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DETERMINATION OF THE ANTIALLERGENIC AGENT, N-[4-(1H-IMIDAZOL-1-YL)BUTYL]-2-(1-METHYLETHYL)-11-OXO-11H-PYRIDO[2,1-*b*]QUINAZOLINE-8-CARBOXAMIDE, IN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS USING FLUOROMETRIC DETECTION

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of the antiallergenic compound N-[4-(1H-imidazol-1-yl)butyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxamide (I), and its major metabolite, 2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxylic acid (I-A), in plasma. The assay involves precipitation of the plasma proteins with acetonitrile—methanol (9:1), followed by the analysis of an aliquot of the protein-free filtrate by reversed-phase ion-pair HPLC with fluorescence detection for quantitation. The analogous compound, N-[6-(1H-imidazol-1-yl)hexyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]-quinazoline-8-carboxamide (II), is used as the internal standard. The overall recovery of compounds I and I-A from plasma is 107.0 ± 8.6% and 107.0 ± 10.0%, respectively. The sensitivity limits of quantitation are 20 ng of I, and 10 ng of I-A per ml of plasma using a 0.5-ml aliquot. The assay was used to monitor the plasma concentrations of I and of I-A in a dog following a 5 mg/kg intravenous infusion of I · 2HCl, a 10 mg/kg oral dose of I · 2HCl and of metabolite I-A.

INTRODUCTION

The compound N-[4-(1H-imidazol-1-yl)butyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxamide·2HCl, I·2HCl, is a member of a series of amide and ester derivatives of 2-substituted pyrido[2,1-*b*]quinazoline-8-carboxylic acids synthesized by Tilley [1] (Fig. 1), of clinical interest as bronchospasmolytic agents [2]. Studies in the dog have shown that compound I is hydrolyzed at the amide bond to yield 2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, compound I-A, as the predomi-

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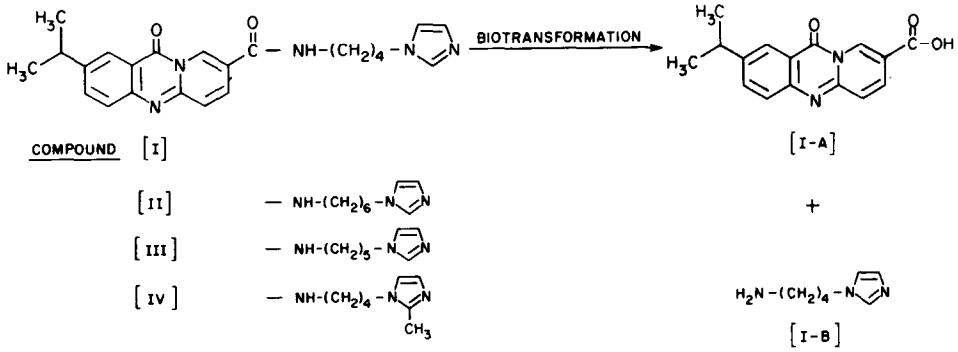

Fig. 1. Chemical structures for the compounds referred to in the text.

nant plasma metabolite. The 2-substituted quinazoline-8-carboxylic acid series of compounds have also been investigated as antiallergenic agents [3—5]. The analogue, 2-methoxy-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, exhibited potent oral activity as an antiallergenic agent [6], and its pharmacokinetics in man have been reported [7]. A high-performance liquid chromatographic (HPLC) assay for this compound was also reported [8].

A rapid, sensitive and selective HPLC assay, with automated injection, was developed for the determination of I and I-A to monitor the biopharmaceutic and pharmacokinetic profile of I in the dog. The parent compound, I, and its major metabolite, I-A, are quantitated in the protein-free filtrate after precipitation of the plasma proteins with acetonitrile—methanol (9:1). An aliquot of the protein-free filtrate is analyzed by reversed-phase ion-pair HPLC with a WISP autoinjector, using fluorescence detection (excitation at 275 nm and emission greater than 418 nm) for quantitation. The assay is selective for the parent drug, I, in the presence of its acid metabolite, I-A. The analogous compound, N-[6-(1H-imidazol-1-yl)hexyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxamide, II, is used as the internal standard in the assay.

