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DETERMINATION OF THE ANTI-ALLERGENIC AGENT, 2-METHOXY-11-OXO-11*H*-PYRIDO[2,1-*b*]QUINAZOLINE-8-CARBOXYLIC ACID, IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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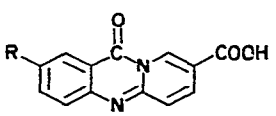
SUMMARY

A rapid, sensitive, and specific high performance liquid chromatographic (HPLC) assay was developed for the determination of 2-methoxy-11-oxo-11*H*-pyrido-[2,1-*b*]quinazoline-8-carboxylic acid (I) from biological fluids. The overall recovery from blood and plasma is 69 ± 10% (S.D.) and 84 ± 6% (S.D.), respectively, and the sensitivity limit of quantitation is 100 ng/ml by UV absorption and 5 ng/ml by fluorescence detection using a 1 ml specimen. The assay was used in the determination of blood levels of compound in the Rhesus monkey following intravenous administration of a 10 mg/kg dose, and of blood and urine levels of compound I in a dog following intravenous and oral administration of a 1 mg/kg dose.

INTRODUCTION

The compound 2-methoxy-11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, compound I (Fig. 1), is a member of a series of 11-oxo-11*H*-pyrido[2,1-*b*]-quinazoline-8-carboxylic acids[1,2] of clinical interest as antiallergenic agents[3].



Compound	R–
I	CH_3O–
I-A	HO–
II	CH_3CH_2O–
III	$CH_3CH_2CH_2O$–
IV	$CH_3CH_2CH_2CH_2O$–

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

The excellent ultraviolet (UV) absorption and intense intrinsic fluorescence (Fig. 2) of compound I prompted the investigation of high-performance liquid chromatography (HPLC) for its analysis, since the technique has been used successfully in the analysis of several pharmaceuticals[4–8], and especially for the sensitive and specific determination of a number of pharmaceuticals from biological media[9–15] using either UV absorption or fluorescence emission for their detection.

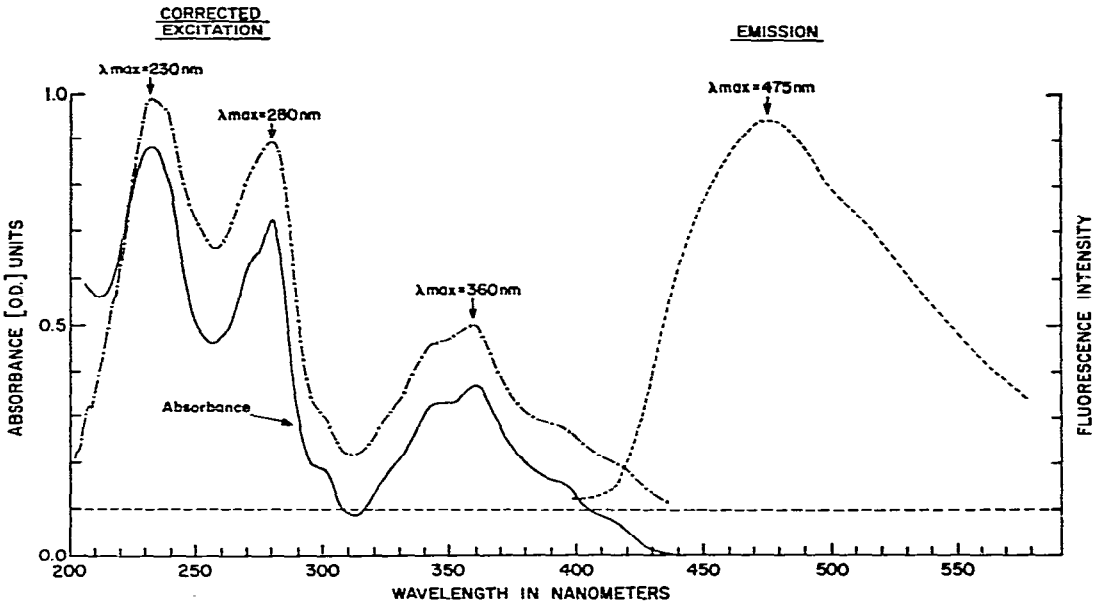


Fig. 2. UV absorption spectrum (7.5 μg/ml) and corrected excitation and fluorescence emission spectra (0.25 μg/ml) of compound I in the mobile phase used for HPLC analysis.

