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RAPID, SENSITIVE AND FULLY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH FLUORESCENCE DETECTION FOR SULMAZOLE AND METABOLITES

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SUMMARY

Sulmazole {2-[(2-methoxy-4-methylsulfinyl)phenyl]-3H-imidazo [4,5-b] pyridine; AR-L 115 BS} and two metabolites (sulfide, sulfone) were quantified from directly injected body fluids (plasma, urine, bile) after high-performance liquid chromatographic separation. No internal standard is needed, which is particularly advantageous when fluorescence detection is established. After automated pre-column enrichment on Corasil C_{18} (37—50 μm), the parent compound and biotransformation products could be backflushed and chromatographed on ODS-Hypersil (5 μm) with a mixture of 0.075 mol/l phosphate buffer—acetonitrile (2:1), an elution rate of 2.0 ml/min and fluorimetric detection (λ_{ex} = 330 nm; λ_{em} = 370 nm). A hydroxylated metabolite of sulmazole which occurs preferentially in urine (and bile) can be quantified in the above-mentioned solvent system diluted 1:1 with water, but with different fluorescence characteristics (λ_{ex} = 345 nm; λ_{em} = 515 nm). The assay was linear in the range 8—1000 ng/ml. The lower limit of detection was about 8 ng/ml or 80 pg with coefficients of variation between 0.4 and 5.8% for sulmazole.

INTRODUCTION

Sulmazole* is a new potent cardiotonic drug which combines a positive inotropic and vasodilatory action in animals [1—4] and man [5—8]. For optimal drug therapy and pharmacokinetic investigations it is necessary to set up a rapid, simple, selective and practicable assay for the pharmacologically active species in body fluids. In general it is also desirable to detect, when possible, simultaneously occurring plasma metabolites to relate any as yet

*Commercial name: Vardax®. Manufacturer: Dr. Karl Thomae GmbH, Biberach/Riss, F.R.G.

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unknown (side) effects to the amounts of metabolites in the body or to assess deviations caused by drug interactions.

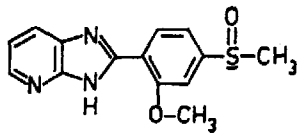
The present paper describes a high-performance liquid chromatographic (HPLC) assay utilising the principle of "alternated pre-column enrichment" which we have described elsewhere [9]. This assay is applicable for routine drug monitoring in pharmacokinetic or clinical investigations. Concentrations of sulmazole and two less-active plasma metabolites, AR-L 114 BS (sulfone of sulmazole) and AR-L 113 BS (sulfide of sulmazole), are simultaneously detected.

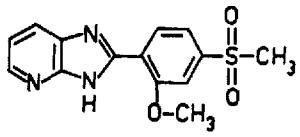
EXPERIMENTAL

Reagents

The amount of 13.8 g disodium hydrogen phosphate dihydrate, molecular weight (MW) 177.99 (Merck No. 6580) and 9.1 g of sodium dihydrogen phosphate, MW 136.0 (Merck No. 4873) were each dissolved in 2000 ml of deionized water. The pH was adjusted to 6.8 with 85% phosphoric acid (Merck No. 573). Acetonitrile LiChrosolv (Merck No. 30) was used as organic solvent. The chromatographic cocktail contained one part of each buffer solution and one part of acetonitrile. Reversed-phase materials for the HPLC columns were Bondapak C_{18} Corasil, 37—50 μm (Waters No. 27248) and ODS-Hypersil, 5 μm (Shandon No. 580X 5).

Reference compounds of sulmazole (Lot No. FNTK 775), and metabolites AR-L 114 BS and AR-L 113 BS, and the hydroxylated metabolite M2 were from Dr. K. Thomae, Biberach, F.R.G. (Fig. 1).





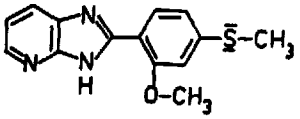


Fig. 1. Formulae of sulmazole and metabolites.

