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DETERMINATION OF THE ANTIALLERGENIC AGENT, *TRANS*-3-[6-(METHYLTHIO)-4-OXO-4H-QUINAZOLIN-3-YL]-2-PROPENOIC ACID, IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of the antiallergenic compound, *trans*-3-[6-(methylthio)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [I], in plasma. The assay involves acetonitrile protein precipitation followed by the analysis of an aliquot of the protein-free fraction by reversed-phase HPLC with fluorescence detection (excitation at 245 nm, with emission greater than 418 nm). The overall recovery of [I] from plasma was 103 ± 10%. The sensitivity limit of the assay was 0.125 μg/ml of plasma. The analogous compound, *trans*-3-[6-[(1-methylethyl)thio]-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [II], is used as the internal standard. The assay was used to monitor the plasma concentration—time fall-off profile of [I] in the dog and in man. The stability of [I] was demonstrated in dog plasma on long-term storage for up to 180 days at —17°C and —70°C.

INTRODUCTION

The compound, *trans*-3-[6-(methylthio)4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [I] (Fig. 1), synthesized by LeMahieu [1], is under investigation as an active mediator release inhibitor for use in the treatment of allergic bronchial asthma [2].

Studies on the biotransformation of [I] in the rat indicated that the compound was converted by S-oxidation to the sulfoxide, *trans*-3-[6-(methylsulfinyl)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [I-A], and by hydrogenation of the side chain to the dihydro compound, 6-(methylthio)-4-oxo-4H-quinazoline-3-propenoic acid, [I-B] (Fig. 1) [3].

A sensitive and selective high-performance liquid chromatographic (HPLC)

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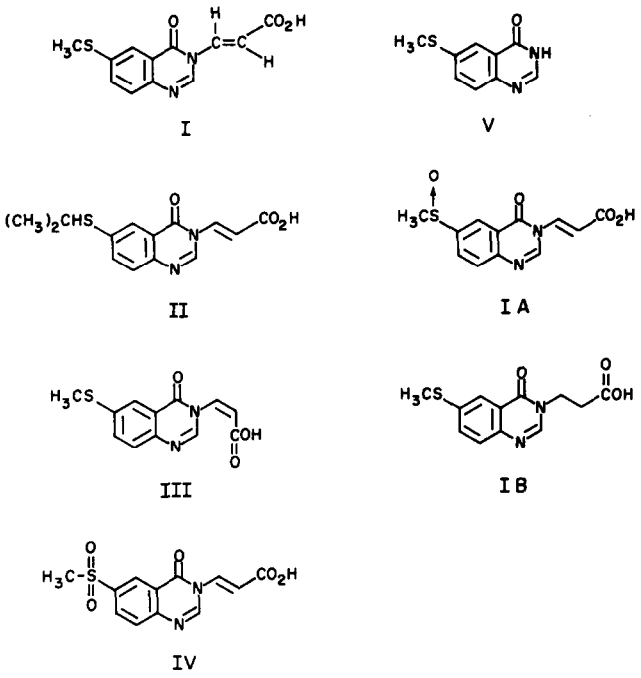

Fig. 1. Chemical structures for the compounds referred to in the text.

assay with automated injection was developed to monitor the biopharmaceutic and pharmacokinetic profile of the drug in the dog. The parent compound [I] and its methylethyl analogue, *trans*-3-[6-[(1-methylethyl)thio]-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid, [II] (Fig. 1) used as the internal standard are quantitated in the protein-free fraction after precipitation of the plasma proteins with acetonitrile. An aliquot of the protein-free fraction is diluted with the HPLC mobile phase, introduced via a WISP auto-injector and analyzed by reversed-phase HPLC using fluorescence detection with excitation at 245 nm and emission greater than 418 nm.

The assay was used to monitor the plasma concentration—time fall-off profile of [I] in the dog and in man. The stability of [I] was demonstrated in dog plasma on long-term storage for up to 180 days at −17°C and −70°C.

EXPERIMENTAL

Column

A prepacked 30 cm × 3.9 mm I.D. column containing μBondapak C_{18}, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.) generating 16,680 plates per meter was used.

