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High-performance liquid chromatographic determination of the H^+/K^+ ATPase inhibitor (BY 1023/SK&F 96 022) and its sulphone metabolite in serum or plasma by direct injection and fully automated pre-column sample clean-up

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ABSTRACT

A fully automated high-performance liquid chromatographic method is described for the determination of the new H^+/K^+ ATPase inhibitor BY 1023/SK&F 96 022 and its major metabolite occurring in dog serum. The method uses direct sample injection of up to 200 μl and a pre-column switching technique. In order to optimize the recovery, pre-column conditions were varied systematically with respect to the pH of the pre-column eluent, its buffering capacity and content of acetonitrile. Optimization resulted in near 100% recovery for both compounds, thus allowing the use of external standardization. The linearity range, precision and detection limits were determined and the method shown to be applicable to both serum and plasma. The method was applied to define the pharmacokinetics in dogs and humans.

INTRODUCTION

Sodium 5-difluoromethoxy-2-[(3,4-dimethoxy-2-pyridyl)methylsulphinyl]-1*H*-benzimidazolate (BY 1023/SK&F 96 022, I [1]) belongs to the benzimidazole sulphoxide class of antisecretory agents [2]. Such compounds, most

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notably omeprazole, have been shown to inhibit the gastric proton-pumping enzyme H^+/K^+ ATPase by covalent binding of an acid-activated intermediate to one or more thiol groups on the enzyme [3,4]. The chemical transformations involved in the activation process have recently been elucidated [5,6].

Compound I is being developed jointly by Byk Gulden and Smith Kline and French as a new therapeutic agent for the treatment and prophylaxis of disease states resulting from excessive gastric acid secretion. It is currently undergoing Phase IIb clinical trial evaluation for oral administration. Compound I and one of its major metabolites in dogs, the corresponding sulphone II (Fig. 1), are determined in serum by high-performance liquid chromatography (HPLC) using the direct automatic injection of up to 200 μl of serum and an on-line pre-column switching technique [7,8]. The assay has been optimized with respect to recovery by variation of the pre-column work-up conditions. Serum was used for this assay because it allows a higher throughput of samples than plasma. However, it was shown that the assay can be applied to plasma in the same way.

EXPERIMENTAL

LiChroprep RP-2, 25–40 μm (Merck), was supplied by BDH (Poole, U.K.) or by E. Merck (Darmstadt, F.R.G.). Empty pre-columns for direct injection (10 mm×4.6 mm I.D.) were obtained from Bischoff (Leonberg, F.R.G.).

Standard compounds were synthesized by Byk Gulden (Konstanz, F.R.G.) and were more than 99% pure by HPLC analysis.

Reagents were supplied by May and Baker (Dagenham, U.K.), BDH or E. Merck, and were AnalaR grade unless otherwise stated.

Preparation of standard drug solutions and serum external standards

Stock standard solutions of the free acid of I or of II at a concentration of 1000 μg/ml were prepared by dissolving 10 mg in 500 μl of ethanol and 50 μl of 1 *M* sodium hydroxide, and then making up to 10 ml with filtered HPLC-grade water.

The aqueous stock solutions were diluted as appropriate to provide solutions

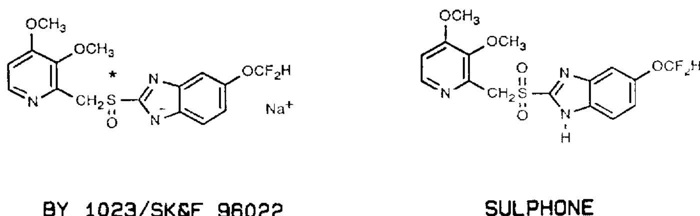

Fig. 1. Structures of I (BY 1023/SK&F 96 022) and II (sulphone metabolite).

for the preparation of the calibration curves. Serum standards were prepared from freshly prepared aqueous standards.

Stock solutions were diluted with filtered HPLC-grade water to give a concentration of 200 μg/ml. The diluted aqueous stock solutions (50 μl) were then added to control serum (950 μl) to give a total volume of 1.0 ml, the final concentration being 10 μg/ml. To avoid precipitation the standard solutions were spiked into control serum as soon as possible after dilution.

