Journal of Chromatography, 571 (1991) 157–168
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6027

High-performance liquid chromatographic method for the routine determination of sulphadimidine, its hydroxy metabolites and N4-acetylsulphadimidine in body fluids and cell culture media

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(First received March 26th, 1991; revised manuscript received May 29th, 1991)

ABSTRACT

A simple high-performance liquid chromatographic method is presented for the determination of trace amounts of sulphadimidine (SDD), its hydroxylated metabolites and N4-acetyl-SDD in blood plasma, urine, hepatocyte culture media and microsomal incubations. The synthesis of 5-hydroxy-SDD and an improved method for the isolation of 4-methylhydroxy-SDD from urine are described and their respective specific absorption coefficients at 265 nm are calculated by on-line radiochemical and ultraviolet detection. The limit of detection of the analytical method is 0.05 μg/ml for SDD and its hydroxy metabolites and 0.2 μg/ml for N4-acetyl-SDD. Linear calibration graphs for SDD and its metabolites were constructed from 0.2 to 50 μg/ml. The method has been applied to biotransformation studies *in vivo* and *in vitro*.

INTRODUCTION

Sulphadimidine [SDD, 4-amino-N-(4,6-dimethyl-2-pyrimidyl)benzene sulphonamide] is extensively used as a prophylactic antibacterial and antiprotozoal drug to treat acute and chronic infections in food-producing animals. The safety of the drug to the consumer has been questioned because of its apparent toxicity and carcinogenicity [1]. During the past fifteen years, concern has increased with respect to SDD residues in milk [2–4], beef [5], pork [5–8] and mutton [9]. Screening for the parent compound only might be insufficient as extensive biotransformation takes place. SDD is metabolized through acetylation and hydroxyla-

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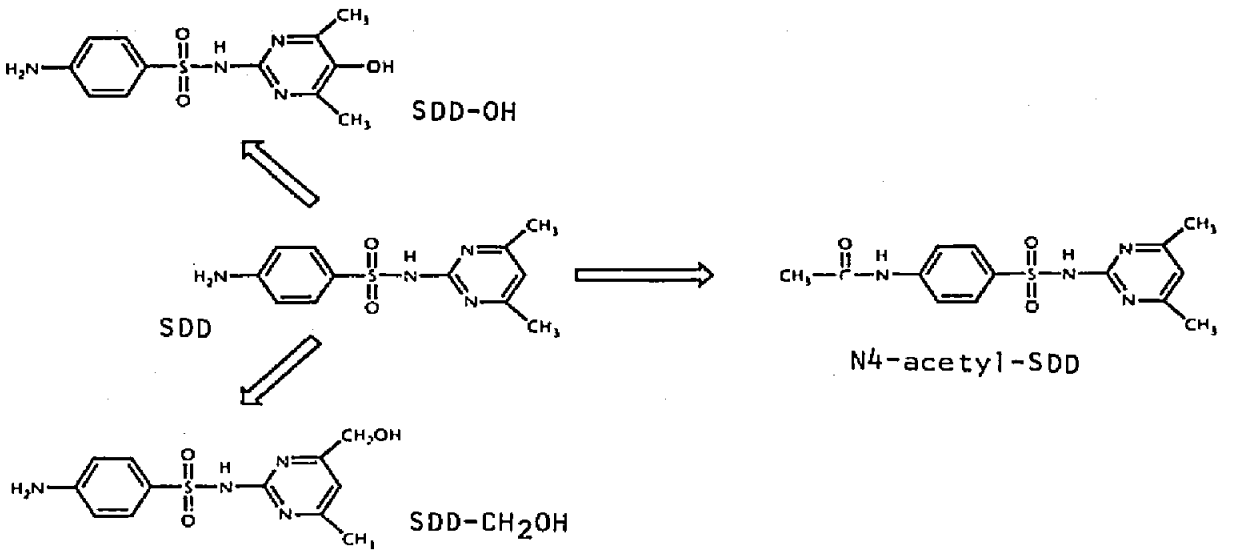

Fig. 1. Hydroxylation and acetylation of sulphadimidine. SDD = sulphadimidine; N4-acetyl-SDD = N4-acetylsulphadimidine; SDD-OH = 5'-hydroxysulphadimidine; SDD-CH_2OH = 4'-methylhydroxy-sulphadimidine.

tion [10] (Fig. 1), the major pathway depending on the animal species and individual genotype [10–12]. Hydroxylation and occasionally the consequent carboxylation of SDD and its N4-acetyl metabolite may be followed by glucuronidation or sulphation.