The method presented, herein, quantitates compound I by either UV absorbance or by fluorescence emission after HPLC separation using ion-pair reversed-phase chromatography. The phenolic analog, 2-hydroxy-11-oxo-11*H*-pyrido[2,1-*b*]-quinazoline-8-carboxylic acid (I-A), an *in vitro* biotransformation product[16] is completely resolved from I. The ethoxy analog, 2-ethoxy-11-oxo-11*H*-pyrido[2,1-*b*]-quinazoline-8-carboxylic acid (II), was selected as the reference standard in the assay instead of the propoxy(III) or butoxy(IV) analog (Fig. 1) because of its favorable retention time on HPLC analysis.

The assay was used in the determination of blood levels of compound I in a Rhesus monkey following a 10 mg/kg intravenous dose, and of blood and urine levels of compound I in a dog following intravenous and oral doses of 1 mg/kg.

EXPERIMENTAL

Column

The column used was a prepacked 30 cm × 4 mm I.D. stainless-steel column containing a μBondapak C_{18} reversed-phase 10 μ microparticulate packing (Waters Assoc., Milford, Mass., U.S.A.).

Instrumental parameters

A Waters high-pressure liquid chromatograph, Model ALC/GPC 204/6000 A, with a Model U6K injection system and a Model 440 UV detector with a 365 nm wavelength kit was used for chromatography. A Schoeffel Model FS 970 fluorescence detector (excitation, 280 nm; emission > 418 nm), (Schoeffel Instrument, Corp. Westwood, N.J., U.S.A.) and a Hitachi Model 204 spectrofluorometer (excitation, 360 nm; emission, 475 nm), (Perkin-Elmer, Norwalk, Conn., U.S.A.) equipped with a 100-μl flow cell, were used as fluorescence detectors.

The mobile phase used for isocratic reversed-phase chromatography consisted of two ampules of Waters PIC reagent A (each containing 0.005 moles tetrabutylammonium phosphate in 14 ml phosphate buffer, pH 7.5) per liter of methanol–water (1:1). The mobile phase is filtered through a 0.6 μm pore diameter polyvic 47-mm diameter membrane filter (Cat. No. BDWP-047-00) (Millipore, Bedford, Mass., U.S.A.) prior to use to remove particulates which might otherwise clog the column. The flow-rate was a constant 2.0 ml/min. Under these conditions the retention times of compounds I, I-A, II, III, and IV were 5.5, 3.0, 9.0, 17, and 25 min, respectively, using either UV absorption (Fig. 3) or fluorescence emission (Fig. 4) for detection. Retention times may vary somewhat from column to column for the same manufacturer. The chart speed on the Leeds and Northrup dual-channel recorder (Model 626) was 30 in./h.

Nearly full-scale response was obtained for 100 ng of compound I both with the UV detector sensitivity set at 1×10^{-2} a.u.f.s. (365 nm), and with either the Schoeffel fluorescence detector set at 1.0 μA or with the Hitachi spectrofluorometer photomultiplier high voltage set at a sensitivity of 8 (648 V), and its amplifier gain set at 1 (550 μA).

