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DETERMINATION OF THE ANTI-TUMOR AGENT, 10-CHLORO-5-(2-DIMETHYLAMINOETHYL)-7H-INDOLO[2,3-C]QUINOLIN-6(5H)-ONE IN BLOOD OR PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific high-performance liquid chromatographic assay was developed for the determination of 10-chloro-5-(2-dimethylaminoethyl)-7H-indolo[2,3-C]quinolin-6(5H)-one [I] in blood or plasma with an overall recovery of 100.3 ± 9.1% and a limit of quantitation of 1.0 ng per ml of blood or plasma. The assay was used to determine blood concentrations of the drug in the rat following oral administration by intubation of a 1.17-mg dose of [I] · HCl.

INTRODUCTION

The compound 10-chloro-5-(2-dimethylaminoethyl)-7H-indolo[2,3-C]-quinolin-6(5H)-one, [I] (Fig. 1), is a member of a series of indoloquinolinones synthesized by Walser and co-workers [1, 2] and is of interest as an anti-tumor agent [3].

A sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of [I] in blood or plasma. The assay was used for the determination of blood concentrations of the drug in the rat following oral administration by intubation of a 1.17-mg dose of [I] · HCl.

The method quantitates compound [I] by fluorescence following normal-phase HPLC separation. The analog, 10-methoxy-5-(dimethylaminoethyl)-7H-indolo[2,3-C]quinolin-6(5H)-one, [II], was used as the internal standard in the assay because of its favorable retention time on HPLC analysis, and comparable luminescence properties.

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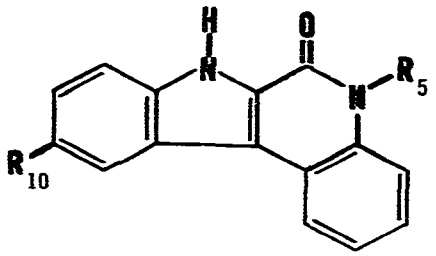

Compound	R_{10}	R_5	Retention time (min)	k'
I	Cl-	$-CH_2-CH_2-N(CH_3)_2$	4.4	2.4
II	CH_3O-	$-CH_2-CH_2-N(CH_3)_2$	5.9	3.5
III	Cl-	$-CH_2-CH_2-NH(CH_3)$	3.9	2.0
IV	Cl-	$-CH_2-CH_2-NH_2$	14	9.8
V	Cl-	$-CH_2-CH_2-OH$	14.5	10.2
VI	Cl-	-H	2.7	1.1

Fig. 1. Chemical structures, retention time and capacity factors (k') of some 10- and 5-substituted 7H-indolo[2,3-C]quinolin-6(5H)-ones.

EXPERIMENTAL

Column

The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing Partisil PXS 10/25 silica gel, 10 μm (Serial No. 1B1709, Whatman, Clifton, NJ, U.S.A.).

Instrumental parameters

A Waters Model 6000A high-pressure liquid chromatography pump, equipped with a Model U6K injection system and a pre-column filter (2-μm) (Waters Assoc., Milford, MA, U.S.A.), was used for chromatography. A Schoeffel Model FS-970 fluorescence detector operated at 258 nm for excitation and emission at wavelengths greater than 340 nm (Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.) was used for fluorimetric detection.

The isocratic mobile phase consisted of hexane—tetrahydrofuran—methanol—concentrated ammonium hydroxide (75 : 15 : 9.75 : 0.25) pumped at a constant flow-rate of 2.0 ml/min. Under these conditions, the retention time of