In ruminant species (cattle, sheep, goats), the hydroxylation pathway, catalysed by cytochrome P450, is predominant [2,10–11]. Within this laboratory, SDD is used in comparative pharmacokinetic studies in goats and rats. These studies include drug metabolism *in vivo* (intact animals) and *in vitro* (isolated liver cells and liver microsomes). Therefore, a routine method for the simultaneous determination of SDD and its main metabolites is needed.

The determination of SDD and its metabolites has been performed using thin-layer chromatography (TLC) combined with either spectrophotometry [9,10,13] or scintillation counting [14]. Gas chromatographic methods have also been published [5,15] but the less laborious reversed-phase high-performance liquid chromatography (HPLC) technique is the method of choice [2–4,7,8.12,15–17]. Although all methods deal with SDD and N4-acetyl-SDD, only two [2,11] include the SDD hydroxy metabolites. Unfortunately, no chromatograms are shown in these papers. The use of the methods described previously [2,11] in combination with some common liquid extractions did not yield acceptable separations in this laboratory.

Neither 5-hydroxy-SDD (SDD-OH) nor 4-methylhydroxy-SDD (SDD-CH_2OH) are commercially available. To the authors' knowledge, no synthesis methods have been described. A brief report on the isolation of the compounds

from the urine of dogs treated with SDD has been published [11] but, unfortunately, this method could not be reproduced in this laboratory.

This paper presents a simple and flexible method for the determination of trace amounts of SDD and its metabolites. The method uses simple sample preparation and can be applied to cell culture media, microsomal incubation mixtures, plasma and urine. A method for the synthesis of SDD-OH and an improved procedure for the isolation of SDD-CH_2OH from urine from dogs is presented. Absorption coefficients for UV detection at 265 nm are calculated, based on the simultaneous UV and radiochemical detection of metabolized [^{14}C]SDD.

EXPERIMENTAL

Reagents

Sulphadimidine sodium, sulphadimethoxine sodium [SDX, internal standard (I.S.)] and sulphaguanidine were purchased from ACF (Maarssen, Netherlands). ^{14}C-Labelled SDD (specific activity 14.4 MBq/mol purified by HPLC before use) was from Amersham (Buckinghamshire, UK). N4-Acetyl-SDD was synthesized according to the method of Nielsen [10].

HPLC-grade methanol was obtained from Rathburn (Walkerburn, UK). Demineralized water was filtered over a Milli Q system (Millipore, Bedford, MA, USA). Potassium dihydrogenphosphate, disodium hydrogenphosphate, diethyl ether, dichloromethane and isopropanol (all p.a. grade) were from J. T. Baker (Deventer, Netherlands).

Limpet acetone powder (*Patella vulgata*) for deglucuronidation and desulphation was purchased from Sigma (St. Louis, MO, USA).

Column chromatography was performed on Kieselgel 60 (<230 mesh) from Merck (Darmstadt, Germany) and silica-bound reversed-phase ODS (RP-C_{18}, average particle size 40 μm) from J. T. Baker. All other chemicals were of analytical-reagent grade.

Apparatus

The HPLC system consisted of a 400S solvent delivery system and a 783A programmable absorbance detector operating at 265 nm from Applied Biosystems (Maarssen, Netherlands), an SP4290 integrator from Spectra-Physics (San Jose, CA, USA) and a Promis autosampler from Spark Holland (Emmen, Netherlands). A 100 mm × 4 mm stainless-steel cartridge column equipped with a 10 mm × 4 mm pre-column, both packed with Hypersil ODS (5 μm, RP-C_{18}) from Knauer (Berlin, Germany) was used.

The isocratic mobile phase contained 10:90 (v/v) methanol–phosphate buffer (0.05 *M*, pH 6.67). The eluent was filtered over 0.45 μm membrane filters (Millipore) and degassed under vacuum before use.

The flow-rate was 0.8 ml/min, resulting in a back-pressure of 80 bar. Analyses were performed at ambient (20°C) temperature.

Occasionally, a 1000S diode-array detector from Applied Biosystems was used to confirm the peak identities. Radiochemical detection was performed using an on-line LB-506C monitor from Berthold (Karlsruhe, Germany). The infusion rate of the scintillation cocktail (J. T. Baker) was 0.5 ml/min.