HPLC equipment

A diagram of the equipment is given in Fig. 2. A single piston pump, Model 410 (Kontron, Switzerland) was used as pump A delivering deionized water

with a flow-rate of 1.5 ml/min. A constant flow pump, Model 600/200 (Gynkotek, F.R.G.) was used as pump B. This pump delivered the eluent for backflush elution of the enriched material from the pre-columns (4 cm × 0.46 cm I.D.; Bischoff, F.R.G.; dry-packed with C_{18} Corasil, particle size 37—50 μm) to the analytical column (12.5 cm × 0.46 cm I.D.; Bischoff; packed with ODS-Hypersil; Shandon, U.K.; particle size 5 μm). A guard column (2 cm × 0.46 mm I.D.; Bischoff) filled with 5-μm ODS-Hypersil was used to protect the analytical column. The composition of the eluent was 0.075 mol/l phosphate buffer—acetonitrile (2:1; v/v) pH 6.8 with a flow-rate of 2.0 ml/min. Samples were injected by a WISP 710 B autosampler (Waters, U.S.A.). An SM-2 (Gynkotek) column switching module was used for the column switching technique.

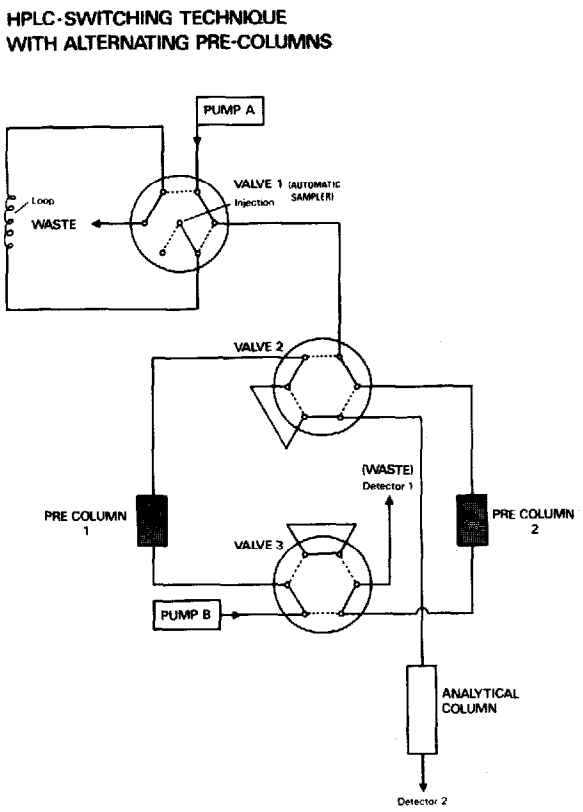


Fig. 2. HPLC column switching technique with alternated pre-column sample enrichment [9].

The compounds were detected with a fluorescence spectrophotometer, Model 650-10 LC (Perkin Elmer, F.R.G.). Wavelengths for sulmazole and metabolites were 330 and 370 nm, and for the hydroxylated metabolite 345 and 515 nm. The detector was adjusted to 5 nm slit width for excitation and emission; output 10 mV, range 1; multiplier gain, response and sensitivity mode normal. Signals were recorded with an integrator/plotter Model 3388 A (Hewlett-Packard, U.S.A.).

Alternated pre-column enrichment system [9]

An autosampler (WISP 710 B, Waters or Model SS 100, Perkin-Elmer), designated valve 1, is connected with a six-way Rheodyne valve (valve 2) which delivers alternately, depending on its switching position, a sample by means of pump A to one of the two pre-columns. These two pre-columns are mounted parallel with another valve (valve 3) which distributes the chromatographic eluent of a second pump (pump B) alternately to one of the pre-columns (backflushing). Both valves are simultaneously activated by a programmable time function of the integrator. This assembly of valves 2 and 3 is now commercially available (e.g. Gynkotek, Switching Module 2).