Instrumental parameters

The HPLC system consisted of a Waters Model 6000 A reciprocating piston pump, a Waters Intelligent Sample Processor (WISP™) Model 710B, and a Schoeffel Model FS-970 LC fluorometer operated at 245 nm for excitation and emission at wavelengths greater than 418 nm (Kratos, Schoeffel Instru-

ments, Westwood, NJ, U.S.A.). The isocratic mobile phase used was a mixture of acetonitrile—methanol—0.001 *M* ascorbic acid (pH 3.25) (36:28:36, v/v) at a pressure of ca. 7 MPa and constant flow-rate of 1 ml/min. The fluorescence detector sensitivity was 1.0 μA full scale and the chart speed on the 10-mV recorder, Model 7132A (Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP auto-injector was programmed to run for 12 min (10 min run time + 2 min purge and rinse) per sample using methanol as the rinse solvent. Under these conditions 25 ng of [I] and 40 ng of [II] injected gave nearly full-scale pen response. The retention times of [I] and [II] were 4.42 min and 6.26 min (Table I) with corresponding capacity factors (k') of 0.55 and 1.19, respectively. The minimum detectable amount of [I] was 1.25 ng injected equivalent to 0.125 μg/ml of plasma.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS (k') OF COMPOUNDS REFERRED TO IN THE TEXT AND IN FIG. 1

Compound	Retention time (min)	k'
[I]	4.42	0.55
[I-A]	3.06	0.07
[I-B]	3.98	0.39
[II]	6.26	1.19
[III]	3.47	0.21
[IV]	3.20	0.10
[V]	3.98	0.40

Reagents

All reagents were of analytical-reagent grade (> 99% purity). They include acetonitrile, methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); 0.001 *M* ascorbic acid, pH 3.25 (0.176 g/l), USP-FCC grade (Roche Chemical Division, Hoffmann-La Roche, Nutley, NJ, U.S.A.); concentrated ammonium hydroxide (29.3% ammonia), Baker analyzed reagent grade (J.T. Baker, Phillipsburg, PA, U.S.A.).

Analytical standards

Compound [I], *trans*-3-[6-(methylthio)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{12}H_{10}N_2O_3S$, MW 262.3, m.p. 271—272°C); compound [I-A], *trans*-3-[6-(methylsulfinyl)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{12}H_{10}N_2O_4S$, MW 278.3, m.p. 276—277°C); compound [I-B], 6-(methylthio)-4-oxo-4H-quinazoline-3-propenoic acid ($C_{12}H_{12}N_2O_3S$, MW 264.3, m.p. 192—193°C); compound [II], *trans*-3-[6-[(1-methylethyl)thio]-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{14}H_{14}N_2O_3S$, MW 290.4, m.p. 238—239°C); compound [III], *cis*-3-[6-(methylthio)-4-oxo-4H-quinazolin-3yl]-2-propenoic acid ($C_{12}H_{10}N_2O_3S$, MW 262.3, m.p. 233—234°C); compound [IV], *trans*-3-[6-(methylsulfonyl)-4-oxo-4H-quinazolin-3-yl]-2-propenoic acid ($C_{12}H_{10}N_2O_5S$, MW 294.3, m.p. 284—290°C) and compound [V], 6-(methylthio)-4-(3H)-quinazolinone ($C_9H_8N_2OS$, MW 192.3, m.p. 203—204.5°C). All compounds

were of pharmaceutical grade purity (> 99%), with the exception of compounds [I-A] and [III] which were approximately 98% pure.

Preparation of analytical standards

Prepare stock solutions of compounds [I] and [II] as described below:

(A) 1 mg of [I] per ml in methanol—conc. ammonium hydroxide (99:1). Dissolve 10 mg of [I] in 10 ml of methanol—conc. ammonium hydroxide (99:1).

(B) 100 μg of [I] per ml in methanol—conc. ammonium hydroxide (99:1). A 1-ml aliquot of Solution A diluted to 10 ml with methanol—ammonium hydroxide (99:1).

(C) 1 mg of [II] per ml in methanol—conc. ammonium hydroxide (99:1). Dissolve 10 mg of [II] in 10 ml of methanol—ammonium hydroxide (99:1).