^{1}H NMR spectra (360 MHz) were recorded in [$^2H_6$]acetone at 25°C relative to $(CH_3)_4Si$ (internal standard), using a Bruker HX 360 (Karlsruhe, Germany) spectrometer. Electron-impact (EI) and chemical-ionization (CI) mass spectra were obtained with a TSQ 45 Finnigan Mat (San Jose, CA, USA) mass spectrometer.

Synthesis of SDD-OH

Based on the synthesis of SDD [18], the principle of this method was a condensation of sulphaguanidine and diacetylcarbinol (3-hydroxy-2,4-pentanedione). Diacetylcarbinol (0.72 g, 6.4 mmol), synthesized as previously described [19], was stirred with sulphaguanidine (1.04 g, 4.8 mmol) and acetic acid (97%, 160 µl) in 10 ml of dry ethanol and refluxed for 17 h. After evaporation to dryness in a rotary evaporator (40°C), the residue was placed on a Kieselgel 60 column and eluted with chloroform–acetone (60:40, v/v). The SDD-OH-containing fraction was further purified over an RP-C_{18} column; the eluent was methanol–aqueous 0.5 *M* sodium chloride (30:70, v/v). Fractions were monitored by TLC and by HPLC with diode-array detection. The fractions containing SDD-OH were concentrated by evaporation, followed by the addition of 75 ml of water and extracted three times with 150 ml of ethyl acetate. The combined organic layers were washed with water that was acidified with hydrochloric acid to pH 3 (2 × 50 ml) and concentrated by evaporation. Drying over potassium hydroxide for two days yielded 61 mg (0.2 mmol) of SDD-OH.

Isolation of SDD-CH_2OH

Two male Beagle dogs (15 kg) received 1.5 g of SDD as an intravenous injection of a sterile 10% solution of sulphadimidine sodium. Urine was collected for 30 h. A 100-ml volume of urine was adjusted to pH 4.5 using sodium acetate and acetic acid and then 1 g of limpet acetone powder, a crude extract with glucuronidase and sulphatase activity, was added. The metabolites were deconjugated for 3 h in a 37°C water-bath. After adjusting the pH to 6.7 using 4 *M* sodium hydroxide solution and 10 ml of 0.5 *M* phosphate buffer, the urine was extracted twice with ethyl acetate (150 ml). The combined organic fractions were evaporated to dryness in a rotary evaporator at 40°C. The residue was dissolved in 10 ml of methanol. A 5-ml volume was used for column chromatography on RP-C_{18} (150 mm × 10 mm); the elution solvent was methanol–water (30:70, v/v). The combined fractions (10 ml) containing SDD-CH_2OH, as monitored by HPLC with diode-array detection, were extracted twice with 15 ml of ethyl acetate and evaporated to dryness. The NMR and mass spectrometric (MS) conditions were as described for the detection of SDD-OH.

Incubations in vitro

Isolated hepatocytes were cultured in Waymouths' MB/751 (Gibco, UK) or in Wiliams' Medium E (Sigma). Cells were incubated with 0.5 m*M* SDD for 20 h. Microsomes in 0.125 *M* phosphate buffer (pH 7.4), supplemented with essential cofactors, were incubated with 0.8 m*M* SDD for 60 min. The details will be published elsewhere [20].

Sample preparation

For the hepatocyte culture medium (plasma or urine), a 2.5-ml (culture medium) or 300-μl (plasma and urine) sample was mixed with 1.0 ml of 0.5 *M* acetate buffer (pH 4.5) and about 20 mg of limpet acetone powder were added. Deconjugation was performed for 3 h in a stoppered tube in a 37°C water-bath. To each tube, 60 μl of sodium hydroxide solution (4 *M*), 1.0 ml of a 0.5 *M* phosphate buffer (pH 6.0) and 200 μl of a solution of 90 mg/l SDX (I.S.) in 15:85 (v/v) methanol–water were added. The samples were saturated with anhydrous ammonium sulphate and extracted with 4.0 ml of diethyl ether–dichloromethane–isopropanol, (60:40:0.5, v/v). Full separation was achieved by centrifugation (10 min, 500 *g*). From each tube, the organic layer was pipetted into a clean tube. After adding 3.0 ml of the mixture, the extraction procedure was repeated. The combined fractions were evaporated to dryness under nitrogen. Each residue was redissolved in 1.0 ml (culture medium and urine) or 200 μl (plasma) of mobile phase. A 20-μl aliquot was injected into the HPLC system.

