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DETERMINATION OF THE QUINIDINE ANALOG, 7'-TRIFLUOROMETHYLDIHYDROCINCHONIDINE·2HCl IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. PAO and J.A.F. de SILVA*

Department of Pharmacokinetics and Biopharmaceutics, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

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SUMMARY

A rapid and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of the antiarrhythmic quinidine analog, 7'-trifluoromethyldihydrocinchonidine·2HCl ([I]·2HCl) in plasma and urine. The overall recovery of [I] from plasma was 86 ± 9% with a sensitivity limit of detection of 0.2 μg/ml.

The assay involves extraction of [I] into benzene—methylene chloride (9:1) from plasma or urine made alkaline with 0.1 *N* sodium hydroxide (pH 13) and saturated sodium chloride, the residue of which is dissolved in methylene chloride, an aliquot of which is analyzed by HPLC using adsorption chromatography on silica gel with UV detection at 254 nm. The mobile phase composed of methylene chloride—methanol—conc. ammonium hydroxide (95.5:4:0.5) yields baseline resolution of quinidine used as the internal (reference) standard, compound [I] and dihydroquinidine, a common contaminant in quinidine.

The assay was applied to the analysis of plasma and urine samples taken from a dog administered a single 20 mg/kg dose via intravenous and oral routes. The stability of [I] in human plasma for up to 37 days of storage at −17°C was also demonstrated.

INTRODUCTION

The compound 7'-trifluoromethyldihydrocinchonidine·dihydrochloride ([I]·2HCl) is a member of a series of synthetic quinidine analogs [1] with antiarrythmic activity [2]. It differs from quinidine mainly in the presence of a trifluoromethyl group on the C'_7 quinoline moiety instead of a methoxy group on C'_6 (Fig. 1). Quinine, quinidine and other quinoline analogs have long been the mainstay of therapeutic agents with antimalarial [3] and antiarrhythmic [4,5] activity.

Numerous publications have appeared on the analysis of quinidine in biolog-

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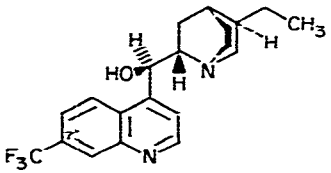
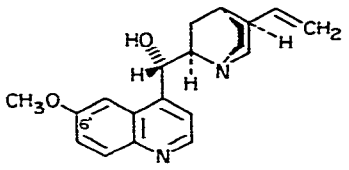
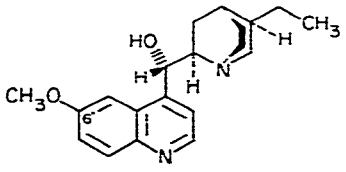


[I] 7'-Trifluoromethyl-Dihydrocinchonidine



[II] Quinidine



[III] Dihydroquinidine

Fig. 1. Chemical structures of the compounds referred to in the text.

