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DETERMINATION OF 4-AMINO-5-ETHYL-3-THIOPHENECARBOXYLIC ACID METHYL ESTER AND ITS ACID METABOLITE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two separate, rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assays were developed for the determination of 4-amino-5-ethyl-3-thiophenecarboxylic acid methyl ester (I) and its acid metabolite, 4-amino-5-ethyl-3-thiophenecarboxylic acid (II), in plasma and urine. The analysis of I is performed directly on a hexane extract of plasma or urine (buffered to pH 11) by normal-phase HPLC analysis using a 10-μm silica gel column with an eluting solvent of hexane–ethanol (95:5) and UV detection of the effluent at 254 nm. A methyl analogue, 4-amino-5-methyl-3-thiophenecarboxylic acid methyl ester, was used as the internal standard. The analysis of II is performed on the residue of either a diethyl-ether-washed protein-free filtrate of plasma or a methylene chloride–isopropanol (95:5) extract of urine (buffered to pH 5.3) using a 10-μm alkyl phenyl (reversed-phase) column with an eluting solvent of water–methanol–1 *M* phosphoric acid, pH 2.5 (70:30:0.05) with UV detection of the effluent at 254 nm. An isopropyl analogue, 4-amino-5-isopropylthiophene-3-carboxylic acid (IV), was used as the internal standard. The assay of compounds I and II were applied to the determination of plasma and urine concentrations of I and II in the dog and in man following oral administration of I · HCl. The data obtained demonstrated the extremely rapid and virtually complete de-esterification of I (ester) to II (acid) in both species.

INTRODUCTION

The compounds 4-amino-5-ethyl-3-thiophenecarboxylic acid methyl ester hydrochloride (I · HCl) and 4-amino-5-ethyl-3-thiophenecarboxylic acid (II) are members of a group of thiophene compounds (Fig. 1) which have been

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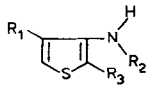

Compound	R_1	R_2	R_3
I	$C(=O)OCH_3$	H	CH_2CH_3
II	COOH	H	CH_2CH_3
III	$C(=O)OCH_3$	H	CH_3
IV	COOH	H	$CH(CH_3)_2$

Fig. 1. Chemical structures of compounds I—IV.

demonstrated to be inhibitors of fatty acid synthesis in rats [1] and are presently under investigation as antiobesity agents in man. Compound I undergoes a rapid presystemic deesterification to the carboxylic acid II in the rat [2] and dog [3] resulting in non-measurable ($\leqslant$ 20 ng/ml) concentrations of I and high concentrations of II in systemic circulation.

Although satisfactory high-performance liquid chromatographic (HPLC) separations of I and II can be obtained by normal-phase or reversed-phase systems to permit the simultaneous quantitation of the two compounds in aqueous solution, differences in their chromatographic properties and pH extraction profile preclude their simultaneous extraction and assay in the same biological sample. This report, therefore, describes two separate and selective HPLC procedures for I and II in individual samples. The assays for I and II were applied to the determination of the concentrations of I and II in the urine and plasma of dog and man following oral administration of I · HCl.

EXPERIMENTAL

Columns

The columns used for determination of I and II were 30 cm × 4 mm I.D. stainless-steel prepacked columns containing either 10-μm μPorasil silica gel or μBondapak phenyl, respectively (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a Model 440 UV detector with a 254-nm wavelength kit (Waters Assoc.). The mobile phases used for the assay of I by normal-phase chromatography and II by reversed-phase chromatography were hexane—ethanol (95:5) and water—methanol—1 *M* phosphoric acid, pH 2.5 (70:30:0.05), respectively. The chromatographic systems were operated at ambient temperature, with flow-rates of 1.0 ml/min and at head pressures of 2.8 and 6.9 MPa (400 and 1000 p.s.i.), respectively. Under the above conditions, the retention times and capacity factors (k') for compounds I and III in the normal-phase system were 4.2 min (k' = 0.46) and 4.7 min (k' = 0.59), respectively (see Fig. 2) and for compounds II and IV in the reversed-phase system were 7 min (k' = 1.9) and 11 min (k' = 3.6), respectively (see Fig. 3). The injection of 20 ng of I and 30 ng of III per 100 μl, and the injection of 100 ng of II and 150 ng of IV per 20 μl on the respective columns yielded approximately full scale response with the detector sensitivity at $1 \cdot 10^{-2}$ a.u.f.s.