Preparation of mixed standard solutions 1—7: aliquots of Solution A, B and C are diluted to 10 ml in methanol as follows:

Solution	Aliquots (μl) of standard			Final concn. (ng per 100 μl of solution)	
	A	B	C	[I]	[II]
1	—	25	80	25	800
2	—	50	80	50	800
3	—	100	80	100	800
4	20	—	80	200	800
5	50	—	80	500	800
6	100	—	80	1000	800
7	—	—	80	0	800

Aliquots (100 μl) of solutions 1, 2, 3, 4, 5 or 6 are added to separate 200-μl specimens of control plasma and processed along with the samples to establish a recovered standard calibration curve for the direct quantitation of unknowns.

Aliquots (50 μl) of the above solutions are added to a 0.5-ml aliquot of the protein-free fraction of control plasma and diluted to 1.5 ml with HPLC mobile phase as the external standard calibration curve to establish the linearity and performance of the HPLC system.

Analytical procedure

The flow diagram of the precipitation procedure is shown in Fig. 2. Into a glass-stoppered 15-ml centrifuge tube, transfer a 100-μl aliquot of solution 7 (equivalent to 800 ng of [II], the internal standard), 700 μl of acetonitrile, and mix for a few seconds on a Vortex super-mixer (Lab-Line Instruments, Melrose Park, IL, U.S.A.). Add 200 μl of unknown plasma (aliquots of less than 200 μl taken of those unknowns with expected concentrations above the highest calibration point are diluted to 200 μl with control plasma) and mix again for 10 sec at the highest speed setting of the Vortex mixer. 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 2100 rpm (1100 *g*). Transfer 0.5 ml of the supernatant protein-free fraction into a standard 4-ml glass vial

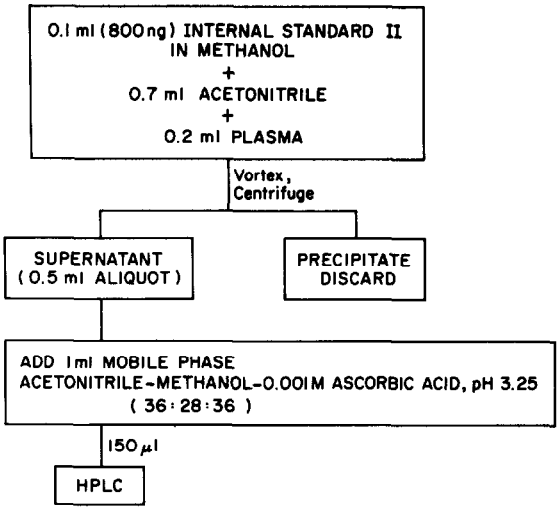

Fig. 2. Flow diagram of the precipitation procedure of Compounds [I] and [II] from plasma.

(Waters part No. 73001). Add 1 ml of the mobile phase, acetonitrile—methanol—0.001 *M* ascorbic acid, pH 3.25 (36:28:36), and seal with an H-style vial cap (Waters part no. 72711) fitted with a PTFE septum (Waters part No. 73005). Program the auto-injector (WISP 710B) to inject 75—150 µl out of a total volume of 1.5 ml for HPLC analysis.

Along with the samples, process seven 200-µl specimens of control plasma, one to be used as a control blank to which 100 µl of methanol are added and six to be used for the preparation of the recovered standards to which 100 µl of solutions 1, 2, 3, 4, 5 or 6, equivalent to 25, 50, 100, 200, 500 ng and 1 µg of [I] and 800 ng of [II] per 200 µl of plasma (representing 0.125, 0.25, 0.5, 1, 2.5 or 5 µg of [I] and 4 µg of [II] per ml of plasma), respectively, are added. These standards are used to establish the recovery curve for the direct quantitation of the unknowns.

In order to verify the linearity and performance of the HPLC assay, a matrix external standard calibration curve is prepared by adding six 200-µl specimens of control plasma to separate mixtures of 100 µl methanol and 700 µl acetonitrile. To 0.5-ml aliquots of the resultant protein-free fractions, 50 µl of solutions 1, 2, 3, 4, 5 or 6 are added along with 0.95 ml of mobile phase. Aliquots (75 or 150/1500 µl) are programmed on the WISP for automated injection. Typical chromatograms are shown in Fig. 3.