The overall recovery of compounds I and I-A from plasma is 107.0 ± 8.6% and 107.0 ± 10.0%, respectively. The sensitivity limits of quantitation are 20 ng of I, and 10 ng of I-A per ml of plasma using a 0.5-ml aliquot.

The assay was used to monitor the plasma concentrations of I and of I-A in a dog following a 5 mg/kg intravenous infusion of I·2HCl, a 10 mg/kg oral dose of I·2HCl, and of metabolite I-A.

EXPERIMENTAL

Analytical standards

Compound I·2HCl ($C_{23}H_{25}N_5O_2 \cdot 2HCl$, MW = 476.4, m.p. = 261—263°C decomposition), compound I-A ($C_{16}H_{14}N_2O_3$, MW = 282.29, m.p. > 310°C), and compound II [$C_{25}H_{29}N_5O_2$, MW = 431.53, m.p. = 135—136°C, of pharmaceutical grade purity (> 99%)] were used as the analytical standards.

Preparation of standard solution

Standard solutions of I, I-A and II were prepared as follows:

Solution A-1. 118 μg of I·2HCl per ml (equivalent to 100 μg of I per ml) in methanol—water—conc. ammonium hydroxide (75:24:1). Dissolve 1.18 mg of I·2HCl in 10.0 ml methanol—water—conc. ammonium hydroxide (75:24:1) in a 10-ml amberized volumetric flask.

Solution A-2. 11.8 μg of I·2HCl per ml (equivalent to 10 μg of I per ml) (1.0-ml aliquot of solution A-1 diluted to 10.0 ml with methanol).

Solution B-1. 100 μg of I-A per ml in methanol—water—conc. ammonium hydroxide (75:24:1). Dissolve 1.0 mg of I-A in 10 ml of methanol—water—conc. ammonium hydroxide (75:24:1).

Solution B-2. 10 μg of I-A per ml in methanol (1.0-ml aliquot of solution B-1 diluted to 10 ml with methanol).

Solution B-3. 1.0 μg of I-A per ml in methanol (1.0 ml aliquot of solution B-2 diluted to 10 ml with methanol).

Solution C. 100 μg of II per ml in methanol—water—conc. ammonium hydroxide (75:24:1). Dissolve 1.0 mg of II in 10 ml of methanol—water—conc. ammonium hydroxide (75:24:1).

Mixed standard solutions Nos. 1—8 are prepared by diluting aliquots of solution A-1, A-2, B-1, B-2, B-3 and C to 10 ml in methanol given in Table I.

TABLE I

PREPARATION OF MIXED STANDARD SOLUTIONS 1—8

Solution no.	Aliquots (μl) of standard						Final concentration (ng per 100 μl of solution)		
	A-1	A-2	B-1	B-2	B-3	C	I	I-A	II
1	—	100	—	—	500	200	10	5	200
2	—	200	—	—	1000	200	20	10	200
3	—	350	—	300	—	200	35	30	200
4	—	1000	—	500	—	200	100	50	200
5	200	—	—	1000	—	200	200	100	200
6	350	—	300	—	—	200	350	300	200
7	1000	—	500	—	—	200	1000	500	200
8	—	—	—	—	—	200	—	—	200

Solutions of I·2HCl, I-A and II are stable for two months when stored at 5°C in their respective solvents.

Aliquots of 100 μl of solution 1, 2, 3, 4, 5, 6 or 7 were added to separate 0.5-ml specimens of control plasma and processed along with the samples to establish a processed (recovered) standard calibration curve for the direct quantitation of unknowns.

Aliquots of 100 μl of the above solutions were added to 0.5 ml of distilled water plus 0.1 ml of 0.25 *M* potassium phosphate buffer, pH 7.0, and diluted with 1.3 ml acetonitrile—methanol (9:1) to establish an external standard calibration curve to determine the linearity and performance of the HPLC system.

