Journal of Chromatography, 423 (1987) 227–237
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3902

SENSITIVE AND SPECIFIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE QUANTIFICATION OF SULFORIDAZINE AND TWO DIASTEREOMERIC SULFORIDAZINE-5-SULFOXIDE METABOLITES IN PLASMA

D.A. GANES and K.K. MIDHA*

College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Canada)

(First received March 26th, 1987; revised manuscript received July 28th, 1987)

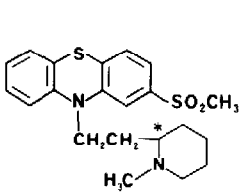
SUMMARY

A sensitive and specific procedure using high-performance liquid chromatography for the quantification of sulforidazine and two diastereomeric sulforidazine-5-sulfoxide metabolites in plasma was developed. Sulforidazine was first extracted from basified plasma using a mixture of pentane and 2-propanol. Sulforidazine-5-sulfoxide metabolites were then extracted from the same basified plasma using a second solvent mixture consisting of methylene chloride, pentane and 2-propanol. Each organic extract was subsequently back-extracted separately with 0.1 *M* hydrochloric acid, basified and re-extracted with the original solvent mixtures. In order to avoid interferences due to hydroxylated metabolites co-eluting with sulforidazine-5-sulfoxides in the more polar extract, this extract was derivatized with N-methyl-(*tert.*-butyldimethylsilyl) trifluoroacetamide. The two extracts were separately chromatographed on a narrow-bore nitrile column using ultraviolet detection. The quantification limits for sulforidazine and two diastereomeric sulforidazine-5-sulfoxide metabolites were 1.0 ng/ml with mean intra-assay coefficients of variation less than 10%. These methods were applied to the analysis of plasma from a dog following the administration of a single oral dose (25 mg of the base) of sulforidazine hydrochloride.

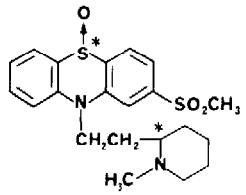
INTRODUCTION

Sulforidazine (I, Fig. 1, Inofal®, F.R.G.) is a potent piperidine ring phenothiazine used in the treatment of schizophrenic disorders [1,2]. Metabolites that have been identified in man include racemic sulforidazine-5-sulfoxide (IIa and IIb, Fig. 1), 7-hydroxysulforidazine and N-desmethyl-7-hydroxysulforidazine [3]. Sulforidazine is also a principal metabolite of mesoridazine (Serentil®, Canada) and thioridazine (Mellaril®, Canada) [3], and is considered to be pharmacologically active with approximately four to ten times the potency of thioridazine and mesoridazine [1,4].

0378-4347/87/$03.50

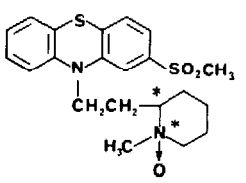


(I)

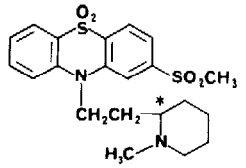


(IIa, fast eluter)

(IIb, slow eluter)



(III, unresolved)



(IV)

Fig. 1. Structures of sulforidazine (I), two diastereomeric sulforidazine-5-sulfoxides (IIa, fast eluter; IIb, slow eluter), the unresolved diastereomeric sulforidazine-N-oxides (III) and sulforidazine-5-sulfone (IV); * = chiral center.

Analytical methods employing high-performance liquid chromatography (HPLC) have been previously reported for the quantification of thioridazine, mesoridazine, sulforidazine and two diastereomeric thioridazine-5-sulfoxides in plasma following the administration of thioridazine [5–11]. There are no known methods to quantify diastereomeric sulforidazine-5-sulfoxides in plasma following the administration of sulforidazine.

A sensitive and specific HPLC assay for the quantification of sulforidazine and two diastereomeric sulforidazine-5-sulfoxide metabolites in plasma has been developed. This method requires a single 2.0-ml plasma sample and can quantify 1.0 ng/ml of each of the analytes I, IIa and IIb with intra-assay coefficients of variation less than 10%.