Apparatus

Some of the measurements were made with HPLC equipment consisting of a Perkin-Elmer Series 4 HPLC system, an ISS-100 autosampler (Perkin-Elmer, Beaconsfield, U.K.) and a Waters Assoc. Model 590 pump (Millipore, Harrow, U.K.). A second system consisted of a Hewlett-Packard liquid chromatograph 1084B (Hewlett-Packard, Waldbronn, F.R.G.). Both systems used a Kratos Spectroflow 783 or 757 detector (Severn Analytical, Shefford, U.K. or Kratos, Karlsruhe, F.R.G.) monitoring at 290 nm. A LiChroprep RP-2 (25–40 μm) pre-column connected across a six-port Rheodyne 7010 (Millipore or Hewlett-Packard column-switching option 79 823A for HP 1084B) was used for extraction. This column-switching arrangement has been described previously [7]. Integration was performed by means of a Beckman Gold chromatography integration system (Beckman RIIC, High Wycombe, U.K.) or the Hewlett-Packard HP 3357 laboratory data system.

The reversed-phase analytical column was pre-packed with Hypersil RP-18 (5 μm, 125 mm$\times$4.6 mm I.D.; Jones Chromatography), fitted with a guard column (12 mm$\times$4.6 mm I.D.) containing the same Hypersil phase. Alternatively, analytical and guard columns of the same dimensions as stated above were packed in the laboratory using an HPLC column-packing unit (Latek, Heidelberg, F.R.G.). For the analytical and guard columns the Hyperchrom system with sieves rather than frits at the column endings was used.

Extraction eluent and analytical column mobile phase

The extraction eluent was sodium acetate buffer (20–300 mmol, pH 5). The flow-rate was 1.5 ml/min, and any dissolved gases were removed by sparging with helium before and during use. No sparging was performed for the second HPLC system.

A gradient using methanol and diammonium hydrogenphosphate buffer (10 mmol, pH 6.5) was used as follows: 0–2 min initial conditions, methanol–buffer 43:57, v/v; 2–19 min methanol increasing linearly to 83:17, v/v; 19–21 min flushing period using 100% methanol; 21–28 min equilibrium period using initial conditions.

Before use, the methanol was filtered through 0.5-μm membrane filters (Type FH) and the phosphate buffer through 0.45-μm membrane filters (Type HA, Millipore). Alternatively, prefiltered, ready-to-use methanol of LiChrosolv®

quality (Merck) and water purified on a Milli-Q purification unit (Millipore, Eschborn, F.R.G.) were used. The flow-rate was 1 ml/min. Any dissolved gases were removed by sparging with helium before and during use, or, alternatively, a 100 cm × 0.02 cm I.D. steel capillary was connected to the outlet of the detector in order to avoid solvent degassing by build-up of back-pressure.

Sample preparation, direct injection and column switching

If necessary, serum samples were centrifuged for 10 min at ca. 2000 *g* prior to analysis to remove solid material. This method did not use an internal standard and all other manipulations were done on-line.

The sample was injected onto the pre-column in a flow of extraction eluent. After 2 min the flow of solvent was switched using a high-pressure switching valve so that the gradient passed through the pre-column in the back-flush mode and the analytical column. The pre-column was dry-packed, and sieves rather than frits were used to avoid blockage. The column was "primed" with injections of 6 × 50 μl or 2 × 200 μl of control serum of spiked serum standards before use. With this system, the pre-column was used for ca. 75 injections of 200 μl of serum each. The pre-column was flushed separately from the analytical column with sodium acetate buffer (150 mmol, pH 5) during the equilibrium time between runs because a gradient was used.

Optimization of recovery

The pH of the spiked serum samples was adjusted off-line to between 4.5 and 8.0 in steps of 0.5 pH units by the addition of small volume fractions (1:10) of 2 *M* aqueous buffer solutions. Acetate was used between 4.5 and 5.5., and phosphate buffer was used for the other pH adjustments. After mixing, the pH of the final sample was measured and used for the plot of peak area versus pH. The concentration of both compounds in the serum standards was 10 μg/ml. The sample (200 μl) was injected onto the RP-2 pre-column. Proteins were flushed off the pre-column with ammonium phosphate buffer (10 m*M*, pH 7) at a flow-rate of 1.5 ml/min for 2 min.