Reagents

All inorganic reagents were analytical reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered

through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.). The inorganic reagents and solutions include: conc. ammonium hydroxide (29.3% ammonia, Baker analyzed reagent grade), 0.25 *M* potassium phosphate buffer, pH 7.0, and 1.0 *M* orthophosphoric acid. The PIC-A (Waters Assoc., Milford, MA, U.S.A.) used for HPLC consists of 0.005 mol of tetrabutylammonium phosphate in phosphate buffer, pH 7.5 (dissolved in approx. 14 ml water). (Note: if the PIC-A reagent is pale yellow to yellow in color and not "water white", it should not be used because it then will contribute significantly to fluorescence background and drastically reduce the sensitivity of the assay.). Acetonitrile and methanol, suitable for spectrophotometry and liquid chromatography, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Instrumental parameters

Column. The column used for reversed-phase HPLC was a pre-packed 30 cm × 3.9 mm I.D. stainless-steel column containing 10-μm Bondapak C_{18} (Waters Assoc.).

Instrument. The HPLC system consisted of a Model 6000A reciprocating piston pump and a Waters Intelligent Sample Processor (WISP^{TM}) Model 710B (Waters Assoc.) and a Schoeffel Model FS-970 LC fluorometer operated at 275 nm for excitation and emission at wavelengths greater than 418 nm (Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.). The isocratic mobile phase consisted of two vials of Waters Pic-A reagent, plus 0.5 ml of 1.0 *M* orthophosphoric acid added to 1 l of a mixture of water—methanol—acetonitrile (350:500:150) and was pumped at a pressure of approx. 14 MPa (2000 p.s.i.) and a constant flow-rate of 1.5 ml/min.

The fluorescence detector range was 0.5 μA full scale and the photomultiplier sensitivity was 580 V. 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 autoinjector was programmed for a 12-min run time per sample using mobile phase as the rinse solvent. Under these conditions 12.5 ng of I and 3.75 ng of I-A injected gave > 75% of full scale pen response, while 2.5 ng of II injected gave approx. 15–20% of scale. The retention times (t_R) of I, I-A and II were 6.1, 3.4 and 8.8 min, with corresponding capacity factors (k') of 1.6, 0.5 and 2.8, respectively (Fig. 2). The minimum detectable amount of I and I-A was 0.125 ng and 0.063 ng injected, equivalent to 20 ng of I and 10 ng of I-A per ml of plasma, respectively, using a 0.5-ml sample aliquot.

Analytical procedure

Into separate 100 × 13 mm disposable borosilicate culture tubes (Cat. No. 14-962-10C, Fisher Scientific, Pittsburgh, PA, U.S.A.) was added a 0.5-ml aliquot of unknown plasma sample (aliquots of less than 0.5 ml taken of those unknowns with expected concentrations above the highest calibration point were diluted to 0.5 ml with drug-free plasma), a 100-μl aliquot of standard solution 8 (equivalent to 200 ng of II, the internal standard) and 0.1 ml of 0.25 *M* potassium phosphate buffer, pH 7. Each sample was mixed for a few seconds on a Vortex mixer. Then 1.3 ml of acetonitrile–methanol (9:1) were added, each tube was stoppered with polyethylene caps (Plugtite Cat. No. 127-0019-

100, Elkay Products, Shrewsbury, MA, U.S.A.) and mixed again for 10—15 sec at the highest speed setting of the Vortex mixer. The samples were centrifuged at 2100 rpm (1100 *g*) for 10 min at 5°C in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC, Needham, MA, U.S.A.). The supernatant protein-free fraction was transferred into a standard 4-ml glass vial (Waters part No. 73001). Each vial was sealed with an H-style vial cap (Waters part No. 72711)) fitted with a PTFE septum (Waters part No. 73005). The autoinjector (WISP 710B) was programmed to inject 25 μl out of a total volume of 2.0 ml for HPLC analysis.