For the microsomal incubation buffer, the samples were not deconjugated and no sodium hydroxide was added. Further processing was identical to that for the cell culture medium.

RESULTS AND DISCUSSION

Chromatographic separation and applications

Fig. 2 shows some real-time chromatograms. Plasma samples (Fig. 2a) were taken from goats treated with SDD (20 mg/kg, intravenously). Chromatograms of samples from goat hepatocyte culture medium, rat microsomal incubation mixture and rat hepatocyte culture medium incubated with SDD are shown in Fig. 2b, c and d, respectively. With all matrices, blank samples (not shown) yielded straight baselines and no interfering peaks after 4 min of analysis.

One of the goals of this study was to develop a simple routine analysis. An isocratic system with a simple buffer–methanol eluent perfectly fitted this aim. Increasing the flow-rate (1.2 ml/min) or column temperature (30°C) is sufficient to decrease the analysis time to 30 min. A good separation was still achieved, especially with samples from microsomal incubations and cell culture media.

Within the scope of this pharmacokinetic study, Fig. 2 shows a good qualitative correlation between *in vivo* (Fig. 2a) and *in vitro* (Fig. 2b) results in goats. In contrast to goats (Fig. 2d) N4-acetylation is predominant in the rat hepato-

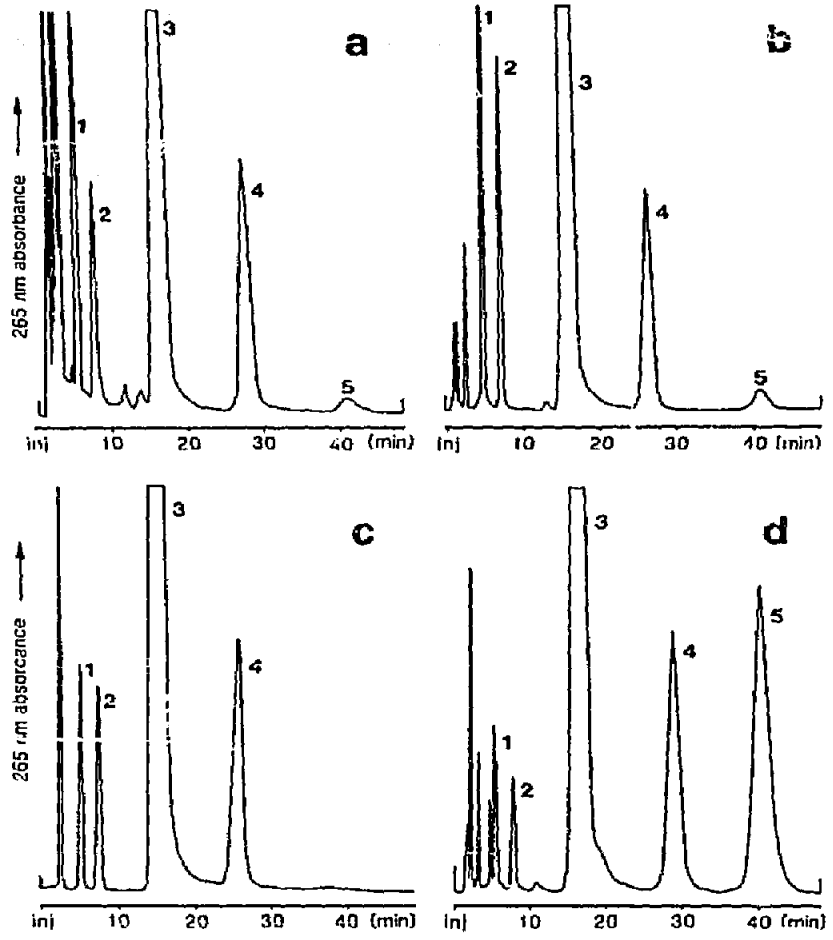

Fig. 2. Representative sample chromatograms from goat plasma (a), goat hepatocyte culture medium (b), rat microsomal incubations (c) and rat hepatocyte culture medium (d). a.u.f.s. = 0.015. Retention time: SDD-CH_2OH (1) 5.5 min; SDD-OH (2) 8 min; SDD (3) 16.5 min; internal standard (4) 25–30 min (depending on pH); N4-acetyl-SDD (5) 41 min.

cytes. No N4-acetyl-SDD is found in microsomal incubations (Fig. 2c) as neither acetyltransferase nor its co-factor is present.