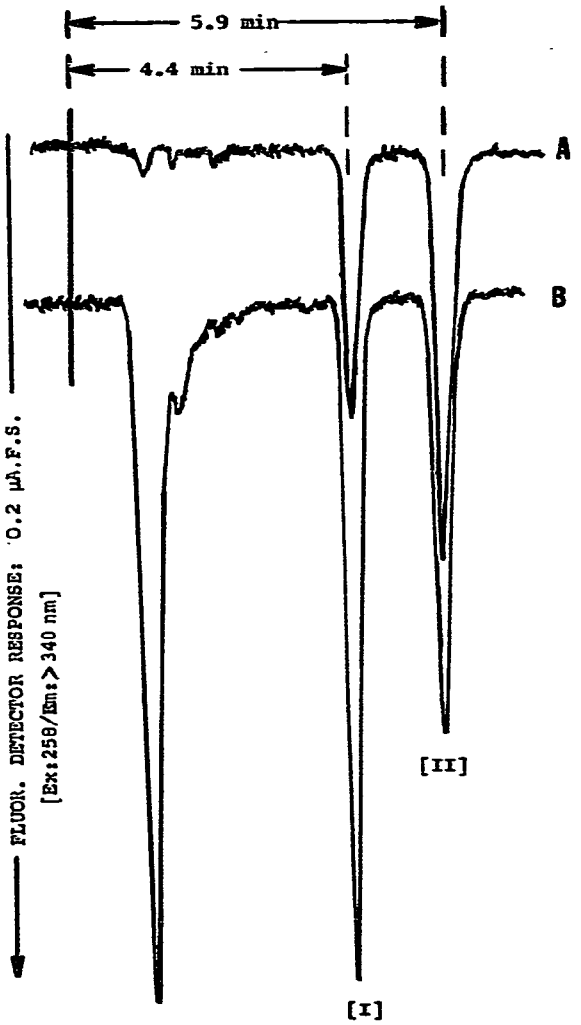

Fig. 2. Chromatograms of (A) authentic standard of 25 ng [I] and 10 ng [II] injected and (B) diethyl ether extract of 0.1 ml rat blood, 30 min post oral dose, containing added authentic [II].

compound [I] was 4.4 min while that of compound [II] was 5.9 min (Fig. 2), with capacity factors (k') of 2.4 and 3.5, respectively. The chart speed of the Hewlett-Packard dual-channel recorder (Model 7132A with option 108) was 0.5 in./min.

Spectrophotometric/fluorimetric instrumentation

Ultraviolet absorbance spectra were recorded using a double-beam ratio-recording spectrophotometer (Coleman Model EPS-3T Hitachi Spectrometer, Coleman Instruments, Maywood, IL, U.S.A.). Corrected luminescence excitation and emission spectra (10 nm bandpass) were recorded using a spectrofluorimeter equipped for direct recording of corrected excitation and corrected

emission spectra (Farrand Mark I, serial No. 947, Farrand Optical Co., Valhalla, NY, U.S.A.). Quantitative analytical fluorimetric data were generated in the uncorrected mode. Cryogenic (77°K) luminescence data were generated using commercially available accessories and equipment, made "in house", which has been previously described [4]. (An analogous cryogenic unit is now available from Farrand Optical Co.)

Reagents

All inorganic reagents were analytical-reagent grade (A.C.S.). All aqueous solutions were prepared in distilled, carbon-filtered, deionized water filtered through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.).

Organic solvents, suitable for spectrophotometry and liquid chromatography were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), with the exception of ethanol (200 proof, absolute) purchased from Pharmco (Publicker Industries, Linfield, PA, U.S.A.) and diethyl ether (anhydrous, absolute, reagent grade) purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Diethyl ether used for extraction of biological samples was used from a freshly opened can before each analysis and was shaken with granular zinc (about 1 g per 450 ml of ether) to reduce the peroxides, which might otherwise decompose low concentrations of [I].

Analytical standard solutions

Stock solutions of compound [I], ($C_{19}H_{18}ClN_3O$, mol. wt. 339.83 (base), m.p. 292—298°C) and compound [II] ($C_{20}H_{21}N_3O_2$, mol. wt. 335.41 (base), m.p. 285—287°C, used as the internal standard) were prepared containing 100 μg/ml of hexane—tetrahydrofuran—methanol (75:15:10). The stock solutions of [I] and [II] were used to prepare mixed standard solutions (Table I) by suitable dilutions in the mobile phase hexane—tetrahydrofuran—methanol—concentrated ammonium hydroxide (75:15:9.75:0.25). Aliquots (250 μl) of these mixed standard solutions were added to control (drug-free) blood or plasma as the processed standards to establish a calibration curve for the determination of the concentrations in the unknowns and for the determination of percent recovery.

TABLE I

STANDARD SOLUTIONS TO BE USED FOR HPLC ANALYSIS

Standard solution	Compound I (ng/250 μl)	Compound II (internal standard) (ng/250 μl)
1	250	10
2	100	10
3	25	10
4	5	10
5	0	10

Analysis of blood or plasma

A 250-μl aliquot of standard solution No. 5 (Table I) was transferred into a 15-ml glass-stoppered centrifuge tube as the internal standard [II] for each unknown. The organic solvent was evaporated at 40°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean dry nitrogen. Then a suitable aliquot (0.1—1.0 ml) of the blood or plasma sample was added and mixed well.