EXPERIMENTAL

Reagents and chemicals

Pentane, 2,2,4-trimethylpentane, methylene chloride, methanol, acetonitrile, 2-propanol, diethylamine, sodium carbonate and antibumping granules were purchased from BDH (Toronto, Canada). Hydrochloric acid (12 *M*) was obtained from Fisher Scientific (Edmonton, Canada). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford,

IL, U.S.A.). Sulforidazine and sulforidazine-5-sulfone were a gift from Sandoz (Basle, Switzerland and East Hanover, NJ, U.S.A.). Racemic sulforidazine-5-sulfoxide and sulforidazine-N-oxide were synthesized in this laboratory and will be reported elsewhere. All chemicals used were of analytical-reagent grade.

High-performance liquid chromatography

The HPLC instruments included: a Waters Assoc. pump (Model M-45) and ultraviolet (UV) absorbance detector (Model 440, Mississauga, Canada); a Rheodyne injector (Model 7125, 20-μl loop, Cotati, CA, U.S.A.); and a Shimadzu integrator (Model C-R3A, Kyoto, Japan). A Beckman Instruments nitrile HPLC column was used (5-μm particle size, 15 cm$\times$2 mm I.D., Berkeley, CA, U.S.A.). The mobile phase was 2,2,4-trimethylpentane–methylene chloride–methanol–acetonitrile–diethylamine (50:20:20:10:0.001, v/v). Other HPLC conditions included a mobile phase flow-rate of 0.5 ml/min, ambient column temperature and UV absorbance measurement at 254 nm.

Extraction

Method A: extraction of sulforidazine. In a PTFE-lined screw-capped glass tube (15 ml), add sample plasma (2 ml), aqueous internal standard amitriptyline (1 ml, 2000 ng), saturated sodium carbonate solution (0.5 ml, pH 12) and a solvent mixture consisting of 5% 2-propanol in pentane (10 ml). Mix (10 min, IKA-Vibrax-VXR shaker, Janke and Kunkel) and centrifuge (1725 *g*, 5 min, Beckman Instruments Model TJ-6RS, Palo Alto, CA, U.S.A.). Transfer the organic layer by pasteur pipette to a clean tube (15 ml) Save the aqueous plasma layer for extraction method B. Add 0.1 *M* hydrochloric acid (0.5 ml) to the separated organic extract, mix (10 min) and then centrifuge (5 min). Discard the organic layer. Add saturated sodium carbonate solution (0.5 ml) to the aqueous acid layer and extract with the same solvent mixture by mixing (10 min) and centrifuging at 1725 *g* (5 min). Transfer the organic layer to a clean tube (15 ml), add a few antibumping granules and evaporate to dryness (dry bath, 40–65°C). Cap the tube and store (−20°C). Reconstitute the sample residue in methanol (10 μl) prior to HPLC analysis.

Method B: extraction of diastereomeric sulforidazine-5-sulfoxides. To the aqueous plasma layer from method A are added aqueous internal standard imipramine (1 ml, 1 μg) and a solvent mixture consisting of methylene chloride–pentane–2-propanol (49:46:5; 10 ml). Extract by mixing (10 min), then centrifuge (5 min). Transfer the organic layer to a clean tube (15 ml). Add 0.1 *M* hydrochloric acid (0.5 ml) to the separated organic extract, mix (10 min) and centrifuge (5 min). Discard the organic layer. Add saturated sodium carbonate solution (0.5 ml) and the same solvent mixture (10 ml) to the aqueous acid layer. Mix (10 min) and centrifuge (5 min). Transfer the organic layer to a clean tube (15 ml), add several antibumping granules and evaporate to dryness (dry bath, 40–65°C). Reconstitute the evaporated residue in dry acetonitrile (100 μl) and add MTBSTFA (25 μl). Mix (30 s) and incubate (dry bath, 65°C, 1 h). Evaporate the reaction mixture to dryness under nitrogen, add saturated sodium carbonate solution (0.5 ml) and the same solvent mixture (10 ml). Mix (10 min) and centrifuge (5 min). Transfer the organic layer to a clean tube (15 ml). Add a few antibumping gran-

ules and evaporate to dryness (dry bath, 40–65°C). Cap the tube and store (−20°C). Reconstitute the sample residue in methanol (10 μl) prior to HPLC analysis.

Extraction efficiencies

Method A. Blank plasma samples spiked with sulforidazine (10 ng/ml) and the internal standard amitriptyline (100 ng/ml) were extracted using method A. Imipramine (200 ng) was used as an external standard in the final evaporation.