Sample extraction and recovery

The described extraction mixture has been developed as a slightly polar solvent with a specific density <1.00. The organic layer may therefore easily be separated from the sample.

Blank samples (plasma, cell culture medium) were spiked with SDD and its metabolites in concentrations ranging from 2 to 40 μg/ml. In this concentration range the extraction procedure yielded absolute recoveries from 70 to 90% (SDD, 90.1 ± 4.5%; SDD-OH, 75.5 ± 4.5%; SDD-CH_2OH, 69.7 ± 3.6%; N4-acetyl-SDD, 82.9 ± 2.8%; I.S., 81.5 ± 3.4%; $n = 5$, results given as mean ± S.D.).

Higher recoveries were found when ethyl acetate or, even better, ethyl acetate–

isopropanol (90:10, v/v) were used, although emulsions were formed upon shaking, which were hard to disperse. A major objection of both alternative solvents was the extensive co-extraction of matrix components.

Internal standard

As only an aliquot of the extraction solvent is taken off as samples for further processing, the use of an internal standard is advisable. SDX was selected from a number of sulphonamide drugs with physicochemical properties more or less like SDD. As for SDD, the SDX spectrum shows a maximum absorption in the range 260–275 nm. The high polarity, relative to SDD, of most sulphonamides resulted in interferences in the peaks of the hydroxylated metabolites. An advantageous feature of SDX is its pH-dependent retention for pH values between 6 and 7. By decreasing the pH of the eluent the capacity factor increases several times. Neither SDD nor its metabolites showed similar changes. Thus the SDX peak may be directed to any position in the chromatogram depending on the matrix and SDD-related peaks can be observed. A mobile phase buffer (pH 6.67) yielded an SDX peak between SDD and N4-acetyl-SDD (Fig. 2). In these studies, no peaks occured in this area.

Linearity and detection limit

For SDD and its metabolites, calibration graphs were constructed in spiked plasma, microsomal incubation buffer and hepatocyte culture medium. Linear correlations (r = 0.990–0.998) were found for concentrations ranging from 0.2 to 50 μg/ml. In the described system, the limit of detection was 0.05 μg/ml for SDD, SDD-OH and SDD-CH_2OH and 0.2 μg/ml for N4-acetyl-SDD.

Synthesis of SDD-OH

^{1}H NMR yielded the following peaks (chemical shifts, δ, in ppm); δ 7.81 (d, J = 8.7 Hz, 2H *ortho* to SO_2), δ 6.69 (d, J = 8.7 Hz, 2H *ortho* to NH_2), δ 2.9 (broad s, probably accounting for OH, NH_2 and traces of water), δ 2.29 (s, 6H, methyl protons), SO_2NH signal not detected.

From CI-MS the molecular mass was deduced to be 294 a.m.u. (calculated 294.31 a.m.u.). The EI spectrum of the synthesis product yields a small molecular ion peak at m/z 295. Peak m/z 230, arising from the loss of the SO_2 moiety, is consistent with the SDD rearrangement ion at m/z 214 [10,24]. This and other fragment ions consistent with the molecular structure are depicted in Fig. 3. Most remaining fragments have been described previously [22].

In contrast to SDD, the UV spectrum of the product (Fig. 4) does not show a second maximum at 240 nm. This may be explained by the increase of the chromophore (hydroxylation in the pyrimidine ring), resulting in a shift to secondary maximum absorption at 250 nm (shoulder).

The results confirm that the synthesis product is SDD-OH. In the HPLC system described, it co-elutes (retention time, t_R = 8.0 min) with a main metabo-