A separate set of standards was prepared by transferring 250-μl aliquots of mixed standard solutions 1, 2, 3, or 4 (Table I) into separate 15-ml centrifuge tubes, evaporating the organic solvent, and adding a suitable aliquot of control blood plasma and mixing well.

The samples were mixed vigorously and 1 ml of 1 *M* ammonium hydroxide and 10 ml of fresh diethyl ether were added. Each tube was stoppered (PTFE No. 13 stopper), and shaken for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes/min. The samples were centrifuged at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, Rotor No. 253; Damon/IEC, Needham, MA, U.S.A.) at 5°C and a 9.0-ml aliquot of the supernatant was transferred into another 15-ml conical centrifuge tube.

The ether extract was evaporated to dryness at 40°C in the N-EVAP evaporator under a stream of clean, dry nitrogen. The residue was dissolved in 250 μl of the mobile phase, 25 μl of which were injected for HPLC analysis using normal-phase (adsorption) chromatography. The Schoeffel fluorescence detector was set between 1.0 and 0.02 μA depending on the sensitivity desired. Typical chromatograms are shown in Fig. 2.

The concentration of [I] in each unknown was determined by interpolation from the calibration curve of the standards processed along with the unknowns, using the peak height ratio (ratio of peak height of compound [I] to the peak height of the internal standard [II] versus concentration) technique.

RESULTS AND DISCUSSION

Compound [I] and its analogs (Fig. 1) are conjugated heteroatomic molecules which possess strong UV absorption and intense luminescence emission characteristics. The spectral characteristics of [I] were determined in selected solvents, including the mobile phase used for HPLC analysis (Table II). The absorption, corrected excitation and luminescence emission spectra of [I] in the mobile phase used for HPLC analysis are shown in Fig. 3. The major UV absorption band of [I] occurs at 250—260 nm for all the solvents listed, the absorptivity (258—260 nm) ranges from 109 to 116 l/g · cm. The corrected fluorescence emission spectra determined at ambient temperature (25°C) show a hypsochromic "blue" shift from a broad, structureless emission (λ_{max} = 465 nm) with a high quantum efficiency at acidic pH; Φ_F = 0.43 in methanol—2 *N* HCl (1:1), to a sharper more structured emission spectrum (λ_{max} = 365, 380 nm) with lower quantum efficiency, Φ_F = 0.075 in the mobile phase, at basic pH. This suggests that at mildly acidic pH the compound exists as an ionized species in the excited state, but not in the ground state, since the UV absorption spectra do not exhibit wavelength shifts with pH. Optimal HPLC separation of [I] and [II] from endogenous interferences could only be achieved how-

TABLE II

UV ABSORPTION, 25°C FLUORESCENCE, AND 77°K LUMINESCENCE CHARACTERISTICS OF COMPOUND I IN SELECTED SOLVENTS

Solvent	UV Absorption		25°C Fluorescence		77°K Total luminescence		77°K Phosphorescence		
	Maximum λ_{ex} (nm)	Absorptivity (l/g · cm)	Maximum λ_{em} (nm)	Quantum efficiency (Φ_F)	Maximum λ_{em} (nm)	Quantum efficiency (Φ_L)*	Maximum λ_{em} (nm)	Approx. efficiency (Φ_P)**	Phosphorescence lifetime (sec)
Methanol—2 *N* HCl (1:1)	220 235 253 258 274 300 313 342 358	116	465	0.43	364, 380, 400, 493, 525	≈0.13	493, 525	≈0.01	1.4
1% Conc. H_2SO_4 in ethanol	253*** 260	115	380, 450	0.28	360, 377, 395, 492, 522	≈0.95	492, 522	≈0.03	1.4

1% 0.1 *N* potassium phosphate buffer (pH 7) in ethanol	253*** 260	109	382	0.23	N.M.§	N.M.	N.M.	N.M.	N.M.
1% Conc. NH_4OH in ethanol	252*** 259	116	365, 380	0.077	360, 375, 395, 480, 510	≈1.0	495, 520	≈0.03	1.3
Hexane—tetrahydrofuran—methanol—conc. NH_4OH (75:15:10:0.25)§§	225 240 253 260 277 300 313 340 357	111	364, 380	0.075	360, 375, 395, 480, 510	≈1.05	480, 510	≈0.055	1.25

* 77°K total luminescence includes both cryogenic fluorescence and phosphorescence. The fluorescence component is in the 350—450 nm spectral region while the phosphorescence component is in the 450—550 nm region. The light scatter from cracked solvent glasses severely limits the accuracy of Φ_L (total luminescence efficiency at 77°K).