Calculations and assay validation

The concentration of [I] in the unknowns was determined by interpolation from a least squares regression equation (power equation: $Y = mX^b$) of the calibration data (processed by a Hewlett-Packard Model 3354B Laboratory Data System) of the recovered standards processed along with the unknowns using peak height ratios (peak height of compound [I] to peak height of internal standard [II]) versus concentration of [I] per ml of plasma. A typical calibration curve as defined by the equation $Y = 0.330X^{0.946}$ is linear from

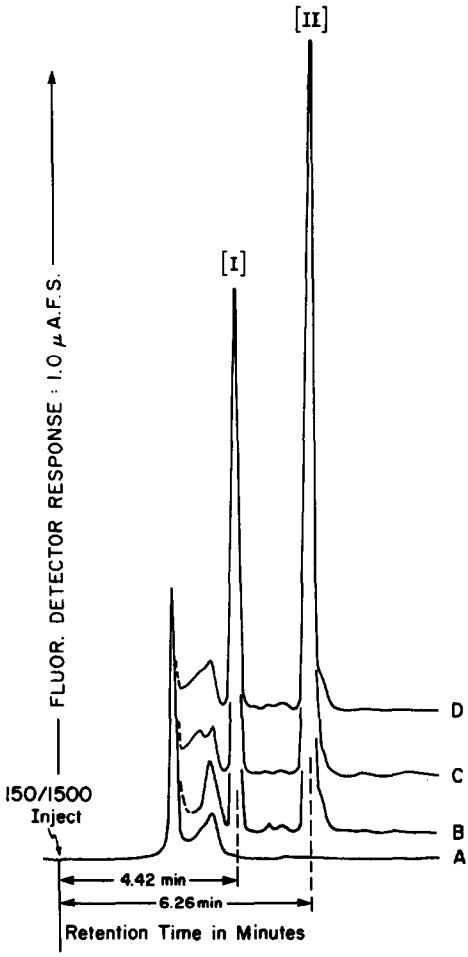

Fig. 3. Chromatograms of Compound [I] and Compound [II], the internal standard. (A) Control (0 h) dog plasma supernatant, (B) 1.5-h dog plasma supernatant following a single 8 mg/kg i.v. dose of [I], (C) authentic standards recovered from control dog plasma, and (D) authentic standards added to the supernatant of control dog plasma (matrix external standards).

0.125—5 μg of [I] per ml of plasma. The correlation coefficient (r) is equal to 0.9997 and the average deviation from the line is 2.71%. Intra- and inter-assay validation data over the linear concentration range (0.125—5.0 μg/ml of plasma) are summarized in Table II. The mean intra- and inter-assay coefficients of variation are 1.7% and 4.0%, respectively.

RESULTS AND DISCUSSION

A rapid, sensitive and selective HPLC assay was developed for the determination of compound [I] from plasma using a WISP auto-injector and fluorescence detection for quantitation. This method enabled the rapid and accurate quantitation of compound [I] with high sample throughput required

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND [I]

Amount added (μg/ml)	Amount found (μg/ml)	n	Coefficient of variation (%)
Intra-assay variability			
0.125	0.131 ± 0.023	3	1.8
0.250	0.243 ± 0.004	3	1.8
0.500	0.480 ± 0.010	3	2.0
1.00	1.00 ± 0.02	3	1.8
2.50	2.49 ± 0.05	4	1.8
5.00	5.11 ± 0.04	3	0.8
		Average	1.7
Inter-assay variability			
0.125	0.130 ± 0.007	14	5.5
0.250	0.246 ± 0.009	13	3.8
0.500	0.481 ± 0.015	13	3.0
1.00	1.00 ± 0.07	13	6.5
2.50	2.54 ± 0.10	14	4.1
5.00	5.05 ± 0.20	12	4.0
		Average	4.0

for pharmacokinetic and biopharmaceutic studies. The corrected excitation and emission spectra of compound [I] occur at 245 and 425 nm, respectively. The Schoeffel Model FS-970 fluorescence detector with excitation at 245 nm and emission greater than 418 nm allowed for quantitation of [I] and [II] in the nanogram range.