Plasma and urine assay for I

Analytical standards. Compound I · HCl ($C_8H_{11}NO_2S$ · HCl, mol. wt. = 221.70, m.p. = 170—171°C) and compound III · HCl, internal standard ($C_7H_9NO_2S$ · HCl, mol. wt. = 207.68, m.p. = 191—192°C) are used as analytical standards.

Prepare stock solutions A and B of compounds I and III, respectively, in separate 10-ml volumetric flasks by dissolving 11.970 mg of I · HCl (equivalent to 10.0 mg of the free base of I) and 12.122 mg of III · HCl (equivalent to 10.0 mg of free base of III) in 10 ml of ethanol. Take appropriate aliquots of stock solution A or B to prepare a working internal standard solution C of 2500 ng of III per ml of hexane and standard solutions 1—5 containing 200, 500, 1000, 2000 and 5000 ng of I and 2500 ng of III per ml of hexane, respectively. Also prepare a series of external standards containing 20, 50, 100, 200 and 500 ng of I and 250 ng of III per ml of hexane. Aliquots (100 μl) of these standards (equivalent to 2, 5, 10, 20 and 50 ng of I and 25 ng of III) are injected into the HPLC column to establish an external standard calibration curve to verify the linearity and performance of the HPLC system.

Reagents. All reagents are of analytical-reagent grade purity and are prepared

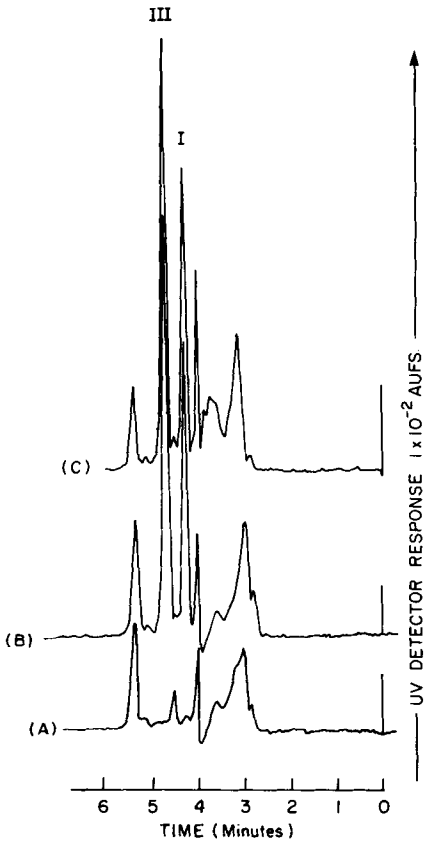

Fig. 2. Chromatograms of the HPLC analysis of hexane extracts of (A) control plasma; (B) dog plasma 30 min post-oral dose of 50 mg/kg I · HCl containing 120 ng/ml I; and (C) control plasma containing 100 ng/ml I and 250 ng/ml III added authentic standards.

in deionized, distilled water. The 1.0 *M* phosphate buffer (pH 11) is prepared by mixing 530 ml of 1 *M* $K_2HPO_4 \cdot 3H_2O$ and 470 ml of a saturated solution of Na_3PO_4 and adjusting the pH to 11.0 with $KH_2PO_4 \cdot 3H_2O$ or a saturated solution of Na_3PO_4 as needed. Other reagents used include ethanol (200 proof, Pharmaco, Publicker Industries, Philadelphia, PA, U.S.A.), hexane (UV grade, Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

Assay procedure. Add 100 μl of solution C (equivalent to 250 ng of III), 1 ml of plasma or urine and 1 ml of 1 *M* phosphate buffer (pH 11) into a 15-ml PTFE-stoppered centrifuge tube, and mix well. Extract the sample with 1 ml of hexane by slowly shaking for 15 min on a reciprocating shaker. Centrifuge the sample in a refrigerated centrifuge at 0—5°C for 10 min at approximately 1500 *g*. Transfer an aliquot (0.5—0.7 ml) of this extract into a tapered 15-ml stoppered centrifuge tube, and inject a 100-μl aliquot using the HPLC parameters described. Typical chromatograms are shown in Fig. 2.