Method B. Blank plasma samples spiked with two diastereomeric sulforidazine-5-sulfoxides (10 ng/ml) and the internal standard imipramine (500 ng/ml) were extracted using method B. Amitriptyline (200 ng) was used as an external standard in the final evaporation.

Application of the methods

One over-night fasting mongrel dog (25 kg) was administered sulforidazine hydrochloride (25 mg of the base) as an aqueous solution (10 ml) by stomach intubation (polypropylene syringe, 10 ml, Plastipak®, Becton Dickinson, Mississauga, Canada; feeding tube, gauge 10, length 1.07 m, Argyle®, Brunswick, St. Louis, MO, U.S.A.). The stomach tube was subsequently washed twice with water (10 ml). Venous blood samples (20 and 10 ml, 0–72 h) were obtained in polypropylene syringes (10 ml) and transferred to heparinized glass tubes (Vacutainer®, Becton Dickinson). The samples were centrifuged (1725 *g*, 10 min, 4°C), and the plasma was stored in glass vials (20 ml, −20°C) until analysis.

RESULTS AND DISCUSSION

Control blank plasma, blank plasma spiked with sulforidazine and two diastereomeric sulforidazine-5-sulfoxides and plasma from a dog dosed orally with sulforidazine hydrochloride were extracted (method A) and analyzed by HPLC. Typical chromatograms (Fig. 2) show that there were no endogenous peaks in the blank plasma extract (Fig. 2A) which had an identical retention time as sulforidazine in either the spiked or dosed plasma extracts (peak b, Fig. 2B and C).

A calibration curve (Table I) for sulforidazine was obtained by fitting the function $y=a+bx+cx^2$ to the values of peak-height ratio (drug/internal standard, y) and plasma drug concentration (1–400 ng/ml, x) using a polynomial regression computer program [12]. Fitting this equation to the unweighted data resulted in a more uniform distribution of residuals and a superior fit for the lower concentrations compared to linear regression. It was also found that there was little difference in the polynomial regression parameters after using unweighted plasma concentrations and peak-height ratio data compared to using data weighted with the inverse of the peak-height ratio. Unknown concentrations (x) were then determined by solving the equation: $x=\{-b+[b^2-4(a-y)c^{1/2}]\}/2c$, where a, b and c were regression coefficients and y was the known peak-height ratio. The intra-assay precision for the quantification of sulforidazine, as approximated by the mean coefficient of variation for the sulforidazine calibration curve, was 4.7% (Table I).

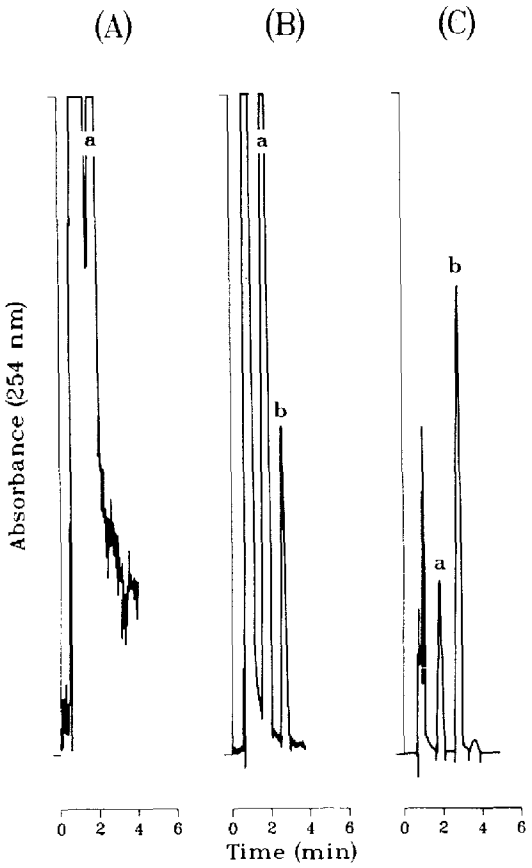


Fig. 2. Chromatograms of plasma extracts (method A): (A) blank plasma; (B) plasma spiked with sulforidazine (peak b, 10 ng/ml); (C) plasma (t=3.5 h) from a dog (25 kg) dosed orally with (25 mg of the base) of sulforidazine hydrochloride. In each chromatogram peak a is the internal standard amitriptyline.