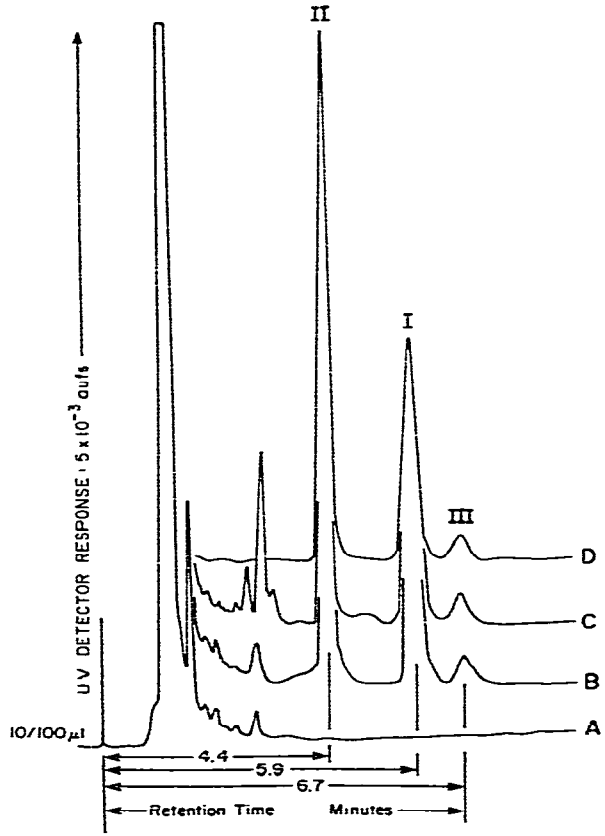


Fig. 2. Chromatograms of compound [I], compound [II], quinidine, used as the internal (reference) standard, and compound [III], dihydroquinidine, an impurity in quinidine. (A) Control dog plasma extract, (B) control dog plasma extract with recovered authentic standards, (C) in vivo dog plasma extract, (D) authentic standards.

ical samples using spectrofluorometric [6,7], thin-layer chromatographic [8—10], gas chromatographic (GC) [7,11,12], gas chromatographic—mass spectrometric (GC—MS) [7,13,14] and high-performance liquid chromatographic (HPLC) [15—24] methods.

The projected clinical dosage of [I]·2HCl is in the multi-milligram per kilogram range; therefore an HPLC assay would provide adequate sensitivity and specificity for quantitation. Quinidine sulfate [II] is a readily available compound, hence it is a logical choice for use as the internal (reference) standard in this assay. However, commercially available quinidine contains a significant percentage of dihydroquinidine [III] as a contaminant [25]. Consequently, the chromatographic resolution of these two components from each other and from [I] and its metabolites is necessary. Baseline resolution of all the components in plasma (Fig. 2) and in urine (Fig. 3) was obtained with the method developed, monitoring the effluent with a fixed-wavelength UV detector at 254 nm.

The assay was applied to the analysis of plasma and urine samples taken from a dog administered a single 20 mg/kg dose via intravenous and oral routes.