Recovered standard curve

Along with the samples, process eight 0.5-ml specimens of control plasma, one to be used as a control blank to which 100 μl of methanol are added and seven to be used for the preparation of the recovered standards to which 100 μl of solutions 1, 2, 3, 4, 5, 6 or 7 equivalent to 0, 10, 20, 35, 100, 200, 350, and 1000 ng of I; 0, 5, 10, 30, 50, 100, 300 and 500 ng of I-A; and 200 ng of II per 0.5 ml of plasma (representing 0, 20, 40, 70, 200, 400, 700 and 2000 ng of I; 0, 10, 20, 60, 100, 200 and 1000 ng of I-A; and 400 ng of II per ml of plasma), respectively, are added. These standards are used to establish the processed (recovered) standard curve for the direct quantitation of the unknowns.

External standard curve

In order to verify the linearity and performance of the HPLC assay, an external standard calibration curve is prepared by adding 100 μl of solutions 1, 2, 3, 4, 5, 6 or 7 to 0.5 ml of distilled water plus 0.1 ml of 0.25 *M* potassium phosphate buffer, pH 7, and 1.3 ml acetonitrile—methanol (9:1) (final volume = 2000 μl) and mixing. Aliquots (25 μl per 2000 μl) are programmed on the WISP for automated injection. Typical chromatograms are shown in Fig. 2.

Calculations

The concentration of I and I-A in the unknowns was determined by interpolation from a least-squares regression equation (weighted linear equation: $Y = a\ X + b$) of the calibration data (processed by a Hewlett-Packard Model 3357B Laboratory Automation System), of the recovered standards processed along with the unknowns using peak height ratios (peak height of compound I or I-A to peak height of internal standard II) versus concentration of I or I-A per ml of plasma.

RESULTS AND DISCUSSION

Compounds I and I-A possess strong ultraviolet (UV) absorption and fluorescence characteristics. The fluorescence excitation maxima of compound I occur at 235, 272, and 360 nm with a broad emission band with peaks at 460 and 480 nm. Compound I-A also has excitation maxima at 235, 272, and 360 nm but with its major emission peak at 450 nm and a smaller one at 475 nm. The Schoeffel Model FS-970 fluorescence detector was used with the excitation set at 275 nm and an emission filter transmitting wavelengths greater than

418 nm and allowed for the quantitation of I and I-A in nanogram concentrations, while avoiding high fluorescence backgrounds from the PIC-A reagent, seen at shorter exciting wavelengths.

Reversed-phase HPLC analysis was the chromatographic method of choice, since it is compatible with an acetonitrile protein precipitation step followed by direct injection of the supernatant. Thus, a rapid sensitive and selective HPLC assay was developed for the determination of compounds I and I-A from plasma using a WISP autoinjector and fluorescence detector for quantitation. The method enabled the accurate quantitation of compounds I and I-A with high sample throughput required for pharmacokinetic and biopharmaceutic studies.

Compound II was chosen as the internal standard in the assay, due to the similarity of its luminescence to that of compound I and its chromatographic resolution from I and I-A.

Chromatographic behavior of I, I-A and II

Compounds I, I-A and II exhibited UV absorbances sufficiently intense for detection at 235 nm. However, the use of PIC-A as an ion-pairing reagent in the mobile phase created a significantly high background UV absorbance, making UV detection impractical. Also, the fact that the UV chromatogram of control plasma (dog and human) contained interfering endogenous peaks made fluorometric detection a necessity for sensitive quantitation.

The HPLC system is flushed initially with methanol, followed by methanol—water (50:50, v/v), to remove deposits from the column accumulated from previous use. The mobile phase is allowed to recycle through the system for at least 2 h at a flow-rate of 1 ml/min to equilibrate the system. Non-equilibration will result in a change in k' values during chromatography. 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 times of $> \pm 0.5$ min for each of the compounds and variation in the separation (α) factors. The preparation of the 100 μg/ml stock solutions in methanol—water—ammonium hydroxide (75:24:1) insures the complete dissolution of the compounds while liberating the free base of I·2HCl, thus the same lots of released material used for dosing were used as the analytical standards in the HPLC analysis of biological samples.