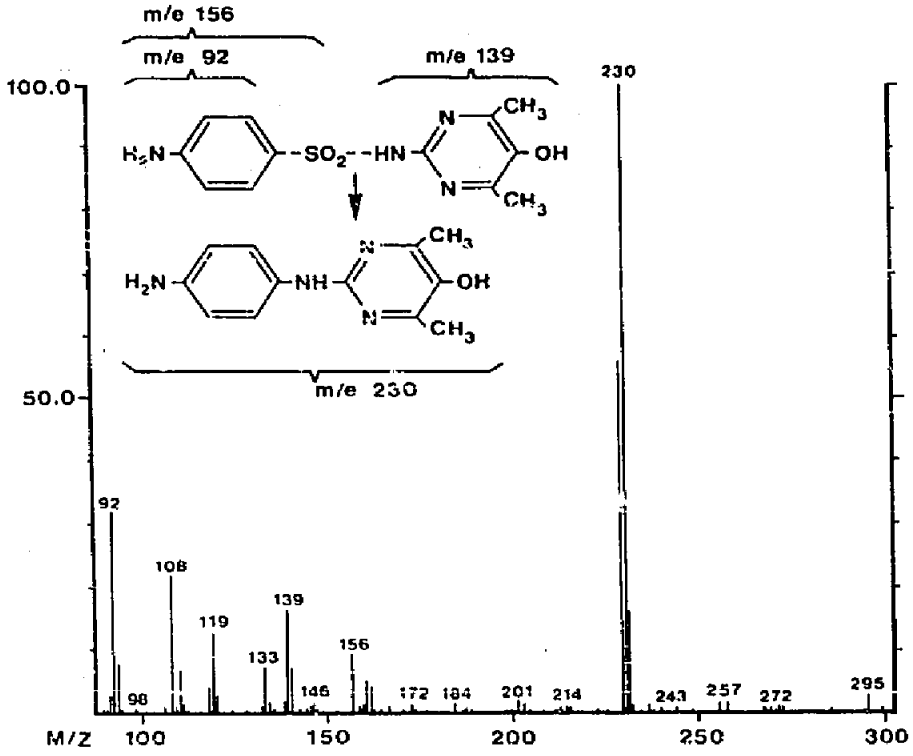

Fig. 3. EI mass spectrum of synthesized SDD-OH. Predominant fragments are shown in the structural formula. Traces of molecular ion are detected at m/z 295 (294 + $^1H^+$). The absolute number of counts is 90 000. MS conditions: EI (2 scans/min) 70 eV; methane 0.40 Torr; emission current 0.20 mA; EMV 1100 V; probe temperature 30–200°C at 60°C/min, then 200–280°C at 5°C/min.

lite in goat plasma (Fig. 2a). Based on NMR and MS analysis, the estimated purity of the product is >90%. HPLC with UV detection at 265 nm yields an apparent purity of >98%.

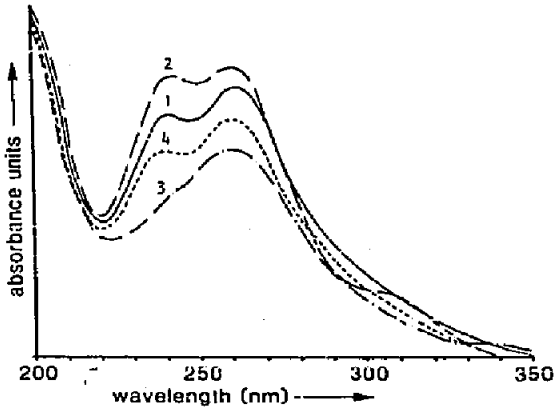

Fig. 4. UV spectra of (1) SDD, (2) N4-acetyl-SDD, (3) SDD-OH and (4) SDD-CH_2OH. The 240-nm maximum in the pyridine ring structure (SDD-OH) shifts to 250 nm on direct hydroxylation.

Isolation of SDD-CH_2OH

As dogs primarily hydroxylate SDD, the starting point for the isolation of SDD-CH_2OH was Beagle dog urine, similar to the procedure described by Vree and Hekster [11], who used TLC for isolation after deconjugation. Unfortunately, very poor yields were found because of the decomposition of the compound during this procedure. Column chromatography as described largely prevented this problem. The SDD-CH_2OH-containing fractions yielded a red–brown residue. The retention volume was approximately 40 ml. The product co-elutes (t_R = 5.5 min, Fig. 2) with a main metabolite in goat plasma. The compound appeared to be slightly unstable and was stored under nitrogen gas at −20°C in darkness.

Chemical shifts in the ^1H NMR spectrum were: δ 7.81, (d, J = 8.9 Hz, 2H *ortho* to SO_2), δ 6.98 (d, $J < 1.0$ Hz, 1H, pyrimidyl proton), δ 6.69 (d, J = 8.9 Hz, 2H *ortho* to NH_2), δ 4.49 (d, $J < 1.0$ Hz, 2H, CH_2OH protons, δ 2.34 (d, $J < 1.0$ HZ, 3H, methyl protons).