Potential metabolites of sulforidazine include (Fig. 1) diastereomeric sulforidazine-5-sulfoxides (IIa and IIb), diastereomeric sulforidazine-N-oxides (III, unresolved) and sulforidazine-5-sulfone (IV). During the development of extraction method B, plasma from a dog orally dosed with sulforidazine was extracted and analyzed by HPLC without prior derivatization with MTBSTFA. Peaks corresponding to either sulforidazine-N-oxide or sulforidazine-5-sulfone were not observed in any of the chromatograms. The detection limit for sulforidazine N-oxide, which had a relative retention time of 0.82 compared to sulforidazine, was 2 ng/ml. The detection limit for sulforidazine-5-sulfone was also 2 ng/ml and had a relative retention time of 1.13 compared to sulforidazine. Large peaks were observed for the two diastereomeric sulforidazine-5-sulfoxide metabolites. However, one diastereomer (IIa) appeared to be present in much greater concentration than the other. Subsequent collection of the HPLC eluent and analysis of the residue using gas–liquid chromatography–mass spectrometry indicated that the diastereomer IIa was co-eluting with several hydroxylated me-

TABLE I

CALIBRATION CURVE DATA FOR SULFORIDAZINE (I)

$y=a+bx+cx^2$; $a=1.580$, $b=1.124$, $c=-0.00008359$; $r^2=0.9998$; $n=6$.

Spiked plasma concentration (ng/ml)	Mean peak- height ratio (×100)	Coefficient of variation (%)
1	1.871	7.6
2	4.386	7.8
5	5.784	3.2
10	12.95	3.8
25	29.31	2.0
50	57.36	6.2
100	118.1	2.9
200	219.4	5.3
400	438.2	3.6
Mean		4.7

tabolites, one being phenolic in nature. The structures of these metabolites will be reported elsewhere. In order to resolve diastereomer IIa from other co-eluting hydroxylated metabolites, the extracts were derivatized with MTBSTFA. This derivatizing agent was used because the sulforidazine-5-sulfoxide metabolites would not react whereas the hydroxylated metabolites would. Additionally, *tert.*-butyldimethylsilyl derivatives are sterically hindered and avoid hydrolysis.

The absorption maximum for sulforidazine in methanol has been reported to be 262 nm [5], whereas the absorption maximum for a mixture of equal proportions of the two diastereomeric sulforidazine-5-sulfoxides in methanol was determined to be 244 nm. Therefore, a fixed-wavelength UV detector with a 254-nm filter which provided adequate sensitivity for each analyte (1 ng/ml) was used in the present study as a suitable compromise between the two wavelength absorption maxima. Also this type of detector is generally readily available in most analytical laboratories.

Control blank plasma, blank plasma spiked with sulforidazine and two diastereomeric sulforidazine-5-sulfoxides and plasma from a dog dosed orally with sulforidazine hydrochloride were extracted (method B) and analyzed by HPLC. Typical chromatograms (Fig. 3) show that there were no endogenous peaks in the blank plasma extract (Fig. 3A) which had identical retention times as the two diastereomeric sulforidazine-5-sulfoxides in either the spiked or dosed plasma extract (peaks c and d, Fig. 3B and C).

Calibration curves (Tables II and III) for the diastereomeric sulforidazine-5-sulfoxides were obtained by fitting the function $y=a+bx+cx^2$ to the values of peak-height ratio (metabolite/internal standard y) and plasma metabolite concentration (1–50 ng/ml, x). Fitting this equation to the unweighted data as well as data weighted with the inverse of the peak-height ratios gave polynomial