Along with the plasma samples, process a 1-ml specimen of control plasma and five 1-ml specimens of control plasma to which are added 100 μl of solutions 1—5 (equivalent to 20, 50, 100, 200 and 500 ng of I and 250 ng of III each per ml of plasma, respectively). Along with the urine samples, process a 1-ml specimen of control urine and four 1-ml specimens of control urine to which are added 100 μl of solutions 2—5 (equivalent to 50, 100, 200 and 500 ng of I and 250 ng of III each per ml of urine, respectively). These standards are used to establish a calibration curve for the direct quantitation of the unknowns in plasma or urine and for the determination of the percentage recovery.

Plasma and urine assay for II

Analytical standards. Compound II ($C_7H_9NO_2S$, mol. wt. = 171.21, m.p. = 132—133°C) and compound IV, internal standard ($C_8H_{11}NO_2S$, mol. wt. = 185.25, m.p. 117—118°C) are used as analytical standards.

Prepare stock solutions A′ and B′ (1.0 mg/ml) of compounds II and IV, respectively, in separate 10-ml volumetric flasks by dissolving 10.0 mg of each compound in 10 ml of methanol. Take appropriate aliquots of stock solutions A′ or B′ to prepare a working internal standard solution C′ containing 100 μg IV and one set of five mixed standard solutions (6—10) containing 0.5, 2.5, 10, 25 and 50 μg of II and 7.5 μg of IV per ml of methanol, respectively. Aliquots (20 μl) of these standards (equivalent to 0.01, 0.05, 0.2, 0.5 and 1.0 μg of II and 0.15 μg of IV) are injected into the HPLC column to establish an external standard calibration curve to verify the linearity and performance of the HPLC system.

Reagents. The 1.0 *M* phosphate buffer (pH 5.3) is prepared by mixing 820 ml of 1 *M* KH_2PO_4 and 180 ml of 1 *M* K_2HPO_4 and adjusting the mixture to pH 5.3 with KH_2PO_4 or K_2HPO_4 as needed. Other reagents used include 6 *M* hydrochloric acid, 6 *M* sodium hydroxide, methanol, acetonitrile, methylene chloride and isopropanol (Burdick & Jackson Labs.) and Glusulase reagent (enzyme preparation containing 100 000 U/ml glucuronidase and 50 000 U/ml sulfatase; Endo Labs., Garden City, Long Island, NY, U.S.A.).

Assay procedure for plasma. Into a 15-ml PTFE-stoppered centrifuge tube, add 0.2 ml of plasma, 15 μl of solution C′ (equivalent to 1.5 μg of IV) and 0.4

ml of acetonitrile and mix on a vortex mixer for 10 s to deproteinate the plasma. Centrifuge the sample in a referigerated centrifuge at 0—5° C for 5 min at approximately 1500 *g*, and transfer the protein-free filtrate into a tapered 15-ml stoppered centrifuge tube. Wash the supernatant with 5 ml of diethyl ether by shaking for 5 min on a reciprocating shaker, centrifuge and aspirate off the diethyl ether phase. Repeat with another 5-ml portion of diethyl ether. Evaporate the acetonitrile protein-free filtrate to dryness at room temperature under a stream of clean, dry nitrogen. Dissolve that residue in 200 μl of methanol, and inject a 20-μl aliquot for HPLC analysis. Typical chromatograms are shown in Fig. 3.

Along with samples, process a 0.2-ml specimen of control plasma and five 0.2-ml specimens of control plasma containing 0.1, 0.5, 2, 5 and 20 μg of II and 1.5 μg of IV, respectively. These standards are used to establish a standard curve for the direct quantitation of the unknowns and for the determination of the percentage recovery.