Analytical standard solutions

Stock solutions of compound I, ($C_{14}H_{10}N_2O_4$, mol.wt. = 270.2, m.p. = 300°), compound I-A ($C_{13}H_8N_2O_4$, mol.wt. = 256.2, m.p. > 300°), and compound II ($C_{15}H_{12}N_2O_4$, mol.wt. = 284.27, m.p. = 297–300°, used as the reference standard) were prepared containing 100 μg of each free acid per ml of methanol: 0.01 *M* pH 7.5 potassium phosphate buffer (1:1). The stock solutions of I and II were used to prepare mixed standard solutions (Table I) by suitable dilutions in methanol–water (1:1). Fresh solutions must be prepared each week and stored refrigerated when not in use.

Aliquots (250 μl) of these mixed standard solutions were added to blood as internal standards to establish a calibration curve for the determination of the unknowns and for the determination of percent recovery.

A calibration (external standard) curve of the peak height ratio of I to II *versus* concentration was constructed. A fresh calibration curve of the external standards and of the recovered internal standards was prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Reagents

All reagents were of analytical reagent grade (>99% purity), and all inorganic reagents were made up in distilled, carbon-filtered, deionized water filtered through a 0.2 μm filter (Type DC system, Hydro-Service and Supplies, Durham, N.C., U.S.A.).

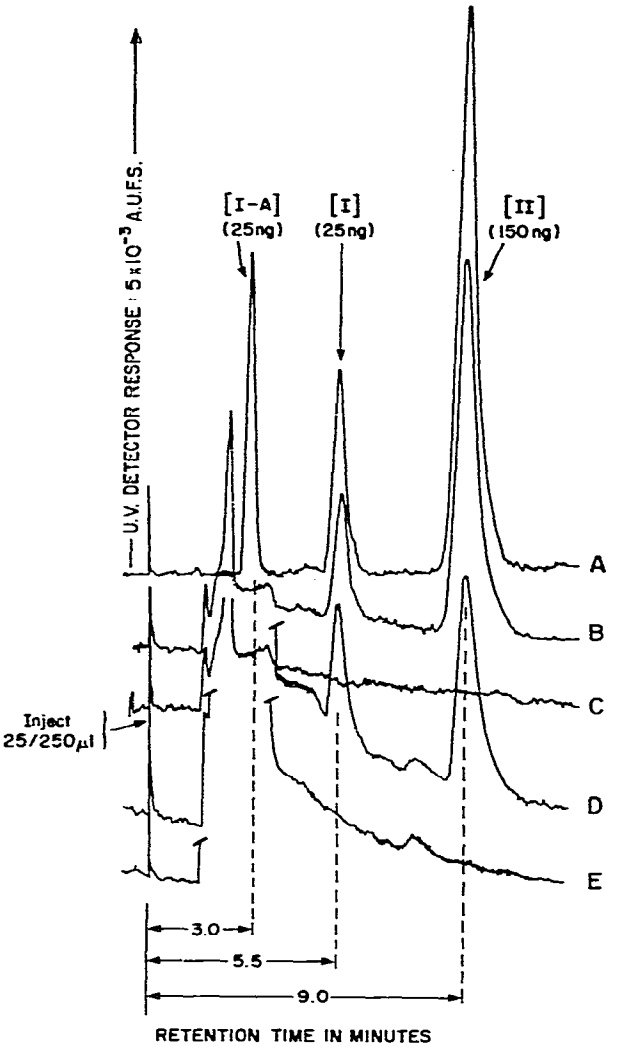


Fig. 3. Chromatograms of (A) authentic standards, and diethyl ether extracts of the protein free filtrates of (B) control blood containing authentic standards of I and II, (C) control blood, (D) control plasma containing authentic standards of I and II and (E) control plasma, using a UV detector at 365 nm.

The 1 *M* potassium phosphate buffer (pH 2.7) was prepared by mixing 1 *M* $K.H_2PO_4$ (136.09 g/l) and 1 *M* H_3PO_4 (68.5 ml of 85% acid/ml) to obtain the correct pH.

Organic solvents were purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.), with the exception of diethyl ether (anhydrous, reagent grade) which was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). The diethyl ether used was taken from a freshly opened can before each analysis and was shaken with granular zinc (about 1 g per lb. of ether) to reduce the peroxides, which might otherwise decompose low concentrations of I.