Reversed-phase HPLC analysis is the method of choice, since it is amenable to an acetonitrile protein precipitation step followed by direct injection of the supernatant after dilution with mobile phase.

Compound [II] was chosen as the internal standard in the assay, because of its similar precipitation and chromatographic behavior to compound [I]. Compound [II] has not been identified as a metabolite of [I].

Percent recovery and sensitivity limits

The overall recovery of [I] was 103 ± 10% (S.D.) over the concentration range of 0.125–5 μg per ml of plasma. The ⩾ 100% recovery was attributed to a matrix enhancement effect, which necessitated that the external standard samples be added to the protein-free fraction of control plasma [4, 5]. The sensitivity limit of the assay was 0.125 μg of [I] per ml of plasma.

Chromatographic behavior of [I] and [II]

Compounds [I] and [II] exhibited UV absorbances sufficiently intense for detection at 254 nm. However, the use of ascorbic acid as a stabilizer/buffer in the mobile phase created a significantly high background UV absorbance, making UV detection impossible. Also, the fact that the UV chromatogram

of the control plasma samples contained significant interfering matrix peaks eluting in the region of compound [I], made fluorometric detection a necessity.

The HPLC system is flushed initially with methanol—water (50:50) to remove deposits from the column accumulated from previous use. The mobile phase [acetonitrile—methanol—0.001 *M* ascorbic acid, pH 3.25 (36:28:36)] is allowed to recycle through the system overnight, or, in some instances, for 48 h or more at 0.5 ml/min until equilibrium is attained when the desired separation and resolution are obtained. Non-equilibration may result in interference from small artifact peaks eluting close to [I] and/or [II], or a change in k' values. Several μBondapak C_{18} columns used during the course of this project showed variation in equilibration time from column to column with concomitant variation in retention time of ± 0.5 min for [I] and ± 1 min for [II].

It was also noted that retention times of [I] and [II] changed significantly when aliquots of mixed standard solutions 1—7 in methanol were injected without the presence of biological matrix. Preparing [I] and [II] in methanol—conc. ammonium hydroxide (99:1) for stock solutions A—C and subsequent preparation of mixed working standard solution 1—7 also in methanol—conc. ammonium hydroxide (99:1) instead of in methanol alone alleviates this discrepancy. However, due to the volatility of the ammonia from the methanol—ammonia solution (which would change its concentration), the working mixed standard solutions 1—7 were prepared in 100% methanol and added to the biological matrix. No adverse effect was observed in quantitation of [I] or [II]. Also, no significant change in chromatography was observed when sample volumes between 10 and 150 μl were injected.

Selectivity of the assay

The biotransformation [3] and the instability of [I] due to isomerization under specific conditions necessitate the use of chromatographic procedures which ensure the stability and selectivity of the assay. Ascorbic acid buffer was chosen in place of the more commonly used phosphate buffer because of its antioxidant property. The resultant chromatography improved the peak shape of compounds [I] and [II] and their resolution. A fresh solution of 0.001 *M* ascorbic acid is prepared every month.

Under the reported procedure, the biotransformed products [I-A], [I-B] and [III] (*cis*-isomer, Table I) were > 90% resolved from each other and from [I] and [II] (Fig. 4). Compound [IV] eluted very soon after [I-A] such that both compounds were not fully resolved from the solvent front. Compound [V] eluted at the same retention time as [I-B], and was not fully resolved from [I] on certain μBondapak C_{18} columns. In this case further equilibration and/or a new C_{18} column was required.

None of these compounds, however, would interfere with the quantitation of [I] (Fig. 4).