Assay procedure for urine. Into a 15-ml PTFE-stoppered centrifuge tube, add 75 μl of standard soltuion C′ (equivalent to 7.5 μg of IV), 0.2 ml of urine and 1 ml of 1 *M* phosphate buffer (pH 5.3), and mix well. Extract the sample

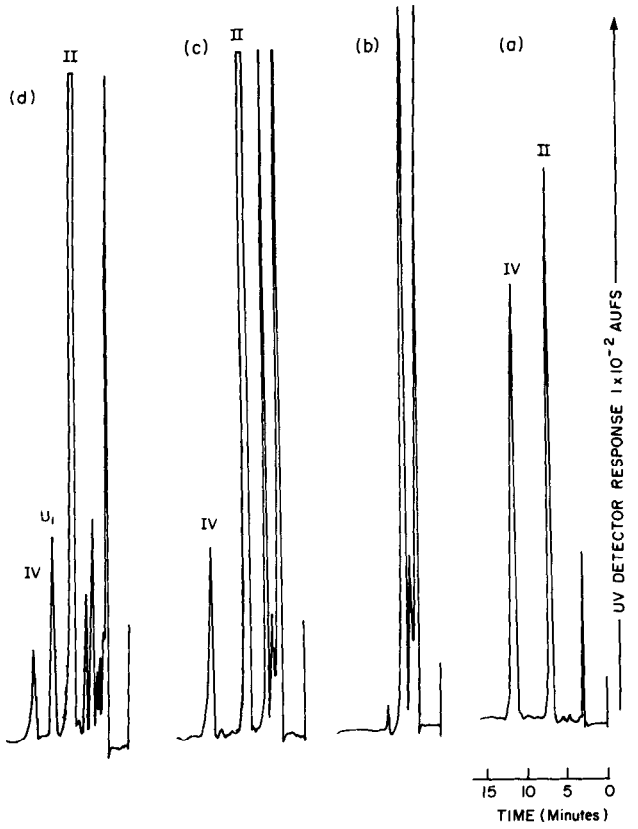

Fig. 3. Chromatograms of the HPLC analysis of (A) authentic standards containing 0.1 μg of II and 0.15 μg of IV injected; (B) protein-free supernatant of control plasma; (C) control plasma containing 20 μg of II and 1.5 μg of IV per 0.2 ml added authentic standards; and (D) dog plasma 45 min post-oral dose of 50 mg/kg I · HCl containing 108 μg/ml II.

with 5 ml of methylene chloride—isopropanol (95:5) by shaking for 15 min on a reciprocating shaker. Centrifuge the sample in a refrigerated centrifuge at 0—5°C for 10 min, at approximately 1500 *g*. (Transfer a 1-ml aliquot from the upper aqueous (urine) phase and save for the determination of conjugated II.) Transfer a 4-ml aliquot from the organic phase into a 15-ml PTFE-stoppered centrifuge tube. Evaporate the extract to dryness under a stream of clean, dry nitrogen at 40–50°C using an N-EVAP evaporator. Dissolve the residue in 500 μl of methanol, and inject a 10-μl aliquot for HPLC analysis. Typical chromatograms are shown in Fig. 4.

Along with the samples process a 0.2-ml specimen of control urine and four 0.2-ml specimens of control urine to which are added 5, 10, 25 and 50 μl of solution A′ and 75 μl of solution C′ (equivalent to 5, 10, 25 and 50 μg of II and 7.5 μg of IV per 0.2 ml of urine, respectively). These standards are used to establish a standard curve for the direct quantitation of unconjugated II in the unknowns and for the determination of the percentage recovery.

To the 1-ml aliquot of the pre-extracted urine sample (pH 5.3) add 10 μl of 1% (v/v) Glusulase enzyme, mix well, stopper loosely with cotton and incubate with shaking in a Dubnoff shaker at 37°C for 2 h. Extract the sample with 5 ml