Selectivity of the assay

The known products of either biotransformation and/or instability of I necessitated the use of chromatographic parameters which ensured the stability and selectivity of the assay. Thus the major biotransformation product, I-A, was resolved from I and II. A second breakdown product/metabolite, 1 H-imidazole-1-butanamine, I-B, has very little UV absorption and no significant fluorescence and is therefore not detected even when injected in microgram quantities.

Several analogues were investigated as candidates for an internal standard, Fig. 1. Of these, compound III had a retention time about 1 min longer than I, while compound IV had a retention time similar to III together with a smaller peak (probably an impurity) at the retention time of I. Compound II, with a

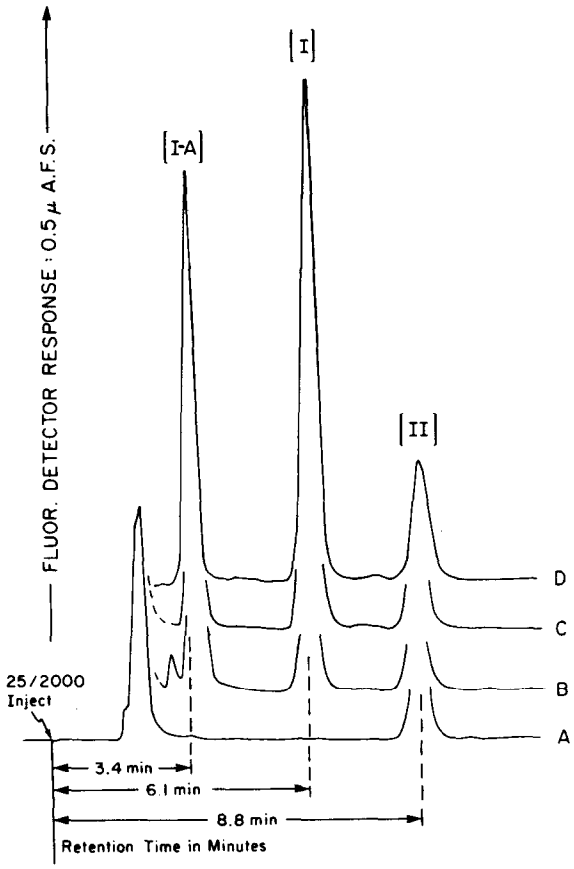

Fig. 2. Chromatograms of the HPLC analysis of the protein-free filtrate of (A) control dog plasma with added internal standard; (B) dog plasma following oral dosing of I·2HCl at 300 mg/kg; (C) authentic standards recovered from control dog plasma; and (D) authentic standards.

retention time of 8.8 min, was selected as the internal standard since it was completely resolved from I and metabolite I-A, Fig. 2.

Assay validation

Calibration curves for I and I-A ($Y = 0.00239X - 0.00058$ and $Y = 0.00568X + 0.0162$) were linear from 20 to 2000 ng of I and 10 to 1000 ng of I-A per ml of plasma, respectively, using a 0.5-ml specimen. The correlation coefficients (r) were 0.9997 and 0.9988 and the average deviation from the line was 5.0% and 5.4% for I and I-A, respectively. Intra- and inter-assay validation data over the linear concentration range of I and I-A yielded mean coefficients of variation of 3.1% and 5.0%, respectively, for I, and 5.1% and 5.8%, respectively, for I-A, Tables II and III.

Percent recovery and sensitivity limits

The overall recovery of I and of I-A from plasma is 107.0 ± 8.6% and 107.0 ± 10.0%, respectively. The sensitivity limit of the assay is 20 ng of I and 10 ng of I-A per ml of plasma, respectively.