To investigate how effective the pre-column eluent was at changing the sample pH, a fixed sample volume (200 μl) of serum was injected onto the pre-column and eluted with four different concentrations of buffer (pH 5): 20, 50, 100 and 300 m*M*. The post-column pH of the eluent was continuously recorded with a combined pH glass electrode (Metrohm, Herisau, Switzerland) coupled to the HP 3357 laboratory data system (Hewlett-Packard) via an A/D converter.

To study the influence of the molarity of the pre-column eluent on the recovery of I and II, 200-μl serum samples (each spiked with 10 μg/ml I and II) were injected onto the pre-column. The flushing eluent was ammonium phosphate buffer (pH 5.0) at concentrations of 20, 50, 100, 150 and 300 m*M*, as

well as pure water. Compounds I and II were eluted from the pre-column onto the analytical column using the gradient conditions described above.

To test the influence of acetonitrile on the recoveries of I and II, 5, 10, 15, 20, 25 or 30% acetonitrile was added to serum spiked with 10 μg/ml of each compound. After injection of 200 μl, the peak areas were recorded and plotted against the acetonitrile concentration. The gradient conditions and the chromatographic separation were as described above. The pre-column was flushed with 10 m*M* ammonium phosphate buffer (pH 5.0).

Preparation of standard curves

Standards for the calibration curve were made up in filtered HPLC-grade water or by spiking control serum at concentrations of 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 μg/ml (0.12–12.3 μM I or 0.13–12.5 μM II). Each standard was injected ten times. Standard curves were constructed by plotting the chromatographic peak areas against known concentrations of I or II in serum or aqueous solution as appropriate. Individual calibration curves were constructed by ordinary least-squares regression analysis, and the concentration of I or II was quantified by relating the respective peak areas to the appropriate curve.

Kinetics of I in dog serum and plasma

A male Beagle dog (12.4 kg) was dosed with 10 mg/kg I orally, as a solution of ca. 120 ml. Blood was collected pre-dose and at 20, 40, 60, 90, 120, 180, 240, 300 and 360 min post-dose. Half of the blood was used for serum assay and half for plasma assay.

Blood samples were taken into tubes not containing anticoagulant, and left for 1 h until clotted. The serum produced was then transferred to a second tube. To obtain plasma, blood samples were collected in heparinized tubes and centrifuged. Both serum and plasma samples were stored at less than $-15\,^{\circ}$C until analysis.

RESULTS AND DISCUSSION

Since the method was designed for external standards to be used for quantification, the recoveries of I and II must be as reproducible as possible. This condition is met particularly well if the recovery is essentially quantitative. Both I and II are protein-bound to a considerable extent, and therefore the aim was to find the optimal pre-column conditions for complete stripping of the analytes off the serum (or plasma) proteins. As was shown previously for a related compound and its corresponding sulphone metabolite [8], recovery from serum on the pre-column was influenced by the pH of the flushing eluent, the percentage of acetonitrile in the flushing eluent and the length of the pre-column. Since the recovery could be optimized by pH adjustment alone, the length of the pre-column was kept constant.

Influence of the sample pH on recovery

As the pH inside the pre-column was anticipated to have some influence on the recovery of I and II, our aim was to adjust the sample pH to its optimum automatically by using an appropriate pre-column eluent. Finding the optimum pH of the pre-column eluent with respect to recovery was achieved quickly by using off-line pH-adjusted spiked serum standards. The off-line pH adjustment was done by using the procedure described in Experimental. The lower end of the pH range was limited at ca. pH 4.5 because of the inherent acid sensitivity of I. An RP-2 pre-column was used rather than more hydrophobic materials in order to use the peak compression effect produced by transferring the analytes to the RP-18 analytical column. Compounds I and II were eluted from the pre-column onto the analytical column using the gradient conditions described in Experimental. As shown in Fig. 2, the peak area of I shows a slight increase between pH 8.5 and 6.5, whereas a four to five-fold increase was observed for II between pH 8 and 5.5. To optimize the recovery of both I and II, pH 5.0 was chosen for the pre-column flushing eluent.