The samples of pure body fluids are directly injected (5—50 μl) by an autosampler into the enrichment pre-column system and washed with water (2—4 min) in order to guarantee separation of the additional water-soluble components such as salts and proteins. These components are flushed into waste under the described conditions. After this loading and enrichment step, the enrichment pre-column system is switched in the eluent stream of pump B, initiated by a programmable internal time event function of the integrator. Now the enriched material is backflushed onto the analytical column in order to obtain proper separation of the components. A second enrichment pre-column (Fig. 2) is mounted such that when one pre-column is loaded with a sample from the autosampler, the second pre-column, which had been loaded before, is in the backflush mode. With the assembly of a set of two pre-columns which are used in an alternated mode, we save time for the analysis. The working cycle of the pre-columns thus consists of an equilibration step, i.e. flushing with the eluent of pump A for 1—2 min in order to equilibrate the reversed-phase material, followed by the loading or enrichment step with an additional flushing period with water for another 2—4 min. After backflushing the enriched material with the eluent of pump B, the pre-column is again switched back into the eluent stream of pump A which delivers water to re-equilibrate the pre-column for the next sample injection.

Technical recommendations for alternating column switching

For the successful operation of alternating column switching from biological fluids, several practical points should be observed:

(1) The steel capillaries from the autoinjector to the enrichment columns should be of an inner diameter (I.D.) of 0.5 mm instead of 0.25 mm, whereas the capillaries from the pre-columns to the analytical column should be of 0.25 mm I.D.

(2) Do not use commercially available inlet frits in capillaries where biological material is transported, since they might block the solvent stream.

(3) Special attention should also be paid to the metal sieves for the pre-columns, which should not be too fine. Sieves of at least 18 μm pore size are recommended with an additional glass fibre filter.

(4) In order to guarantee a homogeneous distribution of the administered plasma on top of the pre-column, crossed grooves should be filed onto the surface of the end fitting which closes the top of each pre-column. This is important to allow immediate distribution of the arriving plasma bolus over the whole top surface of the enrichment column. Without a solvent distribution

device in the connecting nut, plasma may block the frit, because it is focused only to a small area on the sieve.

(5) The analytical column should be protected by a 2—4 cm guard column in order to increase its lifetime.

Collection of samples

Blood samples should be withdrawn with heparinized syringes (e.g. heparinized Monovette, Sarstedt, F.R.G.). Freshly prepared plasma can be immediately injected in the HPLC system. Frozen plasma samples should be brought to ambient temperature, thoroughly shaken and centrifuged in order to prevent blocking of the HPLC capillaries.

Urine or bile can be directly injected (5—10 μl).

RESULTS AND DISCUSSION

Chromatograms

Typical chromatograms of sulmazole and metabolites from directly injected plasma are shown in Fig. 3. The integrator starts drawing the chromatograms immediately on backflush of the enriched drugs from the pre-columns to the analytical column. Therefore the recorded apparent retention times do not reveal the whole time needed for one chromatogram. The time for washing the pre-columns after the plasma injection must be added, in our case 2 min, to obtain the real time needed for a chromatographic run. Thus, sulmazole and metabolites can be chromatographed fully automatically from body fluids in about 6 min. Apparent retention times from plasma are: sulmazole, 1.37 min;