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND I

Amount added (ng/ml)	Amount found (ng/ml)	*n*	Coefficient of variation (%)
(A) Intra-assay variability			
20	17 ± 0.1	2	0.7
40	43 ± 3.3	3	7.6
70	76 ± 2.5	3	3.3
200	195 ± 4.7	3	2.4
400	391 ± 13.2	3	3.4
700	698 ± 15.0	3	2.2
2000	2010 ± 40.0	3	2.0
		Average ±	3.1
(B) Inter-assay variability			
20	18 ± 1.7	5	9.4
40	41 ± 3.8	9	9.2
70	74 ± 2.8	9	3.8
200	200 ± 10.0	9	5.0
400	401 ± 12.0	9	2.9
700	696 ± 20.0	9	2.9
2000	1990 ± 44.0	8	2.2
		Average ±	5.0

TABLE III

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND I-A (METABOLITE)

Amount added (ng/ml)	Amount found (ng/ml)	*n*	Coefficient of variation (%)
(A) Intra-assay variability			
10	10 ± 0.4	3	4.1
20	20 ± 2.5	3	12.5
60	62 ± 0.6	3	1.0
100	95 ± 4.5	3	4.8
200	202 ± 11.0	3	5.5
600	647 ± 22.0	3	3.3
1000	961 ± 41.0	3	4.3
		Average ±	5.1
(B) Inter-assay variability			
10	10 ± 1.2	8	12.1
20	21 ± 1.8	9	8.7
60	61 ± 3.0	9	5.0
100	96 ± 3.6	9	3.7
200	199 ± 9.0	9	4.5
600	624 ± 20.0	9	3.2
1000	972 ± 30.0	9	3.1
		Average ±	5.8

Application of the method to biological specimens: biopharmaceutic/pharmacokinetic studies in the dog

A pilot study was conducted in a dog using the HPLC assay as described, but without the inclusion of II (internal standard) since a pure standard was unavailable at the time. The study involved the intravenous infusion of a single 5 mg/kg dose of I·2HCl and a single oral administration to the same dog of a 10 mg/kg dose of I·2HCl as a solid in a hand-packed gelatin capsule. A 10 mg/kg oral dose of a solution of the metabolite I-A was also administered to the same dog.

Following intravenous (Fig. 3) and oral (Fig. 4) administration, plasma concentrations of I declined rapidly with the major plasma component being the acid metabolite I-A whose concentrations were measurable up to 12 h. These preliminary data, which document the utility of the assay, also suggest first-pass biotransformation of I to yield I-A in the dog, which was substantiated by comparing the plasma concentration versus time profile of I-A following oral administration of I-A.

Plasma concentrations of I and I-A in the above studies were determined using the HPLC assay as described but without the internal standard II. Quantitation was based on absolute peak height versus concentrations of I or I-A. In these experiments the calibration curves for I and I-A (Y = 6.005 X + 23.909 and Y = 12.549 X + 7.098) were linear from 20 to 2000 ng of I and 20 to 600 ng of I-A per ml of plasma, respectively, using a 0.5-ml aliquot. The correlation coefficients (r) were 0.9999 and 0.9987 and the average deviation from the line was 4.0% for I and 5.6% for I-A, respectively. The mean intra-assay (n = 3) coefficients of variation were 3.9% and 5.6% for I