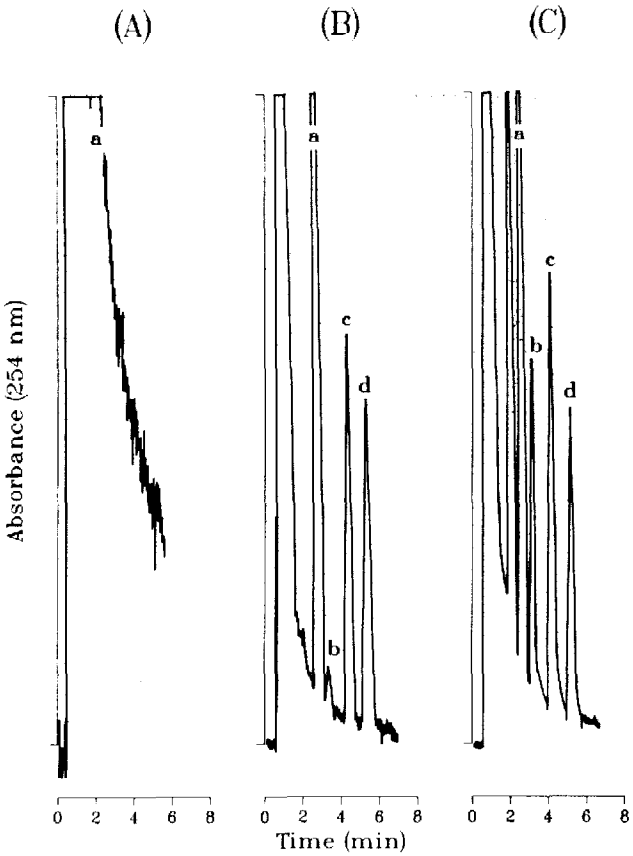


Fig. 3. Chromatograms of plasma extracts (method B): (A) blank plasma; (B) plasma spiked with two diastereomeric sulforidazine-5-sulfoxide metabolites, IIa (peak c) and IIb (peak d), both 10 ng/ml; (C) plasma ($t=2.5$ h) from a dog dosed orally with (25 mg of the base) of sulforidazine hydrochloride. In each chromatogram peak a is the internal standard imipramine. In chromatograms B and C, peak b is residual sulforidazine (method A).

regression parameters which showed little difference. The intra-assay for the quantification of the two diastereomeric sulforidazine-5-sulfoxides (IIa, IIb), as approximated by the mean coefficient of variation for the diastereomers' calibration curves, were 6.5 and 6.1%, respectively (Tables II and III).

Sulforidazine, two diastereomeric sulforidazine-5-sulfoxides and the internal standards amitriptyline and imipramine were added to blank plasma, extracted (methods A and B) and analyzed by HPLC. External standards were added in the final evaporation step. The extraction efficiencies were determined by comparing the peak-height ratio (drug, metabolite or internal standard/external standard) of the extracted sample to that of a non-extracted sample. The mean extraction efficiencies determined for sulforidazine, the diastereomeric sulforidazine-5-sulfoxides and the internal standards amitriptyline and imipramine were 63.5, 79.3, 91.7, 71.3 and 5.3%, respectively (Table IV) with coefficients of variation less than 15% in each case.

The inter-assay coefficients of variation for the quantification of sulforidazine and two diastereomeric sulforidazine-5-sulfoxides, as shown by the coefficients

TABLE II

CALIBRATION CURVE DATA FOR SULFORIDAZINE-5-SULFOXIDE (IIa)

$y=a+bx+cx^2$; $a=-0.1309$, $b=1.214$, $c=-0.006118$; $r^2=0.9994$; $n=6$.

Spiked plasma concentration (ng/ml)	Mean peak- height ratio (×100)	Coefficient of variation (%)
1	1.262	9.2
2	2.608	2.9
5	5.615	8.2
10	10.67	3.0
25	26.85	9.2
50	45.18	6.6
Mean		6.5

TABLE III

CALIBRATION CURVE DATA FOR SULFORIDAZINE-5-SULFOXIDE (IIb)

$y=a+bx+cx^2$; $a=0.0009495$, $b=1.020$, $c=-0.005213$; $r^2=0.9995$; $n=6$.