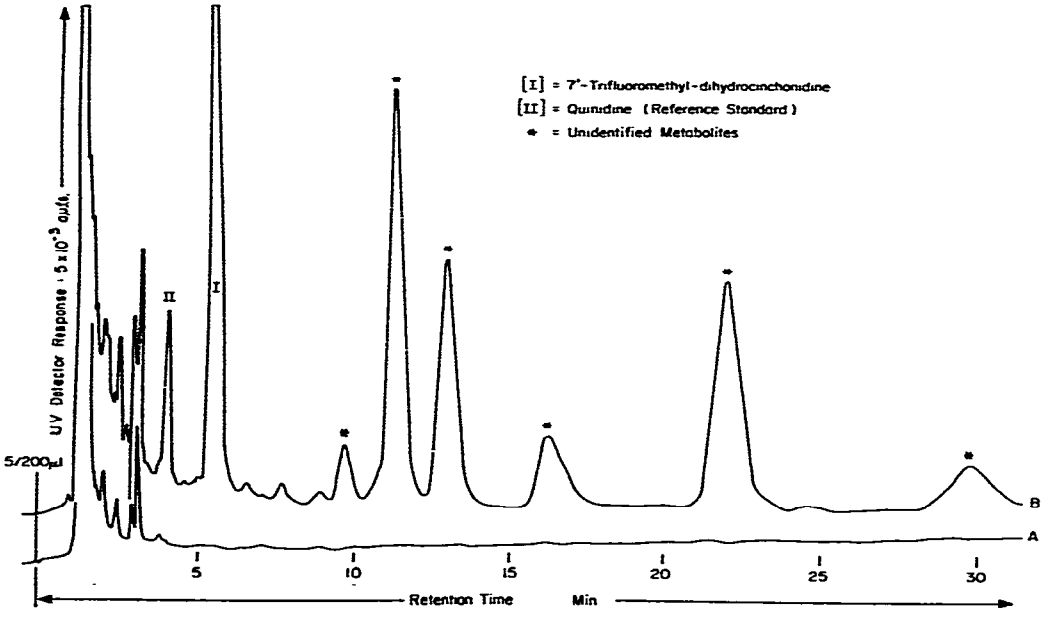


Fig. 3. Chromatograms of 2-ml extracts of the unconjugated fraction of urine (24—48 h post dose) from a dog administered a 20 mg/kg intravenous dose of (A) control urine extract, (B) in vivo dog urine extract.

EXPERIMENTAL

Column

A prepacked 25 cm × 4.6 mm I.D. stainless-steel column containing 10-µm Partisil silica gel, generating 39,100 plates per meter (Whatman, Clifton, NJ, U.S.A.), was used.

Instrument parameters

The HPLC system (Waters, Milford, MA, U.S.A.) consisted of a Model 6000 reciprocating piston pump, a Model U6K loop injector and a Model 440 fixed-wavelength absorbance detector with a UV filter at 254 nm. The isocratic mobile phase used was a mixture of methylene chloride—methanol—conc. ammonium hydroxide (95.5:4:0.5) at a constant flow-rate of 2 ml/min and a column head pressure of ca. 700 p.s.i. at ambient temperature. The detector sensitivity was 5×10^{-3} a.u.f.s. and the chart speed on the Hewlett-Packard recorder (Model 7132 A) was 0.5 in./min at 10 mV input. Under these conditions, 0.2 μg per 10 μl injected of [I], [II] and [III] gave nearly full-scale peak response. The retention times of [I], [II] and [III] were 5.9 min, 4.4 min and 6.7 min (Fig. 2), with corresponding capacity factors (k') of 3.35, 2.15 and 4.00, respectively.

Reagents

0.1 *N* NaOH (pH 13); saturated NaCl solution (1.5 g/ml); benzene, nano-grade (Mallinckrodt, St. Louis, MO, U.S.A.); methylene chloride and methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); conc. ammonium hydroxide (29.3% ammonia), Baker analyzed reagent grade (J.T. Baker, Phillipsburg, PA, U.S.A.).

Analytical standards

Compound [I]·2HCl (M.W. = 437.33, m.p. = >184°C decomp.) and quinidine·sulfate·hydrate ($[II]_2 \cdot H_2SO_4 \cdot H_2O$) (M.W. = 764.95, m.p. = 201—202.5°C) of pharmaceutical grade purity were obtained from the Chemical Research Division, Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.), while dihydroquinidine [III] was obtained from K + K Labs (Plainview, NY, U.S.A.).

Preparation of analytical standards

Weigh out separately 12.00 mg of [I]·2HCl and 11.79 mg of quinidine·sulfate·hydrate (equivalent to 10 mg of free base) and dissolve separately in 10 ml of methanol to yield stock solutions A and B containing 1 mg equivalent of [I] or [II] (free base) per ml, respectively.

Prepare working standard solutions a—f by mixing appropriate aliquots of A and B diluted to 10 ml with methylene chloride as follows:

Solution	Aliquots (μl) of Solution A + Solution B		Final conc./100 μl of solution	
	A	B	[I]	[II]
a	20	200	200 ng	2 μg
b	50	200	500 ng	2 μg
c	100	200	1 μg	2 μg
d	200	200	2 μg	2 μg
e	500	200	5 μg	2 μg
f	–	200	–	2 μg

Aliquots (100 μl) of solutions a,b,c,d or e are added to the residue of separate 1-ml specimens of control plasma as the external calibration standards

and mixed well on a Vortex mixer. Aliquots (10 μl) of these solutions (equivalent to 20, 50, 100, 200 and 500 ng of [I] and 200 ng of [II]) are injected to establish an external standard calibration curve, to verify the linearity and performance of the HPLC system. Addition of the drug to the residue of control plasma is necessary due to a chromatographic "enhancement effect" which compounds [I] and [II] exhibit; i.e., the peak height response is greater when the compounds are chromatographed in the residue of biological extracts than when chromatographed as pure standards, probably due to decreased adsorption losses caused by deactivation of the column by the lipids in the extract.