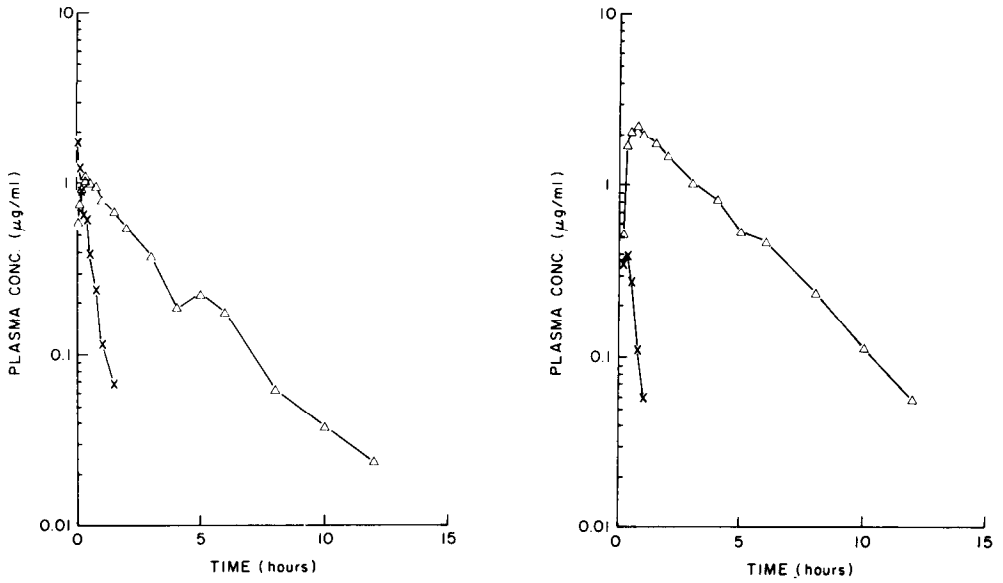

Fig. 3. Plasma concentrations of I (×) and its major metabolite I-A (△) in a dog following a 5 mg/kg intravenous infusion of I·2HCl administered over a 10-min interval.

Fig. 4. Plasma concentrations of I (×) and its major metabolite I-A (△) in a dog following a 10 mg/kg oral dose of I·2HCl (solid) in a hand-packed gelatin capsule.

and I-A, respectively. The mean inter-assay coefficients of variation for eleven experiments were 4.6% for I and 4.7% for I-A, respectively, demonstrating the precision of the assay even without the use of the internal standard.

Stability of I in dog and human plasma on storage at −17°C and −70°C up to 90 days

The stability of I in fresh plasma was evaluated by HPLC analysis at two concentrations (100 and 1000 ng/ml) following storage at −17°C and −70°C for a period of 1, 7, 30, 60 and 90 days. Fresh pools of plasma from the two species were prepared at the respective concentrations by adding 10 μl of solution A-1 and 100 μl of solution A-1 per 10 ml of the respective plasma. Three-ml aliquots of each pool together with a control plasma from each species (total 24) were pipetted into separate 1-dram Wheaton vials. One half of the specimens (12) were stored at −17°C and the other at −70°C.

The stability-indicating plasma samples from day 1, 7, 30, 60 and 90 stored at −17°C and −70°C were analyzed in triplicate as unknowns along with external standards and plasma recovered calibration standards at similar concentrations added to fresh control plasma using the HPLC assay with the internal standard II added. The data tabulated in Table IV were analyzed using a

TABLE IV

STABILITY OF I IN DOG PLASMA AND IN HUMAN PLASMA STORED AT −17°C AND −70°C

Day	Storage temp. (°C)	100 ng/ml added Mean conc. found ± S.D.* (% S.D.)	1000 ng/ml added Mean conc. found ± S.D.* (% S.D.)
(A) Dog plasma			
1	−17	102 ± 2.8 (2.8)	871 ± 11 (1.2)
1	−70	99 ± 3.6 (3.6)	867 ± 47 (5.4)
7	−17	95 ± 2.7 (2.8)	891 ± 16 (1.8)
45	−17	99 ± 3.1 (3.1)	913 ± 24 (2.6)
45	−70	101 ± 3.7 (3.7)	889 ± 20 (2.3)
60	−17	101 ± 2.0 (2.0)	937 ± 117 (12.5)
91	−17	89 ± 3.8 (4.2)	841 ± 42 (5.0)
91	−70	95 ± 1.6 (1.6)	899 ± 10 (1.1)
(B) Human plasma			
1	−17	96 ± 9.7 (10.0)	1143 ± 34 (2.9)
1	−70	101 ± 4.9 (4.9)	1084 ± 57 (5.2)
7	−17	89 ± 1.3 (1.5)	875 ± 32 (3.6)
32	−17	101 ± 2.2 (2.2)	1139 ± 37 (3.2)
32	−70	104 ± 2.6 (2.5)	1108 ± 37 (3.3)
62	−17	97 ± 2.1 (2.1)	1066 ± 49 (4.6)
95	−17	98 ± 3.7 (3.8)	1142 ± 57 (5.0)
95	−70	92 ± 4.6 (5.0)	1086 ± 27 (2.5)

*n = 3 in all cases.

two-tailed *T*-test. The data indicated that I was stable throughout the storage interval at —17°C and —70°C.

The plasma samples collected from dogs in these studies were stored at —70°C prior to analysis.

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