Spiked plasma concentration (ng/ml)	Mean peak- height ratio (×100)	Coefficient of variation (%)
1	1.131	4.8
2	2.264	4.5
5	4.890	8.1
10	9.114	3.4
25	22.57	8.3
50	37.88	7.6
Mean		6.1

TABLE IV

EXTRACTION EFFICIENCIES FOR SULFORIDAZINE, TWO DIASTEREOMERIC SULFORIDAZINE-5-SULFOXIDES AND THE INTERNAL STANDARDS AMITRIPTYLINE AND IMIPRAMINE ($n=6$)

Drug, metabolite or internal standard	Spiked plasma concentration (ng/ml)	Efficiency (%)	Coefficient of variation (%)
Sulforidazine (I)	10	63.5	3.8
Sulforidazine-5-sulfoxide (IIa)	10	79.3	13.8
Sulforidazine-5-sulfoxide (IIb)	10	91.7	14.7
Amitriptyline	100	71.3	5.3
Imipramine	500	5.3	3.9

TABLE V

INTER-ASSAY COEFFICIENTS OF VARIATION FOR ESTIMATED PLASMA CONCENTRATIONS OF SULFORIDAZINE AND TWO DIASTEREOMERIC SULFORIDAZINE-5-SULFOXIDES ($n=5$)

Drug or metabolite	Spiked plasma concentration (ng/ml)	Mean estimated plasma concentration (ng/ml)	Coefficient of variation (%)
Sulforidazine (I)	10	10.0	8.3
Sulforidazine-5-sulfoxide (IIa)	10	9.6	13.1
Sulforidazine-5-sulfoxide (IIb)	10	9.6	14.0

of variation for estimated concentrations of spiked (10 ng/ml) plasma samples for five consecutive assays, were 8.3, 13.1 and 14.0%, respectively (Table V).

Thioridazine, mesoridazine and two separate thioridazine-5-sulfoxide metabolites have been shown to be stable for at least three weeks in frozen plasma and at least 24 h in plasma at room temperature [6]. Analysis of sulforidazine human plasma samples stored at $-20°C$ for two years have been found to be stable. In a separate study carried out in these laboratories [13], human volunteers were dosed with thioridazine and it was observed that the analysis of plasma samples ($n=55$) for sulforidazine (formed from thioridazine) by HPLC and re-analysis of the same samples after storage at $-20°C$ two years later by a newly developed radioimmunoassay gave a good correlation ($r^2=0.926$, $p<0.001$) and satisfactory regression (slope of the least-squares line 0.963). Aqueous solutions of sulforidazine hydrochloride were stable at room temperature for at least four weeks, while methanolic solutions of the two diastereomeric sulforidazine-5-sulfoxides were stable for at least six months at $-20°C$. The stabilities of the two diastereomeric sulforidazine-5-sulfoxides in frozen plasma were not formally ascertained. However, it would be surprising if the 5-sulfoxides of sulforidazine were less stable than the 5-sulfoxides of thioridazine and mesoridazine, considering that the sulfone moiety, unlike the sulfide and sulfoxide moieties, has been shown to be stable to both enzymic and chemical oxidation and reduction [3,13,14].

The application of the present methods to the quantification of sulforidazine and its two diastereomeric sulforidazine-5-sulfoxide metabolites in plasma is demonstrated (Fig. 4). The sensitivities of the HPLC assays were such that the plasma concentrations of both the diastereomers could be followed up to 25 h in this mongrel dog which was administered a single oral dose (25 mg of the base) of sulforidazine hydrochloride. Fig. 4 shows plots of logarithmic plasma concentration versus time for sulforidazine and its two diastereomeric sulforidazine-5-sulfoxide metabolites. Both the diastereomers were quantifiable at 1 h and were present in all the plasma samples up to 25 h in nearly equal concentrations. However, it should be emphasized that the fast eluting diastereomeric 5-sulfoxide (IIa) appeared to be present in slightly greater concentrations than the IIb stereomer in all the plasma samples quantified.