The internal standard [II] is added (100 μl of solution f) to all unknown plasma or urine samples.

Assay procedure

Into a 15-ml (PTFE No. 13) stoppered conical centrifuge tube, transfer a 100-μl aliquot of solution f (equivalent to 2 μg of [II], the internal standard), add 1 ml of unknown plasma or 0.5—2 ml of urine, 1 ml of saturated sodium chloride solution and 2 ml of 0.1 *N* sodium hydroxide (pH 13), mix for a few seconds very gently (at setting 2) on the Super-Mixer (Lab-Line Instruments, Melrose Park, IL, U.S.A.). Extract the samples with 6 ml of benzene—methylene chloride (9:1) by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at ca. 90 strokes/min. Centrifuge the samples in a refrigerated centrifuge (Model PR-J, Rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min at ca. 2600 rpm (1500 *g*), and transfer the supernatant benzene—methylene chloride layer into a conical 15-ml glass stoppered centrifuge tube. Extract the sample a second time with an additional 6 ml of benzene—methylene chloride, centrifuge and combine the extracts. Evaporate to dryness at ca. 50°C in a N-EVAP evaporator (Organomation, Worchester, MA, U.S.A.) under a stream of dried nitrogen. Rinse the walls of the tube with 1—1.5 ml of benzene—methylene chloride (9:1) to concentrate the sample in the tapered end of the tube. (The reconstituted sample residues may be stored overnight at −20°C prior to subsequent analysis.) Evaporate the solution to dryness and dissolve the residue in 100—200 μl of methylene chloride and inject a 5—10-μl aliquot for HPLC analysis.

Along with the samples, process a 1-ml specimen of control plasma or 0.5—2 ml of urine and five 1-ml specimens of control plasma or 0.5—2 ml of urine to which 100 μl of standard solution a, b, c, d or e (equivalent to 200 ng, 500 ng, 1 μg, 2 μg or 5 μg of [I] and 2 μg of [II] each per ml of plasma or urine, respectively) are added; these samples are used to establish the calibration curve for the direct quantitation of the unknowns.

Calculations

The concentration of [I] in the unknowns is determined by interpolation from the calibration curve of the standards processed along with the unknowns using peak area ratios (peak area of [I] to peak area of internal (reference) standard [II] versus concentration). The calibration curve is linear from 200 ng to 5 μg of [I] per ml of plasma or urine.

RESULTS AND DISCUSSION

Percent recovery and sensitivity limits

The overall recovery of [I] and [II] from plasma was 86 ± 9% and 96 ± 6% (S.D.), respectively, over the concentration range of 0.20 to 5.0 μg of [I] per ml of plasma or urine. It was necessary to add the external standards to the residue of control plasma to obtain an apparent recovery of ⩽100% due to the aforementioned "enhancement" effect. The sensitivity limit of the assay was 0.2 μg of [I] per ml of plasma. The ammonia content in the mobile phase was critical to good chromatographic resolution, peak shape and response of these compounds.

The HPLC system is flushed initially with methanol to remove accumulated deposits from previous use. Fresh mobile phase is prepared before each analysis, and allowed to recycle through the system until equilibration with the column is attained, as indicated when a stable baseline is obtained. Several 10-μl aliquots of the mixed standard solution c are then injected until a reproducible response is obtained before the analysis of the biological samples is attempted.

It has been observed that the k' values of the compounds tended to increase throughout the day's run, probably due to a change of the ammonia content of the mobile phase. Prolonged use of the column will also tend to increase k' values.