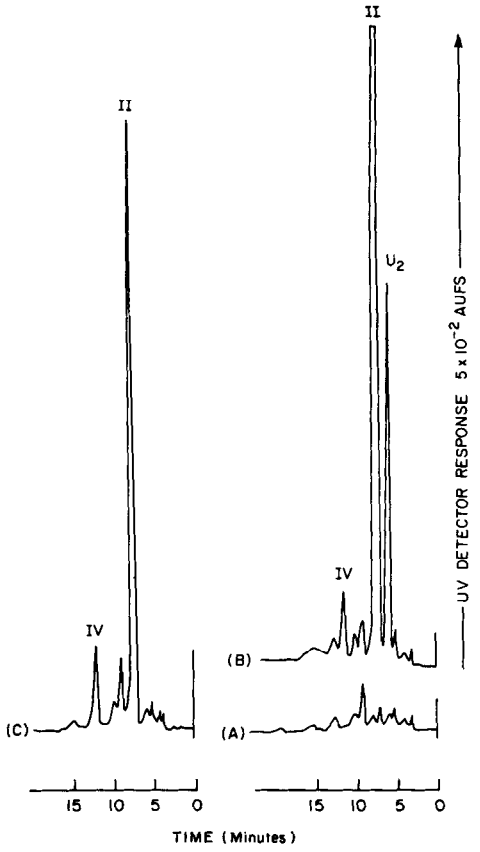

Fig. 4. Chromatograms of HPLC analysis of methylene chloride—isopropanol (95:5) extracts of (A) control urine; (B) 0—24 h dog urine post 50 mg/kg dose and; (C) control urine containing 40 μg of II and 7.5 μg of IV per 0.2 ml added authentic standards.

of methylene chloride—isopropanol (95:5) and process as described above for unconjugated II. Along with the samples, process a 1-ml aliquot of the pre-extracted control urine sample to which 5, 10, 25 and 50 μl of solution A′ and 75 μl of solution C′ are added. These standards are used to establish a standard curve for the direct quantitation of conjugated II in the unknown.

Calculations

The concentration (μg/ml) of I or II in plasma or urine in each respective experimental sample is determined by interpolation from a linear regression analysis of the data from the recovered standards processed along with the exprimental samples using the peak-height ratio (peak height of compound I or II to peak height of the respective internal standard III or IV versus concentration of I or II. The amounts of unconjugated and conjugated II excreted in the urine, expressed as I, are calculated from the concentration (μg/ml) of II found, multiplied by the appropriate molecular-weight factor (F = mol. wt. I/mol. wt. II = 185.2/171.2 = 1.08).

RESULTS AND DISCUSSION

The original goal of this study was to develop a rapid and sensitive HPLC assay suitable for the simultaneous analysis of I and II in both plasma and urine. Initial investigations with compounds I and II utilizing either normal-phase HPLC with silica columns and mobile phase of hexane—ethanol (95:5) and hexane—ethanol—acetic acid or reversed-phase HPLC with μBondapak C_{18} or phenyl columns with a mobile phase of water—methanol—phosphate buffer (or weak acid) as eluting solvents yielded good resolution of I and II. However, the chromatographic properties and polarity of the two compounds were sufficiently different (i.e., I had a long retention time in the reversed-phase mode while II showed a wide tailing peak in the normal-phase mode) that simultaneous assay was not practical and two separate chromatographic assays were required for quantitation. In addition, the polarity and pH of the extraction profiles of the two compounds were so diverse that simultaneous extraction was not possible. Consequently, the extraction of I is performed at pH 11 with a non-polar solvent (hexane), whereas the extraction of II at pH 5 requires a very polar mixed solvent, methylene chloride–isopropanol (95:5). Attempts at sequential extraction initially at pH 11 into hexane to remove I, followed by pH adjustment to 5.3 to extract II into methylene chloride—isopropanol (95:5) was not feasible due to inadequate pH control. Therefore, the two separate HPLC assays were developed.

The assay for I involves extraction into a relatively small volume of organic solvent (1 ml) and direct injection of a relatively large volume of the extract (100 μl). This was required to circumvent a concentration step which would otherwise result in relatively low and variable recoveries of I (25–50%) due to losses upon evaporation of the hexane extract. The plasma assay for II does not utilize methylene chloride—isopropanol (95:5) for extraction at pH 5.3 as described for urine, because the extract requires additional laborious sample clean-up. The formation of a protein-free filtrate of plasma with acetonitrile and a diethyl ether wash of this filtrate yielded an extract which was

devoid of interfering endogenous compounds and was suitable for direct assay using the μphenyl column and the parameters described. Although the volume of the sample assayed is limited to 0.2 ml of plasma the concentrations measured in dog and man are significantly above the limit of quantitation of the assay. The direct extraction into methylene chloride—isopropanol (95:5) can be used for the assay of II in urine due to the high concentrations present.