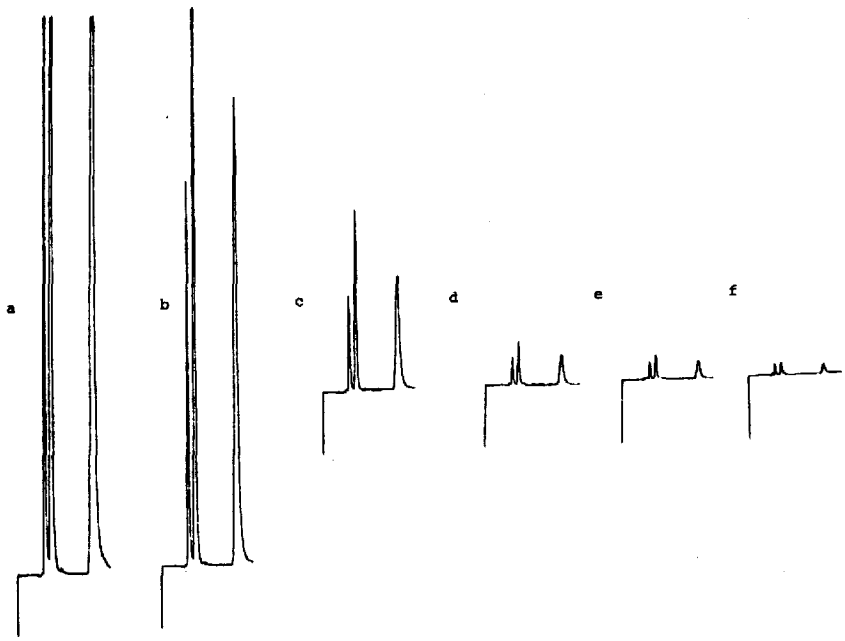


Fig. 3. HPLC chromatogram after automated pre-column sample enrichment of identical concentrations of sulmazole (I) and sulfone and sulfide metabolites (II and III) from 10 μl of directly injected plasma: 1000 ng/ml (a), 500 ng/ml (b), 125 ng/ml (c), 32 ng/ml (d), 16 ng/ml (e), 8 ng/ml (f).

AR-L 114 BS, 1.75 min; AR-L 113 BS, 3.82 min.

Chromatograms from directly injected samples of urine, bile and plasma from a pharmacokinetic investigation are shown in Figs. 4—7. It can be demonstrated that the fluorescence detection enables excellent chromatograms to be obtained from body fluids without any interference from the biological matrix.

Stability in body fluids

Sulmazole and metabolites are stable in various body fluids at 20°C and body temperature [11].

Calibration

Stock solutions of 1 mg/ml and 0.01 mg/ml in ethanol were prepared and kept in the deep freezer. Appropriate aliquots were taken for plasma standards.