Specificity of the assay

The extensive biotransformation of compounds such as [I]·2HCl [26] and quinidine [II] [15,20,24] necessitates the use of chromatographic procedures in order to ensure the specificity and accuracy of quantitation of the many compounds present in biological fluids. Although meaningful data have been reported on the bioavailability [27] and pharmacokinetics [28] of quinidine [II] and of dihydroquinidine [III] [29] using non-specific spectrofluorometric methods, more meaningful pharmacokinetic data on parent drug and metabolite profiles and therapeutic efficacy were obtained using specific chromatographic methods, especially HPLC with either UV absorption [17,18,20] or fluorometric detection [15,21—23,30]. Since therapeutic plasma concentrations of quinidine and its metabolites are high (μg/ml range) and its half-life relatively long (5—12 h), the tandem use of UV and fluorescence detection enables quantitation in plasma over a wide concentration range (e.g. 100 μg/ml to 5 ng/ml), establishing HPLC as the method of choice for the determination of this class of compounds.

Gas chromatographic behavior of [I]

Compound [I] has no intrinsic fluorescence, but its UV absorbance at 254 nm is sufficiently intense to attain a sensitivity limit of 200 ng/ml. The trifluoromethyl group at C_7' is an electron-withdrawing group which tends to delocalize the π electrons in the quinoline ring, reducing its UV absorption and any useful fluorescence. The converse is true with quinidine whose methoxy group at C_6' is electron-donating which enhances the aromaticity of the quinoline nucleus and contributes significantly to its excellent UV absorbance and fluorescence emission characteristics. Efforts at improving the sensitivity

limits of detection of [I] led to the investigation of electron-capture—gas—liquid chromatography (EC—GLC) and gas chromatographic—chemical ionization—mass spectrometric (GC—CI—MS) analysis as alternative methods.

Electron-capture—gas—liquid chromatography

The parent compound [I] and its trimethylsilyl (OTMS) ether (prepared by reaction with bis(trimethylsilyl)trifluoroacetamide + 10% trimethylchlorosilane heated at 60°C for 1 h, the residue of which was dissolved in *n*-hexane for EC—GLC analysis), were chromatographed on 4 ft. × 4 mm I.D. 3% OV-1 and 3% OV-17 columns using a 15 mCi ^{63}Ni EC detector (Tracor Instruments, Austin, TX, U.S.A.) (see Table I).

Compound [I] showed good intrinsic sensitivity to EC detection; the chromatograms however, showed a major tailing peak for [I] and smaller peaks as evidence of either thermal instability or due to small amounts of isomers. Chromatograms of compound [I]-OTMS derivative, however, showed a sharp symmetrical Gaussian-shaped peak of high sensitivity with two minor peaks probably due to trace amounts of the isomers of [I]. The silylation of [I] was confirmed by GC—electron impact (EI)—MS analysis of [I] which gave a molecular ion *m/z* 364 compared to the [I]-OTMS derivative which gave a molecular ion *m/z* 436 and a base peak at *m/z* 138 common to both.

The sensitivity of the response of [I]-OTMS to EC detection was sufficient to determine less than 50 ng/ml.

TABLE I

RETENTION TIMES (min) OF [I] AND ITS TRIMETHYLSILYL ETHER BY EC—GLC

Temperature for GC of [I] 220°C, and for GC of [I]-OTMS 200°C.

GC column	[I]	Smaller peaks		[I]-OTMS	Smaller peaks	
3% OV-1	4.2	3.0	3.5	6.5	10.0	13.5
3% OV-17	6.2	4.2	—	4.6	7.6	8.6

GC—MS analysis

Analysis of [I] and [II] as their respective OTMS derivatives by GC—CI—MS using both positive ion (PI) and negative ion (NI) modes of detection, was also investigated on a Finnigan Model 1015 modified for both PI [31,32] and NI [33] capability. The CI—mass spectra gave more intense ions for both compounds in the PI mode than in the NI mode. This was expected for [II]-OTMS which does not have an electronegative group in the aromatic ring required for good NI response. The major ions for [I]-OTMS in the NI mode were at *m/z* 378 $[M-58]^-$, *m/z* 360 $[M-76]^-$, *m/z* 346 $[M-90]^-$, but were not sufficiently intense for quantitative use.