In summary, the HPLC analytical methods presented are simple, sensitive and

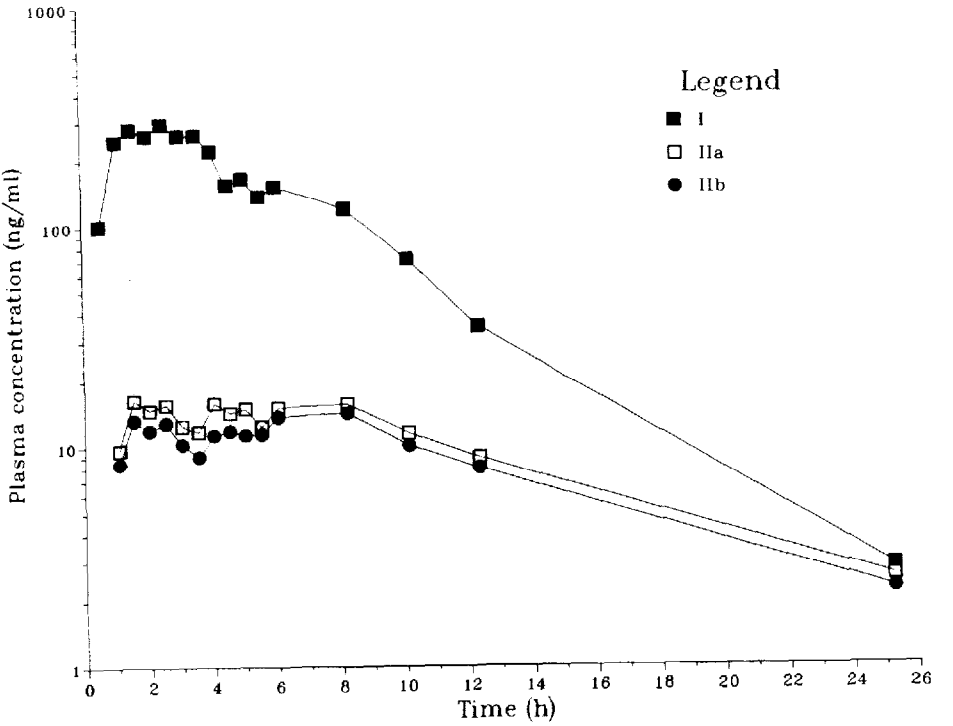


Fig. 4. Profiles of plasma concentration versus time for sulforidazine (I), and two diastereomeric sulforidazine-5-sulfoxide metabolites (IIa and IIb) following oral administration (25 mg of the base) of sulforidazine hydrochloride to one dog.

specific for the quantification of sulforidazine and diastereomeric sulforidazine-5-sulfoxides in plasma. These methods are presently being applied to a study of the effect of route of administration on sulforidazine pharmacokinetics in the dog, with particular emphasis on the effect of route on the extent and stereochemistry of 5-sulfoxidation. These methods could also be applied to studying the pharmacokinetics of sulforidazine and its metabolites in human volunteers and patients.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the Medical Research Council of Canada for the studentships to Derek Ganes and the Program Grant (PG-34). We wish to thank Sandoz (Switzerland and U.S.A.) for the gifts of sulforidazine and sulforidazine-5-sulfone. Drs. J.W. Hubbard, G. McKay and E.M. Hawes are acknowledged for their helpful comments and criticisms.

REFERENCES

1 R. Axelsson, Curr. Ther. Res., 21 (1977) 587.
2 D.M. Niedzwicki, R.B. Mailman and L.X. Cubeddu, J. Pharmacol. Exp. Ther., 228 (1984) 636.
3 A.S. Papadopoulos, J.L. Crammer and D.A. Cowan, Xenobiotica, 15 (1985) 309.
4 C.D. Kilts, D.L. Knight, R.B. Mailman, E. Widerlov and G.R. Breese, J. Pharmacol. Exp. Ther., 231 (1984) 334.
5 C.D. Kilts, K.S. Patrick, G.R. Breese and R.B. Mailman, J. Chromatogr., 231 (1982) 377.
6 C.E. Wells, E.C. Juenge and W.B. Furman, J. Pharm., 72 (1983) 622.
7 P.W. Hale and A. Poklis, J. Chromatogr., 336 (1984) 452.
8 P.W. Hale and A. Poklis, J. Anal. Toxicol., 9 (1985) 197.
9 W.J. Allender, J. Chromatogr. Sci., 23 (1985) 541.
10 G. McKay, J.K. Cooper, T. Gurnsey and K.K. Midha, LC, Liq. Chromatogr. HPLC Mag., 3 (1985) 256.
11 C.N. Svendsen and E.D. Bird, Psychopharmacology, 90 (1986) 316.
12 R. Jennrich and P. Mundle, in W.J. Dixon and M.B. Brown (Editors), Biomedical Computer Programs P Series, University of California Press, Berkeley, CA, 1985, pp. 283–286.
13 B.S. Chakraborty, E.M. Hawes and K.K. Midha, Ther. Drug Monit., in press.
14 J. March, Advanced Organic Chemistry: Reactions, Mechanism and Structure, McGraw-Hill, Toronto, 1977.