On-line adjustment of sample pH

For on-line adjustment of the pH at the pre-column, an eluent of the optimum pH was used. However, owing to its high content of protein, serum exhibits considerable buffering capacity, which must be overcome by using a pre-column buffer of sufficiently high molarity. As described in Experimental, the pH at the pre-column outlet was monitored after injection of a 200-μl serum sample into a series of pre-column eluents (pH 5.0) of increasing molarity. The pH pulse towards the neutral point at the pre-column outlet caused by the injected serum sample is shown in Fig. 3. Data show that a 100-mmol buffer effectively adjusts the serum pH, whereas a 20-mmol buffer results in a deflection approaching the pH of the native sample. This is further substantiated by

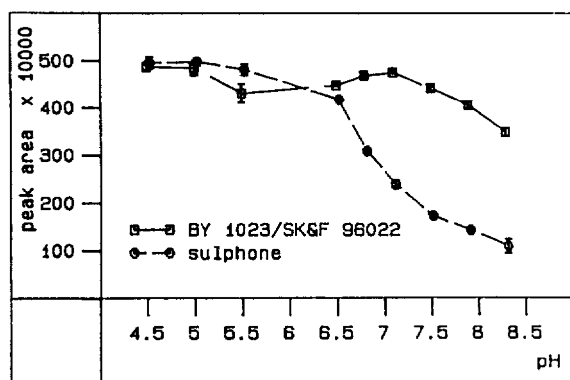

Fig. 2. Influence of the sample pH on the recovery of I (□) and II (○) (spiked serum standards, 10 μg/ml).

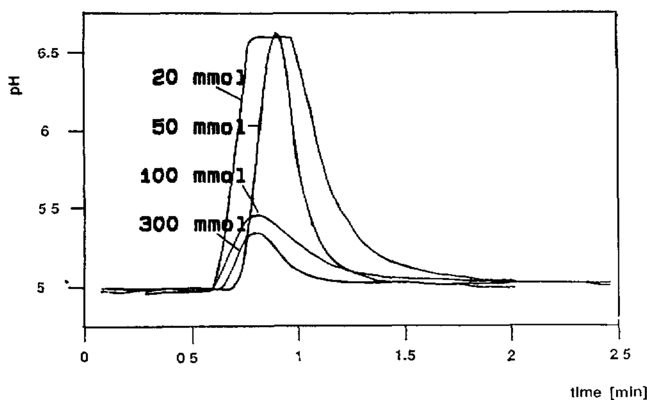

Fig. 3. pH at the pre-column outlet after injection of 200 μl of serum and elution using different buffer molarities.

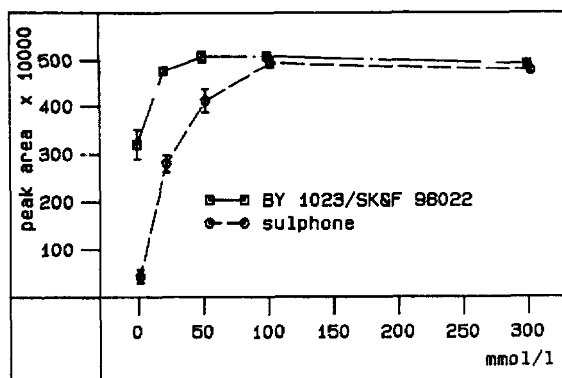

Fig. 4. Plot of peak areas for I (□) and II (○), when the pre-column elution buffer strength was increased from 20 to 300 mmol (spiked serum standards, 10 μg/ml).

the results shown in Fig. 4, which show increases of peak areas of I and II with increasing molarity of the pre-column buffer.

Influence of the percentage of acetonitrile on recovery

Acetonitrile may be used as a modifier to decrease the extent of drug–protein binding in serum and hence to increase the recovery on the pre-column [8, 9]. As for the pH, the influence of acetonitrile on recovery was studied by using off-line adjusted samples as outlined in Experimental. From Fig. 5 it can be seen that the recovery of I is almost unchanged, but somewhat improved for II after addition of 5% acetonitrile. However, at concentrations above 5% the effect was adverse, leading to losses from the pre-column owing to the increasing elution strength of the pre-column eluent.