From CI-MS the molecular mass was deduced to be 294 a.m.u. (calculated 294.31 a.m.u.). As with SDD-OH, the predominant peak is the m/z 230 rearrangement ion arising from the loss of the SO_2 moiety (Fig. 5). Further frag-

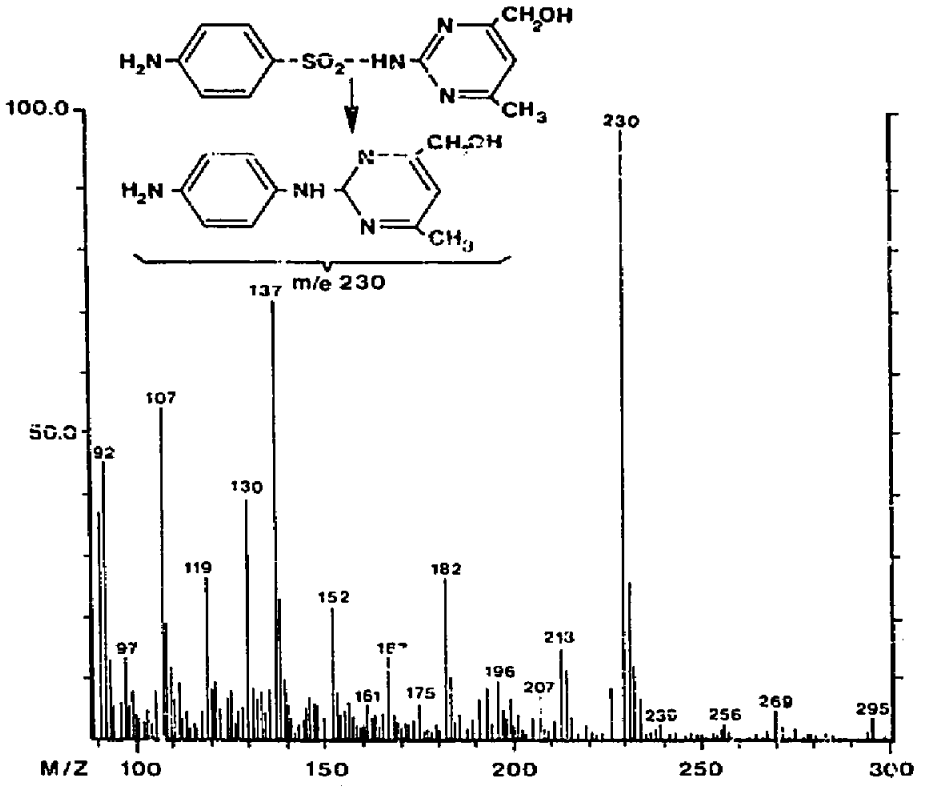

Fig. 5. EI mass spectrum of isolated SDD-CH_2OH. Traces of molecular ion are detected at m/z 295 (294 + $^1H^+$). Rearrangement yield the m/z 230 peak. Further fragmentation and rearrangement of the pyrimidine structure to m/z 137 and 107, differing from SDD-OH (m/z 139 and 109), is unknown. Absolute number of counts 9000. MS conditions as in Fig. 3.

mentation, yielding peaks *m/z* 137 and *m/z* 107 *versus* *m/z* 139 and *m/z* 108, is apparently different from SDD-OH.

Side-chain hydroxylation does not influence the UV spectrum, as observed with SDD-OH (Fig. 4).

Combined NMR and MS spectral analyses confirm the identity of the isolation product to be SDD-4-CH_2OH. Although the apparent purity of the product in this HPLC system was >93%, the MS spectrum (Fig. 5) shows some low mass impurities. To obtain a product of higher purity, efforts have been put into the synthesis of SDD-CH_2OH, which is similar to that of SDD-OH. Unfortunately, it was not possible to synthesize acceptable yields of pure 1-hydroxy-2,4-pentanedione. Condensation with sulphaguanidine resulted in low yields of impure SDD-CH_2OH.

Specific absorption of hydroxylated SDD metabolites

Until now, calculations on SDD metabolism were performed without considering the differences in specific absorptions for SDD and its metabolites. The assumption that all these compounds should show equal absorption coefficients was questionable, especially for SDD-OH. For SDD-OH, the hydroxylation takes place in part of the chromophore and is reflected in a spectral shift (the secondary maximum of the pyrimidine structure at 240 nm disappears, as shown in Fig. 4). Calculations based on the simultaneous quantification of [^{14}C]SDD metabolites by UV detection at 265 nm and on-line radiochemical detection yield specific absorptions of 96 ± 3 and 105 ± 2%, relative to SDD, for SDD-CH_2OH and SDD-OH, respectively (n = 5, mean ± S.D.). In these studies, these small differences in specific absorption were neglected.