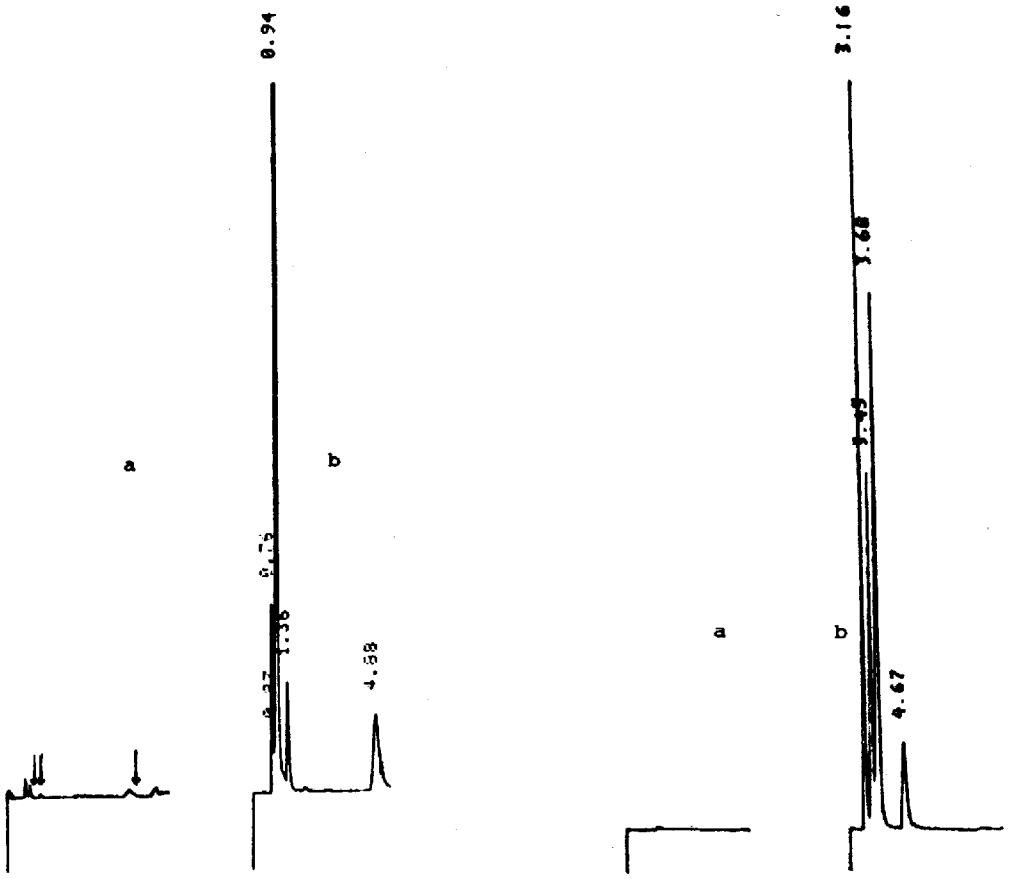


Fig. 4. HPLC chromatograms after automated pre-column sample enrichment of 5 µl blank human urine (a) and 5 µl of human urine (b) after administration of sulmazole (solvent system: 850 ml eluent + 150 ml water). Apparent retention time of 1.36 min represents parent compound.

Fig. 5. HPLC chromatograms after automated pre-column sample enrichment of blank rat bile (a) and rat bile (b) after administration of sulmazole (5 µl injected). Apparent retention time of 3.68 min represents parent compound (solvent system: 1000 ml eluent + 250 ml water).