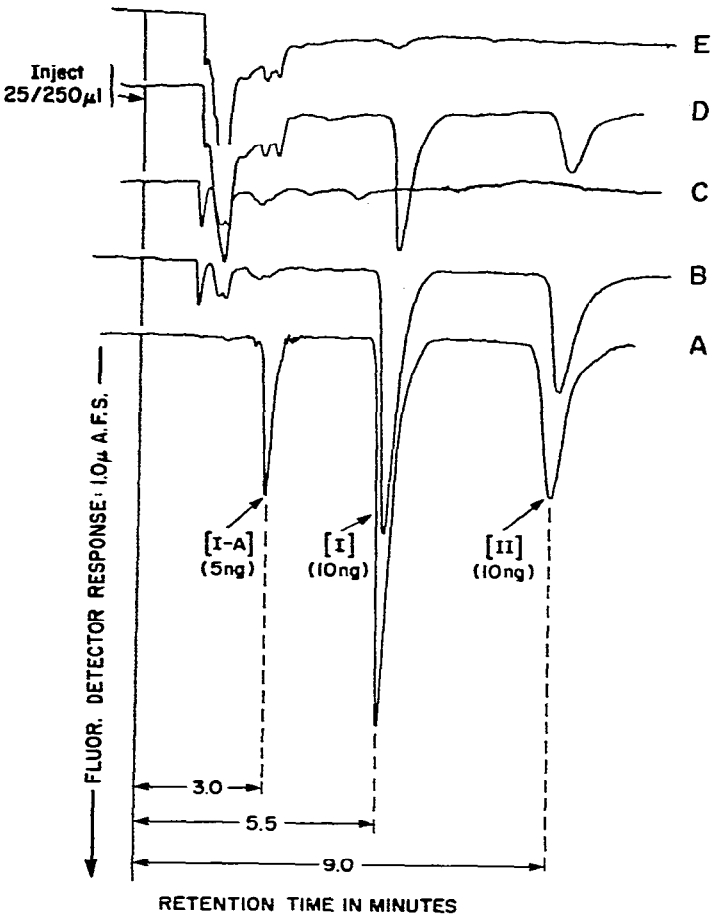


Fig. 4. Chromatograms of (A) authentic standards, and of diethyl ether extracts of the protein free filtrates of (B) control blood containing authentic standards of I and II, (C) control blood, (D) control plasma containing authentic standards of I and II and (E) control plasma, using a fluorescence detector, excitation: 280 nm/emission: > 418 nm.

TABLE I

STANDARD SOLUTIONS TO BE USED WITH UV AND FLUORESCENCE DETECTORS

Standard	*Compound I (µg/250 µl)*	*Compound II (µg/250 µl)*
UV detector		
1	2.00	1.5
2	0.75	1.5
3	0.25	1.5
4	—	1.5
Fluorescence detector		
5	0.250	0.10
6	0.100	0.10
7	0.050	0.10
8	—	0.10

Analysis of whole blood

A 250-μl aliquot of either standard solution No. 4 or No. 8 (Table I) was transferred into a 15-ml glass-stoppered centrifuge tube to provide a reference standard of II for each blood unknown. Two ml of water was added to dilute the methanolic standard, then a suitable aliquot; (0.1–1.0 ml) of the blood sample was added and mixed well.

A separate set of internal standards was prepared by transferring 250 μl aliquots of either mixed standard solutions 1, 2, and 3 for UV or no. 5, 6, and 7 for fluorometric determination (Table I) into separate 15-ml centrifuge tubes, and directly diluting with 2 ml water followed by an appropriate aliquot of control blood.

The samples were mixed vigorously and 8 ml of acetone–methanol (75:25) were added. The tubes were stoppered (PTFE no. 13 stopper) and shaken for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.). The samples were centrifuged at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, Rotor No. 253; Damon/IEC, Needham, Mass., U.S.A.) at 5° and the supernatant was transferred into another 15-ml conical centrifuge tube.