Application of the HPLC method

Biopharmaceutic and pharmacokinetic studies in the dog. The HPLC method was applied to a pilot study of single 2 mg/kg doses of [I] administered intra-

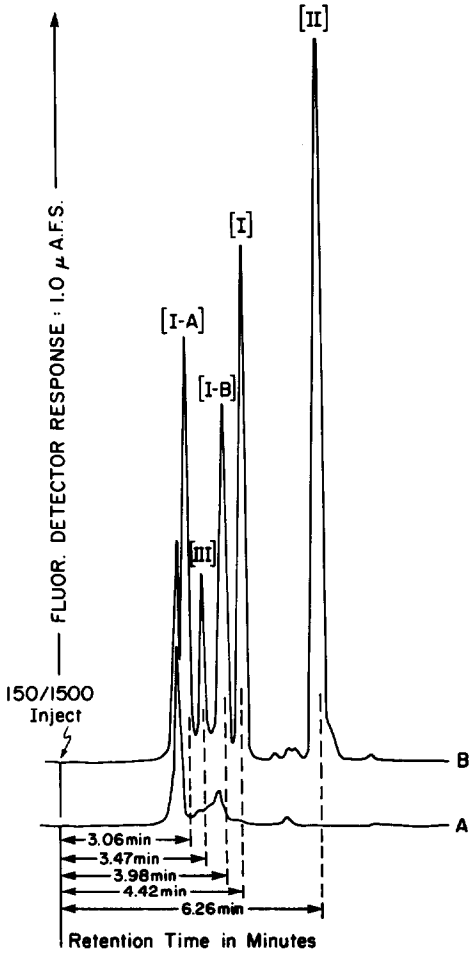

Fig. 4. Chromatogram of Compounds [I], [I-A], [I-B], [II] and [III]. (A) Control dog plasma supernatant and (B) authentic standards recovered from control dog plasma.

venously (i.v.) and orally as a solution and capsule (handpacked) in the dog. Following this dosing regimen plasma concentrations were measurable up to 4 h.

Studies in man. Plasma concentrations of [I] were determined in four normal volunteers following the oral administration of a single 350-mg dose of the drug in suspension. Blood samples were collected at appropriate time points in heparinized tubes and centrifuged; the plasma was separated and stored frozen at −70°C until analysis. The mean data plotted in Fig. 5 suggest that peak concentrations of about 7 μg/ml occur at about 2 h and that the plasma concentrations decline, being measurable at 9 h, but not 12 h post dosing.

Stability of [I] in plasma

Stability of [I] in dog and human plasma at ambient temperature. Compound [I] added to fresh control dog plasma was analyzed by HPLC after

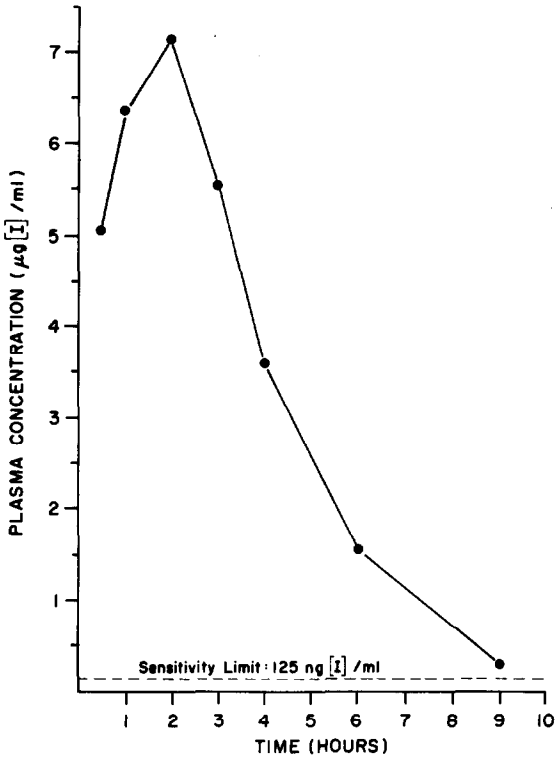

Fig. 5. Mean plasma concentrations of [I] following the oral administration of a single 350-mg dose to four subjects.

remaining at room temperature (23°C) from 0—48 h, and found to be stable for up to 6 h. The recovery dropped to 93 and 86% at 24 and 48 h, respectively, indicating apparent instability. Similar results were obtained for compound [I] in fresh human plasma. Under these conditions, Compound [I] is stable under the sample preparation techniques described with no additional precautions required.