Other metabolites of sulphadimidine

In addition to SDD-OH, SDD-CH_2OH and N4-acetyl-SDD, metabolites resulting from the combined acetylation and hydroxylation of SDD and a carboxylated metabolite resulting from further oxidation of SDD-CH_2OH have been reported in body fluids [11].

Samples from microsomal incubations with N4-acetyl-SDD were analysed using HPLC with diode-array detection. Peaks with spectra similar to SDD-OH and SDD-CH_2OH were designated N4AcOH and N4AcCH_2OH, respectively. The corresponding retention times were 12 and 14 min.

In microsomal incubations with SDD, only SDD-OH and SDD-CH_2OH were found (Figs. 2c and 6). No peak corresponding to carboxy-SDD could be detected. The formation of carboxy-SDD is possibly a minor route of SDD metabolism in the rat and goat, although extraction procedures such as that reported here may be unsuitable for this compound, which may be charged over the whole pH range. The compound was not included further in this work.

The deamination of SDD [23–26] is a route of non-enzymatic SDD decomposition, rather than a biotransformation pathway. The deamination product is,

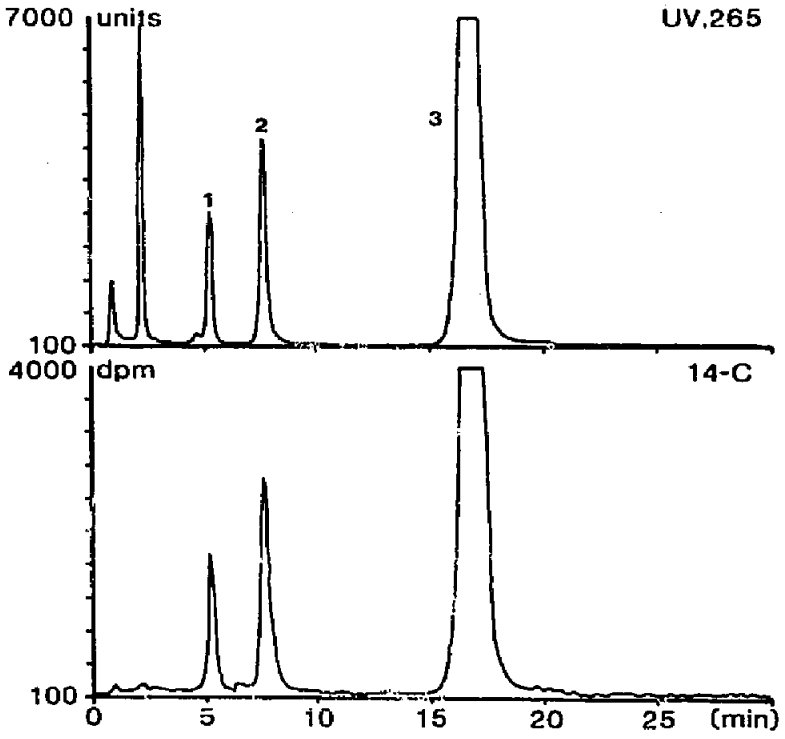

Fig. 6. Comparative analysis using simultaneous UV detection at 265 nm and radiochemical detection. Samples were taken after incubating goat microsomes with 0.2 m*M* [^{14}C]SDD, diluted with non-labelled SDD to an activity of 20 kBq. Peaks: 1 = SDD-OH; 2 = SDD-CH_2OH; 3 = SDD.

therefore, not of primary interest in drug metabolism studies, although significant amounts may be deteted in food of animal origin.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the outstanding technical assistance of R. D. van Ooyen (NIDDR, Utrecht, Netherland), F. A. W. Koeman (Department of Bio-organic Chemistry, Utrecht, Netherlands) and H. J. Kappert (Department of Animal Nutrition, Utrecht, Netherlands). The authors thank Dr. L. A. P. Hoogenboom (RIKILT, Wageningen, Netherlands), Dr. P. A. Soons, Professor J. Noordhoek (RITOX, Utrecht, Netherlands) and Professor A. S. J. P. A. M. van Miert (Department of Veterinary Pharmacology, Utrecht, Netherlands) for valuable discussions and cooperation.

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