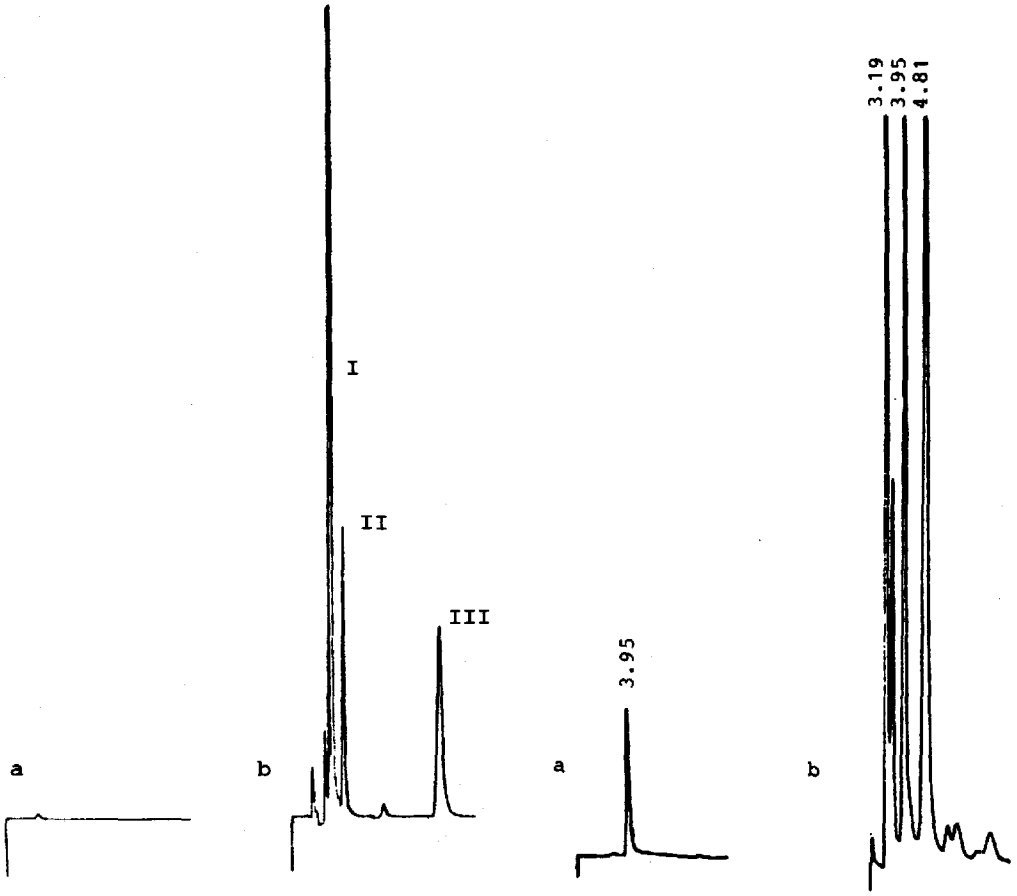


Fig. 6. HPLC chromatograms after automated pre-column sample enrichment of blank human plasma (a) and human plasma (b) after administration of sulmazole. Concentrations: I = 1745 ng/ml sulmazole, II = 137 ng/ml sulfon, and III 223 ng/ml sulfide.

Fig. 7. HPLC chromatograms after automated pre-column sample enrichment of 10 µl blank rat bile (a) and 10 µl of pooled rat bile (b) 0—2 h after intraduodenal administration of sulmazole. Retention time of 4.81 = metabolite M2 (= 6-hydroxy AR-L 115 BS). λ_{ex} = 345 nm; λ_{em} = 515 nm. Eluent = solvent system for pump B, diluted 1:1 with deionized water.

Concentrations of sulmazole and metabolites, AR-L 114 BS and AR-L 113 BS, were automatically calculated and recorded by calibrating the printer/plotter computer where peak area was used as a measurement of drug and metabolite concentration. The calibration curves were constructed from five replicate measurements of concentrations between 7.8 and 1000 ng/ml plasma (Table I), unless otherwise stated.

Machine standard

Pooled human plasma spiked with known concentrations of the three compounds was used as external and instrument standard. The standard or control samples were run between unknown plasma samples to assess precision and accuracy of the system during continuous chromatography (Table I).

TABLE I

CONCENTRATION–PEAK AREA CALIBRATION DATA OF AR-L 115 BS (SULMAZOLE) AND METABOLITES AR-L 114 BS AND AR-L 113 BS AFTER HPLC SEPARATION AND FLUORIMETRIC DETECTION FROM DIRECTLY INJECTED BODY FLUIDS

Volume injected = 10 μl.

CONCENTRATION (NG/ML)	AR-L 115 BS (VARDAX) AREA UNITS	C.V. %	METABOLITE AR-L 114 BS AREA UNITS	C.V. %	METABOLITE AR-L 113 BS AREA UNITS	C.V. %
1000	3567 3358 3314 3299 3275	0.35	8536 8460 8311 8221 8093	2.14	11948 11599 11345 11213 11168	2.82
500	1642 1629 1637 1634 1645	0.39	4074 4036 3999 3947 3937	1.45	5520 5444 5468 5369 5319	1.47
250	800 807 800 812 819	1.01	1948 1944 1928 1933 1912	0.74	2614 2625 2550 2540 2547	1.58
125	398 404 414 406 420	2.12	943 934 949 938 898	2.15	1276 1236 1231 1227 1235	1.60
62.5	197 195 210 216 223	5.8	465 467 455 466 468	1.13	633 609 607 647 643	2.99
31.3	788* 775 794	1.3	233 229 230 228 232	0.9	314 314 313 314 324	1.46
15.6	381* 377 386	1.2	110 110 110 115 121	4.34	177 161 185 151 -	9.11
7.8	226* 227 241	3.6	61 55 57 61 63	6.23	- - - - -	-
Y = a . X r = CORRELATION COEFFICIENT	a = 0.2991 r = 0.9995		a = 0.1215 r = 0.9996		a = 0.0886 r = 0.9992	

*Volume injected = 50 μl.