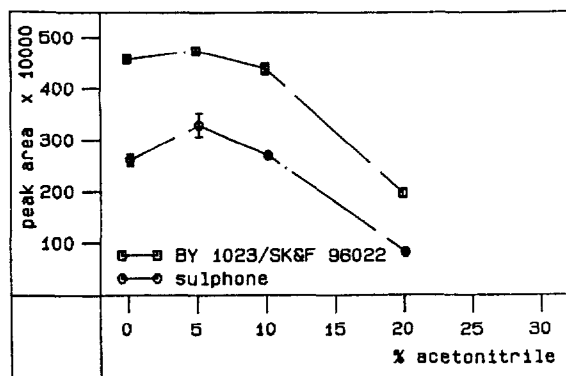

Fig. 5. Influence of the percentage of acetonitrile in the serum sample on recoveries of I (□) and II (○) (spiked serum standards, 10 μg/ml).

TABLE I

PRECISION, ACCURACY, AND RECOVERY OF I AND II FROM SPIKED SERUM SAMPLES

Concentration (μg/ml)	Compound	Mean area ($n=10$)[a]		Accuracy %	Recovery %
		Solution	Serum		
5.0	I	49 520 (6.8)	51 900 (<1)	<1	105
	II	52 760 (2.3)	50 620 (2.3)	<1	96
2.0	I	19 310 (6.7)	20 780 (4.2)	<2.0	108
	II	21 230 (2.2)	19 860 (7.4)	<1.9	94
0.5	I	4 460 (8.3)	4 960 (2.2)	<4.4	111
	II	5 180 (3.2)	4 810 (3.8)	<4.9	93

[a]Values in parentheses are C.V. (%).

Recovery, linearity, precision and accuracy under optimized conditions

Mean peak areas and percentage recovery values from serum for I and II are listed in Table I. The recovery of I was quantitative, and that of II well above 90% in the concentration range 0.5–5.0 μg/ml.

Linearity was proved by injection of both aqueous and spiked serum standards at a series of concentrations in the range 0.05–5.0 μg/ml (see Experimental). Each standard was injected ten times, and the calibration curve was plotted to determine both the linearity and the smallest amount of drug that could be detected on the column. The latter value was calculated to be 2 ng of each on-column for aqueous and 4 ng/ml for serum standards. The calibration was described by straight lines represented by the equations $y=9.94x-0.32$

(I) and $y=10.57x-0.045$ (II), the coefficients of regression being 0.9999 and 1.0000, respectively.

Serum samples spiked with I and II were assayed on three separate days, with a total of ten replicates at each concentration. The between-day precision was estimated by calculating coefficients of variation (C.V.) for all three calibrations combined, at each concentration ($n=10$ over three days). The combined peak areas were fitted by ordinary least-squares regression analysis, and the individual areas at each concentration were entered as unknowns. The accuracy was calculated as the percentage difference between these estimated values and the true values. Values for precision and accuracy are listed in Table I.

Sensitivity

The limit of detection for the assay is defined as twice the noise level. The limit of quantification is defined as the lowest concentration at which the assay gives acceptable precision. The limit of detection is 4 ng/ml (0.01 μM) for an injection of 200 μl of serum. The limit of quantification is dependent on the amount of interference present, but is less than or equal to 0.05 μg/ml (0.13 μM).

Column stability

With continuous usage, the performance of the Hypersil RP-18 column deteriorates after ca. 2500–3000 injections of 50 μl of serum, or after 700–1000 injections of 200 μl of serum. The deterioration is characterized by an increase in back-pressure or peak-broadening. To extend the lifetime of the column, the guard column was changed after every 120–150 samples. The pre-column was exchanged after ca. 40–50 injections of 200 μl of serum, which means replacing it once a day for routine measurements.

Stability of I and II in stock solution and serum

Aqueous stock solutions of I and II (100 μg/ml) as described in Experimental, as well as dilute solutions (5.0 μg/ml), were analysed five times on days 0, 1, 3 and 7 of storage at 4°C in the dark. Mean peak areas and C.V. were calculated in each case and expressed as percentages of the fresh standard area (Table II). The C.V. of the peak areas were less than 3.1%. Stock solutions of I and II were stable for at least seven days when stored at 4°C in the dark. No extra peaks were observed in the chromatograms after seven days storage.