The PI—CI—mass spectra for both compounds are shown in Fig. 4. The major ions for [I]-OTMS (Fig. 4A) were at *m/z* 477 $[M+41]^+$, *m/z* 465 $[M+29]^+$, *m/z* 437 $[M+H]^+$, *m/z* 421 $[M-15]^+$ and *m/z* 417 $[M-19]^+$, while those for [II]-OTMS (Fig. 4B) were at *m/z* 438 $[M+42]^+$, *m/z* 425 $[M+29]^+$, *m/z* 397 $[M+H]^+$, *m/z* 381 $[M-16]^+$. The additional ions at *m/z* 477 and *m/z* 465 for

[I]-OTMS and at *m/z* 438 and 425 for [II]-OTMS, respectively, are adducts due to methane used as the reagent gas. The intense $[M+H]^+$ ions at *m/z* 437 and *m/z* 397 are used for the quantitation of [I]-OTMS, using [II]-OTMS as the internal standard and carrier. The total ion chromatograms (see inset in Fig. 4) for [I]-OTMS gave two distinct peaks indicating the possible presence of an isomer as an impurity in the sample, while that for [II]-OTMS gave a single major peak, and a minor peak for [III]-OTMS, a known impurity.

Plasma samples spiked with known amounts of [I] and [II] were extracted as described, the residue silylated and analyzed by GC—CI—PI—MS. Linear calibration curves for both authentic pure standards and the compound recovered from plasma were obtained over a concentration range of 25—200 ng of [I]-OTMS injected, on a 4 ft. × 2 mm I.D. packed column containing 3% SE-30 on 120—140 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) at 230°C using methane as both the carrier and reagent gas. The $[M+H]^+$ ions at *m/z* 437 and *m/z* 397 were monitored at retention times of 1.75 and 3.42 min for [I]-OTMS and [II]-OTMS, respectively. Improvement in sensitivity may yet be obtained in either PI or NI modes using either isobutane or methane—ammonia as the reagent gas. These leads can be further developed to yield a more sensitive and specific assay for [I] using the deuterated analog of [I] as the internal standard, if needed for use in future clinical pharmacokinetic studies.