Precision and linearity

The coefficient of variation (C.V.) for the within-day precision of the automated assay for sulmazole, calculated on the basis of five repetitive injections per concentration, was 0.35% (1000 ng/ml), 0.4% (500 ng/ml), 1.0% (250 ng/ml), 1.3% (31 ng/ml), 1.2% (16 ng/ml) and 3.6% (8 ng/ml).

Between-day precision, characterized by the coefficient of variation (C.V. %) after five replicates in each concentration over a period of twelve months was 2.8% (1000 ng/ml), 1% (500 ng/ml) and 2% (100 ng/ml).

Pre-column enrichment — extraction efficiency

It is desirable that the HPLC assay measures total (i.e. free and bound) drug and metabolites. This requires that the pre-column strips all bound drug from plasma proteins in addition to adsorbing free drug. The total amount of drug in plasma is the sum of free drug and protein-bound drug, where the extent of reversible protein binding at equilibrium is dependent on the affinity of the drug molecule to the plasma proteins.

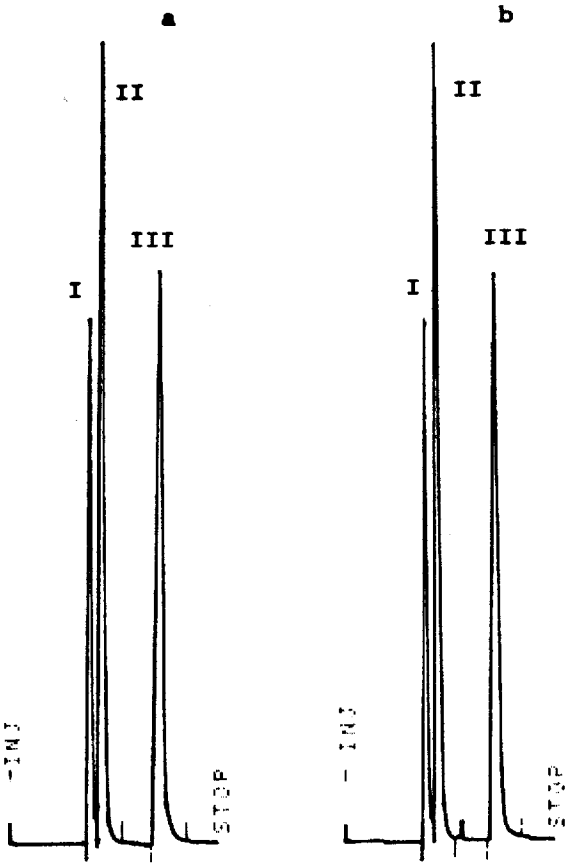


Fig. 8. HPLC chromatograms after automated pre-column sample enrichment of sulmazole (I) and sulfone and sulfide metabolites (II and III) in spiked water (a) and in human plasma (b) of 1000 ng/ml of each compound. Conditions see Fig. 7.