Stability of [I] in dog plasma on storage at −17°C and −70°C up to 180 days. The stability of [I] in dog plasma was evaluated by HPLC analysis at three concentrations following storage at −17°C and −70°C for a period of 7, 30, 61, 90 and 180 days. The plasma samples were prepared as follows: transfer 12.5 μl of solution B (see Experimental section) into a 10-ml glass stoppered amberized volumetric flask, dilute to 10 ml by adding fresh control dog plasma slowly, mix well by sonication to yield a plasma stock solution 1 whose concentration is 0.125 μg/ml (representing the sensitivity limit of the assay). Transfer 10 μl of solution A into a 10-ml glass stoppered amberized volumetric flask, dilute to volume with plasma to yield plasma solution 2 whose concentration is 1 μg/ml (representing a mid-point value on the calibration curve). Transfer 50 μl of solution A into a 10-ml glass stoppered amberized volumetric flask, dilute to volume with plasma to yield plasma solution 3 whose concentration is 5 μg/ml (representing the upper limit on the calibration curve).

Storage. Transfer 0.8-ml aliquots of plasma solutions 1, 2, and 3 (0.125, 1, and 5 μg/ml, respectively) into ten 1-dram vials for each concentration (30 vials). Five vials at each concentration (total of 15) were stored at −17°C with the remaining five vials at each concentration (total of 15) stored at −70°C.

The stability-indicating plasma samples from days 0, 7, 30, 61, 90 and 180 stored at −17°C and −70°C were analyzed in triplicate as unknowns along with the external standards and recovered calibration standards at similar concentrations added to fresh control plasma. The data are tabulated in Table III and were analyzed by least-squares regression. The data indicated that [I] was stable throughout the storage interval at −17°C and −70°C, although the recovery of samples stored at the higher temperature was consistently a few percentage points lower though not statistically significant. The coefficients of correlation of all samples as compared to day 0 were ⩾ 0.999, indicating overall stability.

TABLE III

STABILITY OF [I] IN DOG PLASMA STORED AT −17°C AND −70°C FOR UP TO 180 DAYS

n = 3 for all samples except those denoted with asterisk.

Day	Mean concn. found ± S.D. (% S.D.) (0.125 μg/ml added)	Mean concn. found ± S.D. (% S.D.) (1.00 μg/ml added)	Mean concn. found ± S.D. (% S.D.) (5.00 μg/ml added)
Stability of [I] stored at −17°C			
0	0.124 ± 0.004 (3.3)	1.01 ± 0.02 (1.6)	5.24 ± 0.08 (1.5)
7	0.123 ± 0.000 (0.0)	0.85 ± 0.01 (0.6)	4.58 ± 0.03 (0.6)
30	0.138 ± 0.004 (3.0)	0.96 ± 0.03 (3.0)	4.80 ± 0.05 (1.1)
61	0.132 ± 0.000 (0.0)	0.86 ± 0.01 (1.4)*	4.48 ± 0.03 (0.6)
90	0.130 ± 0.000 (0.0)	0.89 ± 0.01 (1.1)	4.48 ± 0.09 (2.0)
180	0.114 ± 0.000 (0.0)	0.85 ± 0.05 (5.8)	4.55 ± 0.07 (1.5)
Stability of [I] stored at −70°C			
0	0.124 ± 0.004 (3.3)	1.01 ± 0.02 (1.6)	5.24 ± 0.08 (1.5)
7	0.123 ± 0.000 (0.0)	0.91 ± 0.02 (2.0)	4.81 ± 0.19 (3.9)*
30	0.143 ± 0.000 (0.0)	1.00 ± 0.01 (0.9)	5.03 ± 0.04 (0.8)
61	0.132 ± 0.000 (0.0)*	0.89 ± 0.02 (2.0)	4.71 ± 0.23 (4.9)
90	0.130 ± 0.000 (0.0)	0.91 ± 0.02 (1.6)	4.77 ± 0.04 (0.9)
180	0.114 ± 0.000 (0.0)	0.89 ± 0.02 (2.1)	4.75 ± 0.09 (1.9)

*n = 2 for these samples.

The plasma samples collected from dogs in the biopharmaceutic and pharmacokinetic studies and from man in the single-dose pharmacokinetic study were stored at −70°C prior to analysis.

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