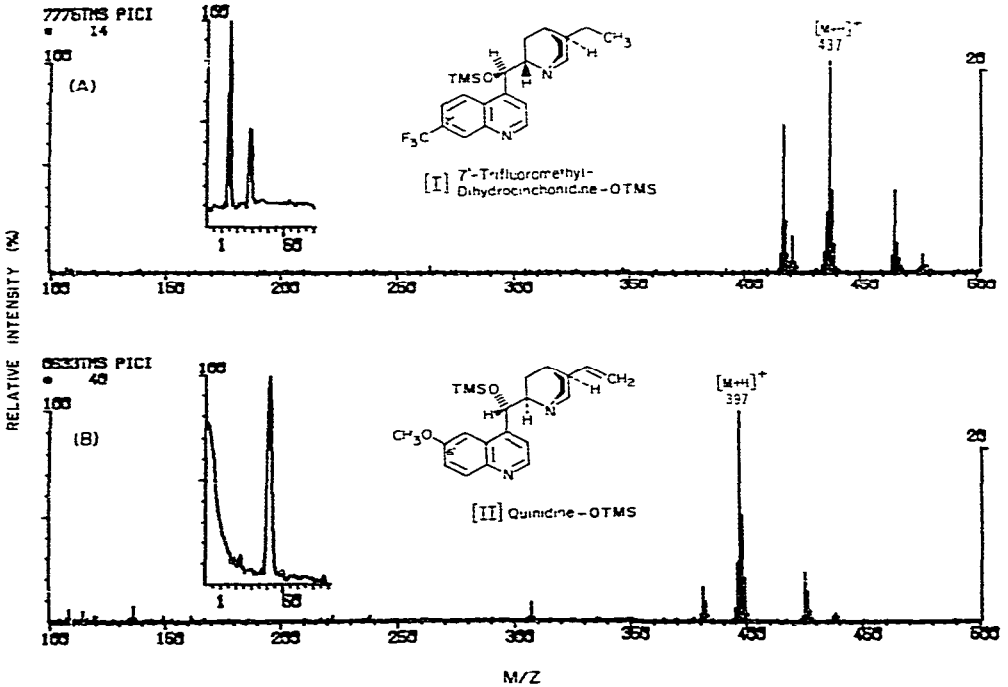


Fig. 4. GC—CI—PI—mass spectra of (A) compound [I] and (B) quinidine [II] as their respective trimethylsilyl derivatives. The inset in each panel is a total ion chromatogram for the compound.

Application of the HPLC method to pharmacokinetic studies in the dog

Plasma analysis. Plasma concentrations of [I] were determined in a dog (Table II) following the intravenous and oral administration of single doses of an aqueous solution of [I]·2HCl equivalent to 20 mg (free base) per kg. Following intravenous administration, a plasma concentration of 7.85 μg/ml at 5 min declined to a plateau of about 4.4 μg/ml from 30 min to 30 h, then declined to 1.41 μg/ml at 72 h. Following oral administration of the drug, a peak plasma concentration of 9.52 μg/ml was observed at 26 h, declining to 1.11 μg/ml at 100 h.

TABLE II

PLASMA CONCENTRATIONS OF [I] IN A DOG FOLLOWING THE ADMINISTRATION OF SINGLE DOSES OF AN AQUEOUS SOLUTION OF THE 2 HCl SALT EQUIVALENT TO 20 mg (FREE BASE)/kg BY INTRAVENOUS AND ORAL ROUTES

NST = No sample taken. NM = Non-measurable, < 0.2 μg/ml.

Time	Intravenous (μg/ml)	Oral (μg/ml)
5 min	7.85	NST
7.5 min	6.34	NST
10.0 min	5.84	NM
12.5 min	5.54	NST
15 min	5.64	NST
20 min	5.31	1.31
30 min	4.66	2.41
45 min	4.52	2.54
1 h	4.93	2.78
1.5 h	4.05	3.83
2 h	4.44	3.53
3 h	4.09	3.88
4 h	4.15	4.39
6 h	4.00	5.34
8 h	4.18	5.36
10 h	4.73	5.91
24 h	4.83	8.11
26 h	NST	9.52
28 h	NST	8.45
30 h	4.70	NST
32 h	NST	7.05
48 h	3.01	5.62
52 h	NST	5.09
56 h	NST	4.34
72 h	1.41	2.61
76 h	NST	2.42
80 h	NST	2.02
96 h	NST	1.20
100 h	NST	1.11

Urine analysis. The 0—24 h and 24—48 h urine samples from the dog administered a single intravenous [20 mg (free base)/kg] dose of the 2HCl salt equivalent to 224 mg of free base [I] were analyzed. The parent compound [I] in the unconjugated form represented 2.4% of the dose (Table III). Significant peaks representing at least 6 metabolites were seen in the unconjugated form in both the 0—24 h and 24—48 h urine samples (Fig. 3); the same peaks were also seen in lesser amounts in the glucuronide conjugate fraction. These metabolites were completely resolved from the parent drug, hence do not interfere with the specificity of the assay.

Studies on the in vitro metabolism of [I] have yielded five hydroxylated metabolites [26], which were characterized by NMR and mass spectrometry. The identity of the in vivo metabolites seen in urine must await the synthesis of authentic reference compounds.