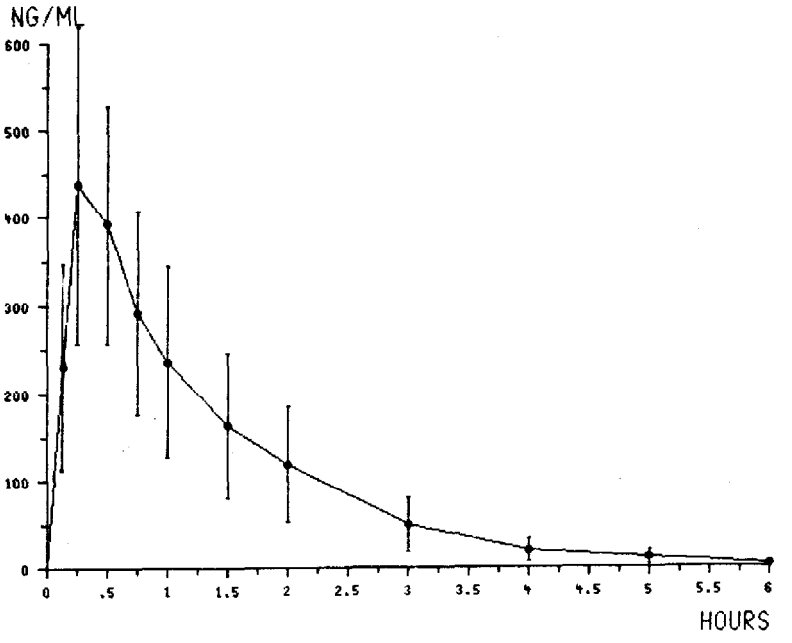


Fig. 9. Time course of sulmazole in humans (n = 8) after oral administration of a 50 mg solution.

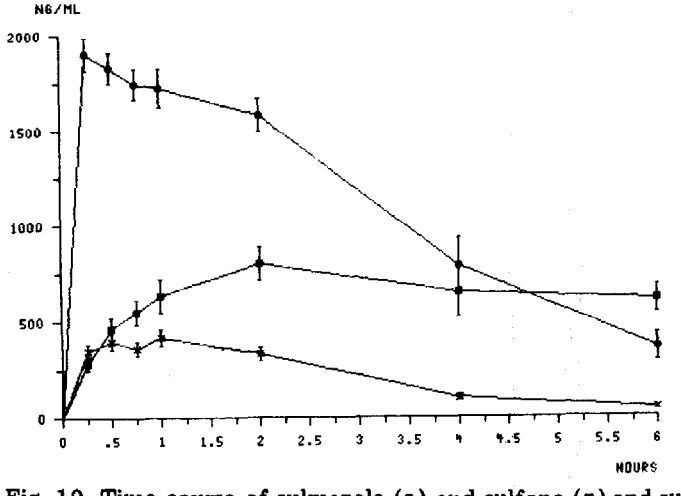


Fig. 10. Time course of sulmazole (•) and sulfone (▪) and sulfide (×) metabolites after oral administration of a 15 mg/kg aqueous solution to rats (n = 5; mean ± S.D.).

It is therefore important to check the partition characteristics of drug between binding sites on plasma proteins and on the lipophilic reversed-phase material under the conditions of the HPLC assay. The lipophilic alkyl brushes of the reversed-phase modified silica matrix should interact with the drug–protein complex and should adsorb free and protein-bound drug simultaneously to the reversed-phase matrix of the enrichment column.

In the case of the pre-column enrichment technique in automated HPLC

from directly injected plasma, it is therefore important to confirm that the binding of the reversed-phase matrix competes effectively for the plasma protein binding. This can be achieved by comparing the peak signals of spiked buffer and spiked plasma injections of the same total drug concentrations (Fig. 8).

The data show that protein-bound and free sulmazole and metabolites are quantitatively partitioned from plasma onto the pre-columns, indicating that their binding to the reversed-phase matrix is effectively infinitely greater than plasma protein interactions under these specific conditions.

Plasma level

The time course in plasma of sulmazole and metabolites, detected with the above described HPLC methodology is depicted in Figs. 9 and 10.

CONCLUSIONS

In addition to the previously reported HPLC assay of sulmazole with classical sample pre-treatment [10], we have developed an HPLC methodology which works without any manual sample pre-treatment step prior to HPLC analysis. High-performance liquid chromatographic separation with automatic alternated pre-column enrichment technique and fluorimetric detection is able to quantify sulmazole and two plasma metabolites, AR-L 114 BS (sulfone of sulmazole) and AR-L 113 BS (sulfide of sulmazole), in one HPLC run from directly injected plasma, urine or bile. Metabolite M2, as one representative of a hydroxylated sulmazole species, was detectable in rat bile and in dog urine [11].

The method is easy to handle, needs no sample pre-treatment, has a high precision and accuracy, is ideally suited for pharmacokinetic drug monitoring and thus can be immediately used for a fast individual dose titration.

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