** Φ_P is calculated from uncorrected spectra and must be considered approximate only.

*** Only the two most intense maxima are recorded here, the rest of the UV absorption spectrum is closely similar to that recorded for the mobile phase.

§ N.M. = not measured. Upon freezing this solvent scatters exciting light to the extent that the luminescence is masked, leading to erroneous measurements.

§§ Mobile phase for HPLC analysis; forms a snowy matrix upon freezing, thus Φ_L and Φ_P are highly approximate.

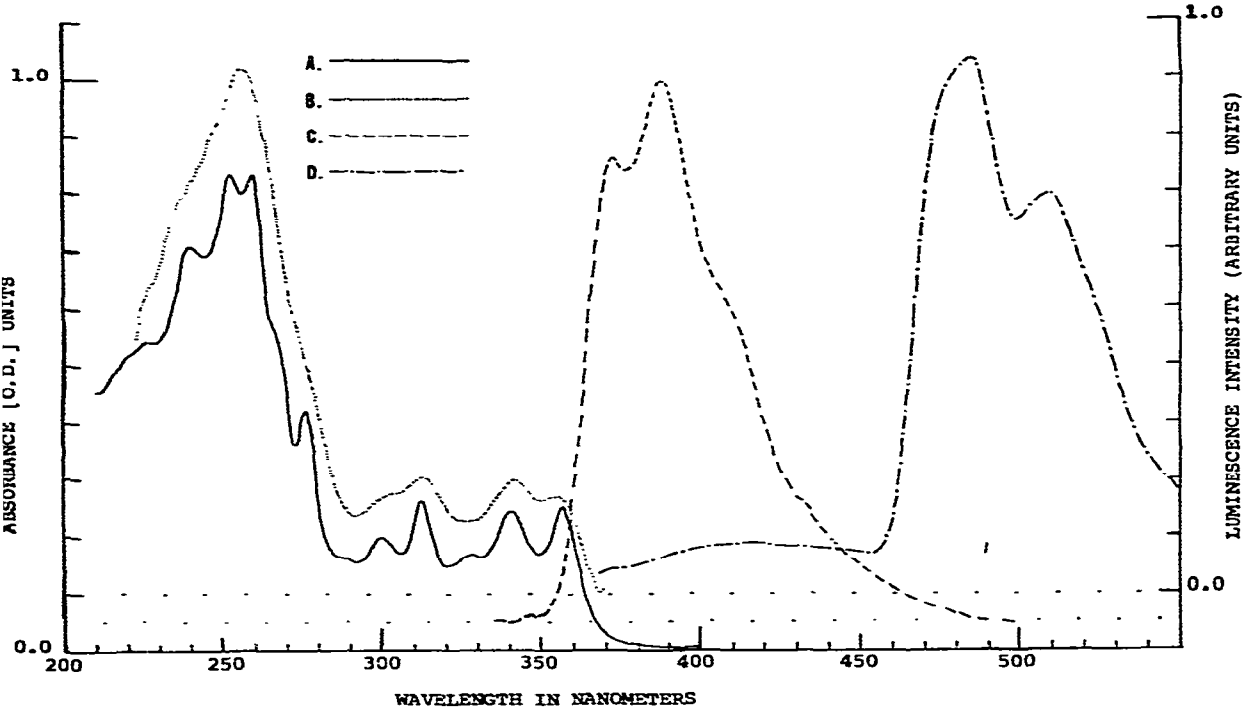

Fig. 3. (A) UV absorption spectrum (7.5 μg/ml); (B) corrected fluorescence excitation spectrum (0.25 μg/ml, λ_{em} = 380 nm, 10 × attenuation); (C) corrected fluorescence emission spectrum (0.25 μg/ml, λ_{ex} = 258 nm, 10 × attenuation); and (D) uncorrected phosphorescence emission spectrum (0.25 μg/ml, λ_{ex} = 258 nm, 3 × attenuation) of compound [I] in the mobile phase used for normal-phase HPLC analysis.

ever at basic pH, at the expense of higher sensitivity. Although the UV absorbance of [I] at 260 nm is sufficiently intense for quantitation in biological fluids using a tubeable UV detector, fluorescence detection was preferred due to higher sensitivity, specificity, and better overall background signal from the sample extract.