TABLE III

URINARY EXCRETION OF UNCONJUGATED [I] (μg/ml) IN A DOG FOLLOWING THE INTRAVENOUS ADMINISTRATION OF AN AQUEOUS SOLUTION OF THE HCl SALT EQUIVALENT TO 224 mg (20 mg/kg) OF FREE BASE

Excretion period (h)	Volume voided (ml)	μg/ml	Total excreted (mg)	% of dose
0—24	140	25.90	3.63	1.6
24—48	410	4.43	1.82	0.8
		Total	5.45	2.4

Stability of [I] in human plasma on prolonged storage

The stability of [I] in plasma (determined by HPLC analysis) was evaluated at three concentrations following storage at −17°C and −70°C for a period of 7 and 37 days. The plasma samples were prepared as follows: Transfer a 0.5-ml aliquot of stock solution A (see Experimental section) into a 50-ml glass stoppered volumetric flask, dilute to 50 ml with human plasma, mix well in a sonic bath, to yield a plasma stock solution 1 whose concentration is 10 μg/ml (representing an upper limiting value on the calibration curve). Transfer 5 ml of solution 1 into a 25-ml volumetric flask, dilute to mark with plasma, and mix well, to yield plasma solution 2 whose concentration is 2 μg/ml (representing a mid-point value on the calibration curve). Transfer 0.5 ml of plasma stock solution 1 into a 25-ml volumetric flask, dilute to mark with plasma, to yield plasma solution 3 whose concentration is 0.2 μg/ml (representing the sensitivity limit of the assay).

Storage

Transfer four 3.5-ml aliquots of solutions 1, 2 and 3 (10, 2 and 0.2 μg/ml, respectively) into 7.5-ml glass vials with plastic caps for storage at −17°C (12 samples).

Additionally, transfer two 3.5-ml aliquots of solutions 1, 2 and 3 into 7.5-ml glass vials for storage at −70°C (6 samples).

The stored stability indicating plasma samples are analyzed as unknowns in triplicate on appropriate days along with the external standards and internal standards added to fresh control plasma as described previously.

The mixed standard solutions a—e prepared fresh on day 0 are stored at 5—10°C for evaluation of solution stability. A 10-μl aliquot of each standard is injected and compared to the response of a corresponding 10-μl injection from a set of freshly prepared mixed-standard solutions on each day of analysis.

The stability indicating plasma samples from day 0, days 7 and 37 stored at −17°C were analyzed and the data tabulated in Table IV. A correlation coefficient (determined by the method of least squares) of at least 0.95 was arbitrarily selected as the lower limit of acceptable stability of the compound under storage. The data indicated that [I] was stable throughout the storage interval at −17°C, with an overall coefficient of correlation between day 0 to 7 and day 0 to 37 of 0.9999. In the event that the samples stored at −17°C showed instability, then the analysis of the samples stored at −70°C would have been undertaken, using the same statistical criteria.

TABLE IV

STABILITY OF [I] IN PLASMA STORED AT −17° FOR 37 DAYS

Day 0 compared to day 7: coefficient of determination = 0.9998; coefficient of correlation = 0.9999. Day 0 compared to day 37: coefficient of determination = 0.9999; coefficient of correlation = 0.9999.

Sample conc. (μg/ml)	Day	*n*	Mean conc. found (μg/ml)	S.D.	S.D. (%)
0.2	0	3	0.26	± 0.01	4.5
2.0	0	3	1.98	± 0.05	2.4
10.0	0	2	9.52	± 0.01	0.1
0.2	7	3	0.29	± 0.02	8.1
2.0	7	3	2.01	± 0.10	4.9
10.0	7	3	10.14	± 0.27	2.7
0.2	37	3	0.20	± 0.02	12.3
2.0	37	3	2.03	± 0.09	4.4
10.0	37	3	10.55	± 0.17	1.6

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