Control serum and serum obtained from a dog dosed with 50 mg/kg I intravenously were assayed immediately and after storage at less than −15°C for eleven months. Data in Table III show that compounds I and II are stable under these conditions. The stability of I and II in serum over 14.5 h at room temperature was investigated in a similar manner. The results indicated that the measured concentrations ($n=10$ for 5.0, 2.0 and 0.5 μg/ml each) were within

TABLE II

STABILITIES OF I AND II IN AQUEOUS SOLUTION AT 4°C

Day	Average peak area ($n=5$)	Percentage difference from day 0
5 μg/ml (50 μl injected)		
0	12.735	0
1	12 343	3.1
3	12.786	<1
7	12.854	<1
100 μg/ml (25 μl injected)		
0	131.088	0
1	129.266	1.4
3	133.614	1.9
7	133.202	1.6

TABLE III

LONG-TERM STABILITY OF I AND II IN SERUM

Samples taken from a dog dosed with 50 mg/kg intravenously for five days and stored at less than −15°C for eleven months.

Time (h)	Concentration of I (μg/ml)		Difference (%)	Concentration of II (μg/ml)		Difference (%)
	First measurement	Second measurement		First measurement	Second measurement	
0.5	96.89	98.92	2.1	11.50	11.50	0.0
0.67	73.73	74.19	0.6	13.70	13.26	−3.2
1.50	53.11	53.11	0.0	23.05	22.81	−1.0
2.50	40.05	39.44	−1.5	32.15	31.59	−1.7
4.00	23.27	23.51	1.0	36.19	36.74	1.5
6.00	7.78	7.74	−0.5	28.48	28.60	0.4
12.00	0.75	0.77	2.7	18.69	19.01	1.7
18.00	0.085	0.085	0.0	9.71	9.95	2.5
24.00	N.D.[a]	N.D.[a]	–	6.19	6.35	2.6

[a]N.D., not detectable.

the typical variations of the analytical procedure, the C.V. being between 0.7 and 2.3% for I and between 1.8% and 3.8% for II.

Chromatography and application to kinetic measurements in humans

Fig. 6 shows some typical chromatograms for I and II obtained from various serum samples using optimized pre-column conditions. Besides a chromato-

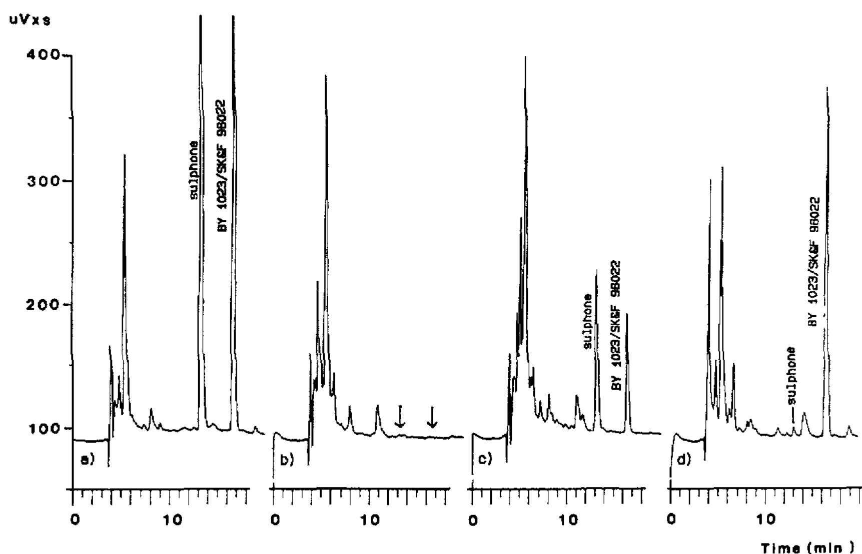

Fig. 6. Chromatograms of (a) a serum standard spiked with 3 μg/ml I and II each, (b) a blank serum sample, (c) a serum sample from a dog 1.5 h after dosing 5 mg/kg I orally and (d) a serum sample from a human 1.5 h after a single intravenous dose of 80 mg of I. Chromatographic conditions as described in Experimental.

