containing 4- and 5-hydroxysulfadiazine [8]. Other reported conjugates of sulfadiazine included the deaminated metabolites [9]. O-Glucuronides can be hydrolysed by β-glucuronidases; of the four β-glucuronidase systems tested, systems C and D were the most active for O-deglucuronidation.

The 5-hydroxy group in the pyrimidine ring in the *meta* position to the nitrogen atoms acts as a phenolic hydroxyl group and is extremely suscep-

Table 7
Intra-day coefficients of variation (C.V.) for spiked sulfadiazine and its metabolites in human urine ($n = 4$, in vitro)

Compound	Concentration added (μg/ml)	Concentration measured (μg/ml)	Precision (C.V., %)	Accuracy (%)
4OH	116.0	113.0	1.5	2.2
5OH	89.0	88.4	0.9	0.7
5OHgluc	118.0	115.0	0.9	2.5
5OHsulf	70.0	64.3	1.0	8.1
S	215.0	214.0	0.9	0.2
N_4	178.0	171.0	0.7	3.9
4OH	58.0	58.4	1.1	0.7
5OH	44.5	45.7	1.5	2.7
5OHgluc	59.0	59.0	1.2	0.0
5OHsulf	35.0	33.8	0.9	3.4
S	108.0	111.0	1.0	3.3
N_4	89.0	88.4	0.4	0.7
4OH	14.5	15.3	2.5	5.5
5OH	11.1	11.4	3.7	2.7
5OHgluc	14.8	14.7	2.9	0.7
5OHsulf	8.8	9.1	1.3	3.4
S	26.9	27.7	1.8	3.0
N_4	22.3	22.5	1.2	0.9
4OH	5.8	6.1	5.4	5.2
5OH	4.5	4.5	10.5	0.0
5OHgluc	5.9	6.7	4.8	13.6
5Osulf	3.5	3.2	5.9	8.6
S	10.8	11.0	2.2	1.9
N_4	8.9	8.7	1.7	2.2

tible to conjugation. The 4-hydroxy group is not accessible for conjugation in man, monkey and turtle.

Differences in the metabolism of sulfonamides between humans and monkeys may be related to differences in the rates of metabolism and differences between the urine pH in the two species. In man, the urine pH varies between 5 and 7, resulting in renal excretion of the parent drug (45%), acetylation (35%) and oxidation of sulfadiazine (13%). Monkey urine pH varies between 8 and 9.5, resulting in a similar renal excretion of the parent drug (40%), but minimal acetylation (8%), which is compensated for by enhanced oxidation (50%). A similar difference in the N-glucuronidation was reported earlier; human urine contained the N_1-glucuronide of sulfamethoxazole [10], which is absent in monkey urine [11]. Also in humans sulfadimethoxine is mainly glucuronidated at the N_1-position [12], whereas in pigs it is only acetylated at the N_4-position [13].

Addition of $AcONH_4$ to the eluent is essential for a stable retention time of sulfadiazine-O-sulfate. Without $AcONH_4$, the retention time and the peak shape of the sulfate depend on the concentration. A similar behaviour was observed for paracetamol sulfate [14].

In conclusion, the hydroxy metabolites of sulfadiazine, 4-hydroxysulfadiazine and 5-hydroxysulfadiazine with its corresponding sulfate and glucuronide conjugate were isolated from

Table 8
Pharmacokinetic parameters for sulfadiazine and its metabolites and conjugates from a healthy human subject and from a Rhesus monkey after a single oral administration of sulfadiazine

Parameter	Value			
Subject	A			Monkey
Gender	Male			Female
Body mass (kg)	85			10
Acetylator status	Slow			
Dose (mg)	500			50[a]
Compound	S	N_4	4OH	S
Bioavailability, F (%)	94.2[b]	35.1	13.3	100
C_{max} (μg/ml)	16.9	1.54	0.64	46.2
t_{max} (h)		3.7	10.1	7.738
$t_{1/2\ absorption}$ (h)	0.49			1.25
$t_{1/2\ formation}$ (h)		2.7	2.0	
$t_{1/2}$ (h)	16.6	20.7	17.5	5.9
MRT S (h)	25.9	34.7	28.8	10.6
Total body clearance (l/h)	1.06	3.84	3.11	0.084[a]
Volume of distribution (l)	25.3	84.7	78.2	0.71[a]
Renal clearance (l/h)	0.61	3.35	3.44	0.071
Percentage of dose excreted (mol-%)	45.8	35.1	13.3	39.7
Total (%)	94.2[b]			100[b]

[a] mg/kg, values/kg.
[b] Renal excretion (mol-%) 4OH 19.9%, 5OH 17.4%, 5OHgluc 9.1%, 5OHsulf 5.9%, S 39.7%, N_4 8.0%.

monkey urine and their structures could be confirmed as corresponding to those reported in the literature.

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