Statistical evaluation of the methods

The inter-assay precision of the two methods was evaluated in dog plasma over concentration ranges of 0.020—2.00 μg/ml I and 0.50—100 μg/ml II (Tables I and II). The mean inter-assay coefficients of variation for I and II over their respective concentration ranges were 4.6 and 4.7%, respectively. The recoveries of compounds I and II were 79.4 ± 3.3% (S.D.) and 72.5 ± 9.0%

TABLE I

INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND I IN DOG PLASMA

Concentration added (μg/ml)	*n*	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
0.020	7	0.021 ± 0.002	7.6
0.050	6	0.051 ± 0.002	3.9
0.100	9	0.098 ± 0.004	4.1
0.250	4	0.269 ± 0.016	5.9
0.300	7	0.300 ± 0.015	5.0
0.500	8	0.474 ± 0.039	8.2
1.00	6	0.990 ± 0.025	2.5
2.00	4	2.00 ± 0.03	1.3
Mean			4.6

TABLE II

INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND II IN DOG PLASMA

Concentration added (μg/ml)	*n*	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
0.50	3	0.48 ± 0.02	4.2
1.00	4	0.97 ± 0.05	5.1
2.50	5	2.66 ± 0.16	6.1
5.00	5	4.90 ± 0.04	8.1
7.50	2	7.64 ± 0.06	0.8
10.0	6	10.4 ± 0.9	8.6
25.0	4	23.6 ± 0.7	2.9
50.0	3	51.7 ± 2.2	4.3
75.0	2	75.3 ± 3.7	4.9
100	6	99.7 ± 2.2	2.2
Mean			4.7

(S.D.), respectively, with a limit of quantitation of 0.02 μg/ml I using a 1-ml specimen and 0.5 μg/ml II using a 0.2-ml specimen.

Similar data were obtained in human plasma over the identical concentration ranges of I and II.

The inter-assay precision in dog and human urine over a concentration range of 0.05—1.0 μg/ml I and 25.0—100 μg/ml II was similar to that obtained in plasma.

Stability of I and II in biological specimens

Stability of I. Short-term stability experiments in which I was added to fresh human plasma at 37, 25 and 0°C showed that approximately 47.4, 66.3 and 91.8% of the parent compound was recovered after a 3-h interval, respectively (Table III). The half-lives of degradation were approximately 3 and 5 h, at 37°C and 25°C, respectively. Similar data were obtained upon incubation of the drug in dog plasma at 37°C.

TABLE III

STABILITY OF I · HCl IN HUMAN PLASMA

Time (h)	Recovery (%)		
	37°C	25°C	0°C
0.25	88.5	94.7	93.3
0.50	84.3	93.8	95.5
1	77.3	85.7	86.7
2	60.4	72.0	93.5
3	47.4	66.3	91.8
4	37.8	63.7	—
5	31.5	56.8	—
6	25.9	—	—
19	—	11.2	—
22	2.6	—	—

Long-term stability experiments in which I was added to ice-cooled fresh human blood were also performed. The plasma obtained from this blood sample was sub-divided and stored at −17°C for a period of two months and showed approximately 10% degradation.

Stability of II. The short-term stability data of II in plasma at room temperature (23°C) indicated that minimal degradation occurred over a 5.5-h period.

The long-term stability data of II in plasma were demonstrated by the re-analysis of twenty plasma samples from four subjects which had been stored at −17°C for over five months. No significant difference from the original assay was found upon re-assay ($y = 1.1198x - 0.197$ and $r = 0.9889$), where y and x are equivalent to re-assay and original concentrations, respectively, indicating stability of compound II under conditions of storage.

Sample handling recommendations. The stability of I during long-term storage (−17°C) and sample handling can be enhanced if the blood specimens are kept in an ice bath immediately after collection and then centrifuged under refrigeration shortly thereafter to obtain the plasma. Samples should be stored at −17°C if analysis is to be completed within two months of collection. For

periods of storage greater than two months, storage at −70° C is recommended. The frozen plasma samples should be thawed gradually by keeping the samples in an ice bath with repeated mixing on a vortex mixer prior to the assay. The assay procedures should be performed at 0° C whenever possible.