The cryogenic (77°K) luminescence characteristics demonstrate similar emission maxima regardless of pH and contain both fluorescence (singlet—singlet emission) in the 350—450 nm region and phosphorescence (triplet—singlet emission) in the 450—550 nm region. Total luminescence quantum efficiency, Φ_L, ranged from 0.13 at acidic pH to about 1.0 at basic pH. The phosphorescence quantum efficiency, Φ_P increased five-fold from acidic pH to basic pH, based on estimations from uncorrected spectra obtained in the phosphorescence mode. Phosphorescence lifetimes ranged from 1.40 to 1.25 sec over the pH range studied. The mobile phase forms a "snowy" matrix at 77°K, impairing the accuracy of the determination of Φ_L and Φ_P due to scatter effects.

The luminescence characteristics of the compound were used initially, to develop a thin-layer chromatographic (TLC)—spectrofluorimetric assay based on the intense fluorescence emission of [I] in acidic alcohol solutions. The analysis of [I] in blood or urine involved buffering the sample with 1 *M*

K_2HPO_4 (pH 8.8—9.1), double-extraction into diethyl ether, concentration of the combined ether extract, transfer of the ether concentrate containing [I] onto a 20 × 20 cm, E. Merck (Darmstadt, G.F.R.) pre-coated 60-μm silica gel F-254 TLC plate and ascending chromatography in benzene—methanol (1:1) as the developing solvent (Fig. 4). Compound [I] (R_F = 0.50) was then eluted from the silica gel with 5 ml of methanol, the residue of which was dissolved in 5 ml of methanol—2 *N* HCl (1:1) and quantitated in a 1-cm quartz cuvette by spectrofluorimetry at 460 nm, with excitation at 350 nm. The overall recovery of [I] by this procedure was 65 ± 7%, with a sensitivity limit of 70 ng/ml of sample.

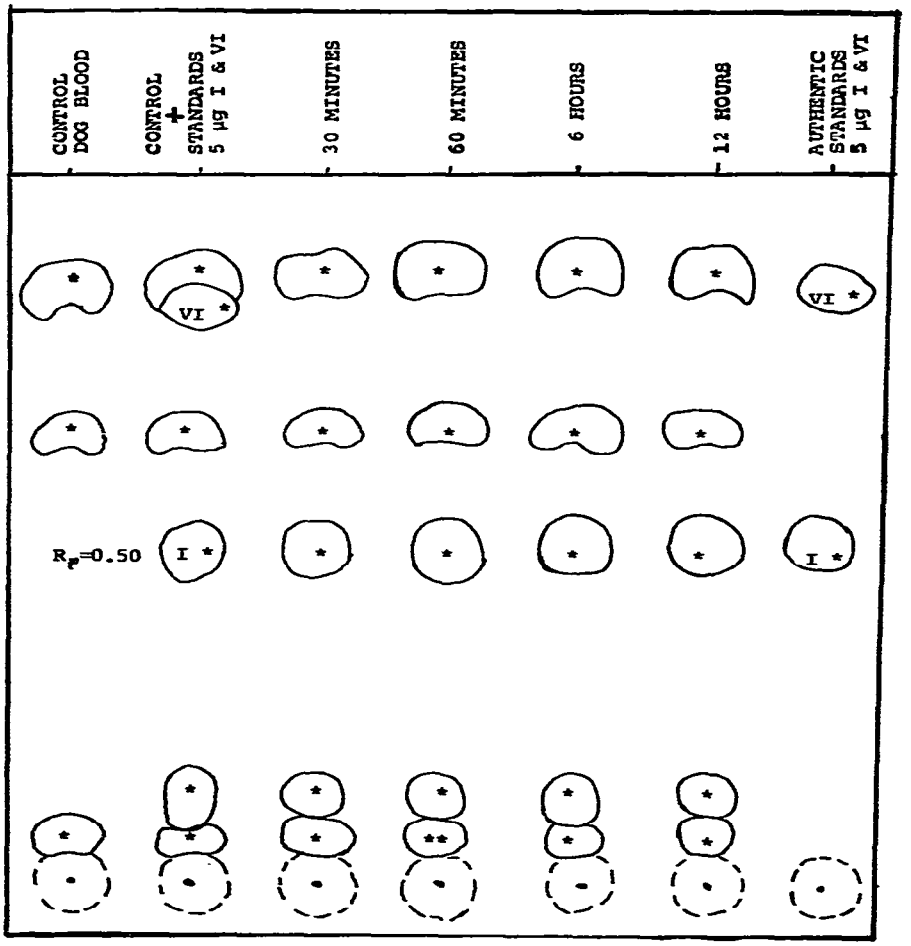

Fig. 4. TLC of ether extracts of dog blood after intravenous administration of 10 mg [I]/kg. Solvent system benzene—methanol (50:50). * = Fluorescent material.