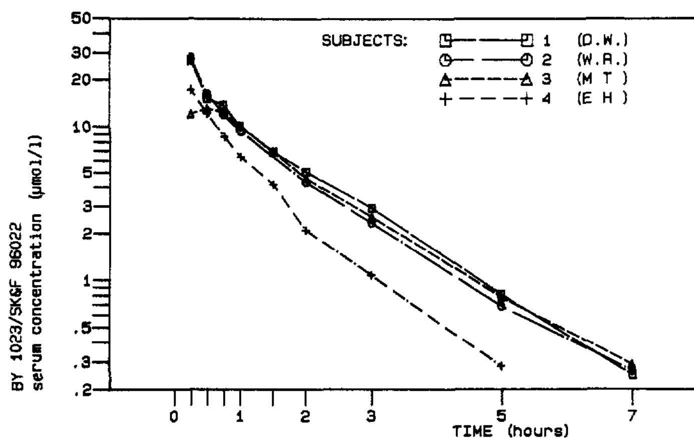

Fig. 7. Individual serum profiles of I in a human after a single intravenous 15-min infusion of 60 mg of I.

gram of a spiked serum standard, chromatograms are shown of serum samples from a dog 1.5 h after an oral dose of 5 mg/kg I and of a serum sample from a human volunteer 1.5 h after starting a 15-min infusion of 80 mg of I. Retention times under the conditions described were ca. 16.5 and 13 min for I and II, respectively.

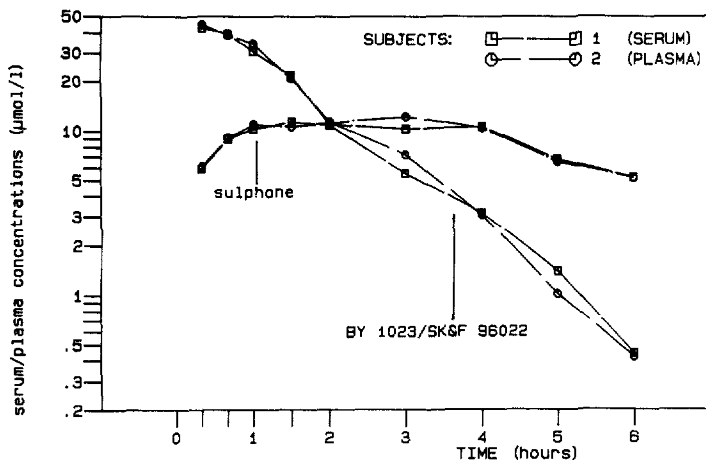

Fig. 8. Comparison of serum (□) and plasma (○) concentrations of I and II, measured following a single oral dose of 10 mg/kg to a dog.

The applicability of the assay to determine the kinetic profile of I in humans is demonstrated by Fig. 7, which shows individual kinetic profiles of I in four healthy male volunteers after a single, intravenous 15-min infusion of 60 mg of I.

Comparison of the assay of I and II in dog serum and plasma

In order to investigate whether the assay optimized for measurement of serum samples gives the same results when using plasma samples, concentration–time profiles of I and II in dog serum and plasma were obtained. The results show that there was no difference between serum and plasma for the circulating concentrations of I and II (Fig. 8).

CONCLUSIONS

This method demonstrates the applicability of direct injection and HPLC column-switching to the assay of I and II in serum. The conditions for recovery on an RP-2 pre-column have been optimized for both compounds. The pH selected for the pre-column flushing buffer was 5.0, as this represented a point of reproducible and nearly quantitative recovery. pH Values lower than 4.5 could not be used because these substituted benzimidazoles of the sulphoxide type are too unstable. A buffer concentration of 150 m*M* was required to adjust 200-µl samples of serum or plasma sample on-line. However, if a smaller sample is injected then a less concentrated buffer can be used. Although the addition of acetonitrile has been shown to improve the recovery of other substituted benzimidazoles [8], this was not found to be advantageous for the assay of I

and II, and so acetonitrile was not added to the flushing eluent. The between-day precision of the assay shows that it is repeatable within one laboratory, and the method has been shown to be reproducible when used in both Byk Gulden (F.R.G.) and Smith Kline and French (U.K.). The demonstrated reproducibility and sensitivity of this assay make it suitable for the investigation of the pharmacokinetics of I, as was shown with samples from dogs and humans after dosing with I.

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