The special precautions described above for sample handling for the measurement of concentrations of I should only be employed in those instances in which concentrations of I are anticipated to be above the limit of detection of 20 ng/ml. In all clinical studies conducted to date no measured plasma concentrations of I have been determined in man following oral doses of as much as 400 mg (see below). Routine clinical pharmacokinetic studies are presently designed to measure only the major de-esterified metabolite II and as such no special sample handling procedures are required.

Application of the method to biological specimens

The HPLC assays for I and II were applied to the determination of I and the de-esterified biotransformation product II (acid) in dog and human plasma and urine, following oral doses of 50 mg/kg and 400 mg I · HCl (Table IV).

Following oral administration to the dog, maximum concentrations of 0.12 μg/ml I and 144 μg/ml II in plasma were determined at 30 and 20 min, respectively, and declined to non-measurable concentrations after 2 and 10 h with estimated elimination half-lives ($t_{½\beta}$) of 0.62 and 2.0 h for I and II, respectively. The urinary excretion of the parent drug I accounted for less than 1% of the dose recovered in the 0—24 h excretion period. The concentration of metabolite II in the unconjugated and conjugated forms (calculated as I equivalents) accounted for 16.0 and 11.2%, respectively (total of 27.2%), during the same excretion interval. Concentrations of II were non-measurable

TABLE IV

PLASMA CONCENTRATIONS OF I AND II IN DOG AND MAN FOLLOWING ORAL ADMINISTRATION OF A 50 mg/kg AND 400 mg DOSE OF I · HCl, RESPECTIVELY

Time	Concentration in dog (μg/ml)		Concentration in man (μg/ml)	
	I	II	I	II
10 min	0.034	95.5	—*	—
20 min	0.070	144	—	—
30 min	0.12	139	NM**	16.1
45 min	0.12	108	—	—
1 h	0.10	87.8	NM	10.6
1.5 h	0.04	61.3	NM	7.6
2 h	0.02	44.7	NM	5.6
3 h	NM	28.8	NM	3.3
4 h	NM	12.6	NM	2.7
6 h	NM	6.8	NM	1.1
8 h	NM	3.4	NM	0.7
10 h	NM	1.5	—	—
12 h	NM	NM	NM	NM

*No sample taken.

**NM = Non-measurable; concentration of I ⩽ 0.02 μg/ml; concentration of II ⩽ 0.5 μg/ml.

($\leqslant$ 5 μg/ml) in the 24—48 h excretion period. During routine analysis of II in dog plasma and urine, two possible metabolite peaks [U_1 (k' = 2.4) and U_2 (k' = 1.1)] were oberved in the chromatograms (Figs. 3D and 4B, respectively). The confirmation of these possible metabolites will have to await structural elucidation and the synthesis of authentic reference compounds.

Following oral administration to man non-measurable ($\leqslant$ 0.02 μg/ml) concentrations of I were obtained over the entire 12-h sampling period. A maximum concentration of II of 16.1 μg/ml was obtained at 0.5 h which declined to 0.7 μg/ml at 8 h with an estimated elimination half-life ($t_{1/2\beta}$) of 2.0 h. The urinary excretion of parent drug I accounted for less than 0.1% of the dose recovered in the 0—24 h excretion period. The concentration of II in the unconjugated and conjugated form over the 0—24 h excretion period accounted for less than 1 and 17.9% (calculated as I equivalents), respectively.

REFERENCES

1 J. Triscari and A.C. Sullivan, Life Sci., 34 (1984) 2433—2442.
2 F.-J. Leinweber, A.J. Szuna, A.C. Loh, T.H. Williams, G.J. Sasso, I. Bekersky and J. Triscari, J. Pharmacol. Exp. Ther., submitted for publication.
3 A.J. Szuna, A.C. Loh, J.J. Carbone, T.H. Williams, G.J. Sasso, E. Baggiolini and F.-J. Leinweber, Pharmacologist, 27 (1985) 122.