The TLC—spectrofluorimetric assay was used in the early phases of pre-clinical development of the drug. The assay was tedious, time-consuming and was replaced by the HPLC assay described. The HPLC assay is a simpler more straightforward three-step operation which involves selective extraction, sample concentration, and direct analysis by HPLC with quantitation by spectrofluori-

metric detection. It provides better sensitivity, precision and higher sample throughput than the TLC procedure, hence it is the method of choice.

Recovery, precision and sensitivity limits of the HPLC assay

The mean recovery of compound [I] from human plasma over the concentration range 1.0—250 ng/ml is 100.3 ± 9.1% (S.D.) (Table III). The inter-assay precision over this concentration range showed a mean relative standard deviation of about ± 7.4%. The sensitivity limit of quantitation is 1.0 ng of [I] per ml of blood or plasma using a 1-ml sample per assay.

TABLE III

RECOVERY AND PRECISION OF ASSAY FOR COMPOUND [I] IN HUMAN PLASMA

Compound [I] added (ng/ml)	Replicates (n)	Average amount of [I] recovered* (ng/ml)	Mean recovery** (%)	Relative standard deviation** (± %)
1.0***	3	1.047	104.7	8.6
5.0	4	4.88	97.6	7.9
25	5	23.08	92.3	6.0
100	5	98.9	98.9	6.4
250	5	272.5	109.0	8.0

*Corrected for 9/10 ether aliquot in extraction procedure; calculated from a least squares regression analysis of HPLC response to external standards of [I], based on a logarithmic curve: $Y = aX^b = 0.0688\ X^{0.9761}$ with a correlation coefficient of 0.9983. Average percent deviation of the curve fit is ± 8.5%.

**Overall average recovery = 100.3% with an overall relative standard deviation of ± 9.1% ($n = 22$).

*** Limit of quantitation.

Analysis of biological specimens

The initial TLC—spectrofluorimetric assay was used to determine compound [I] in a pilot study in a dog following the intravenous administration of a 10 mg/kg dose (total dose = 80 mg [I] administered as the free base). Blood concentrations of [I] were measured over 48 h (Table IV), with an apparent half-life of elimination [$t_{\frac{1}{2}\beta}$] of about 5.4 h. The blood samples were also analyzed for compound [VI] as a potential metabolite (Figs. 1 and 4), however, none was found (sensitivity limit ≈ 0.07 μg/ml). The assay was specific by virtue of the TLC separation step.

The HPLC assay was subsequently used to determine compound [I] in a pilot study in one rat (400—450 g) following the oral administration by intubation of 1.5 ml of an ethanol—water (50:50) solution containing 1.17 mg of [I] · HCl. Blood concentrations of [I] were observed over 1.5 h sampled via caudal venipuncture (Table V). None of the analogs III, IV, V, or VI considered to be potential metabolites (all of which were chromatographically resolved) (Fig. 1) were detected in these samples.

The sensitivity and specificity of the HPLC assay should be sufficient for future clinical pharmacokinetic studies.

TABLE IV

BLOOD CONCENTRATIONS OF COMPOUND [I] IN THE DOG FOLLOWING A SINGLE INTRAVENOUS ADMINISTRATION OF A 10 mg [I]/kg DOSE (TOTAL = 80 mg)

Time	Concentration* (μg/ml)
2.5 min	1.8
5.0 min	2.5
10 min	1.7
20 min	2.1
30 min	1.8
45 min	1.3
1.0 h	1.0
1.5 h	0.75
2 h	1.2
3 h	0.85
4 h	0.90
6 h	0.55
7 h	0.40
11 h	0.30
24 h	0.07
48 h	—**
72 h	—

* Determined by TLC—fluorimetric procedure.
** — = below the limit of quantitation (0.07 μg/ml of blood).

TABLE V

BLOOD CONCENTRATION OF COMPOUND [I] IN THE RAT FOLLOWING A SINGLE ORAL ADMINISTRATION OF 1.17 mg [I] - HCl BY INTUBATION

Time (min)	Concentration* (μg/ml)
15	2.3
30	0.62
45	0.68
60	0.42
75	0.83
90	0.25

* Determined by HPLC analysis with fluorimetric detection.

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