The precipitate was re-extracted by adding 0.5 ml of water and 3 ml of acetone–methanol, resuspending the precipitate by stirring it with a clean microspatula, shaking for 10 min and centrifuging. The second extract was combined with the first, and the combined organic phase was evaporated at 60° for 20 min in a N-EVAP evaporator (Organomation Assoc., Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen, until a fixed volume of aqueous material remained.

To each of the concentrated extracts was added 3 ml of 1 *M* pH 2.7 phosphate buffer (prepared from 1 *M* H_3PO_4 and 1 *M* KH_2PO_4) and 7.5 ml of fresh diethyl ether. Each tube was stoppered (PTFE No. 13 stopper), and shaken on a reciprocating shaker for 10 min at a moderate speed (80–100 strokes/min). The samples were centrifuged for 5 min at 2300 rpm (1300 *g*) at 0–5° in a refrigerated centrifuge. The diethyl ether layer was carefully removed using a 10-ml serological pipette and transferred into a 15-ml conical centrifuge tube. The sample was re-extracted with a second 7-ml aliquot of diethyl ether as described above and was combined with the first ether extract.

The combined ether extract was evaporated to dryness at 40° in a N-EVAP evaporator under a stream of clean, dry nitrogen, and the residue was dissolved in 250 μl of methanol–water (1:1). Each sample was then filtered through a Millipore 0.6-μm pore diameter polyvic 13-mm diameter membrane filter (Cat. No. BDWP013/00), using a 1-ml hypodermic syringe, to remove any colloidal particulates. A 25-μl aliquot of the filtrate was then injected for HPLC analysis using reversed-phase chromatography. The UV detector was set at either 5×10^{-3} a.u.f.s. or 2×10^{-2} a.u.f.s. depending on the sensitivity desired. The Schoeffel fluorescence detector was set at the 0.2–0.02 μA range, depending on the sensitivity desired with the excitation monochromator at 280 nm and the emission filter chosen to pass wavelengths greater than 418 nm.

Analysis of plasma and urine

For plasma, 250 μl of either standard solution 4 or 8 was used to provide the reference standard (II). A separate set of internal standards was prepared as in the blood procedure.

Urine was assayed without the addition of the reference standard. A separate set of internal standards consisting of a "control" and three separate control specimens to which 1 μg, 5 μg or 25 μg standards of I was added directly (without evaporation) to comprise the set of internal standards.

Plasma and urine samples were diluted with 5 ml pH 2.7 phosphate buffer and extracted directly with diethyl ether without prior treatment. The ether extract is then processed according to the blood procedure. For urine, a 10/1000 μl aliquot of the final residue was injected for HPLC analysis. Generally, the plasma and urine extracts are sufficiently free of colloidal particulates to enable direct injection without the membrane filtration step required for whole blood extracts.

Calculations

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

RESULTS AND DISCUSSION

A rapid, sensitive, and specific HPLC assay was developed for the determination of I from 1.0 ml of oxalated whole blood, plasma or urine.

Reversed-phase chromatography on μBondapak C_{18} with 2 vials of PIC A (28 ml) per liter of methanol–water (1:1) completely resolved compounds I to IV. The analogous compounds II, III and IV were investigated as possible reference standards in the assay, and II was selected due to its favorable retention time of 9.0 min relative to I under the HPLC conditions described. The major UV absorption bands of compound I in methanol were at 230–240, 275–285, and at 355–365 nm, respectively (Fig. 2).

The Waters Model 440 absorbance detector is capable of UV measurements at 254, 280, 315 and 365 nm, respectively, depending on the filters selected. Measurement at 365 nm was preferred in order to minimize interference from extracted endogenous impurities. Greater sensitivity and specificity is achieved by using a fluorescence detector in tandem with the UV detector. The corrected excitation and emission spectra of compound I were determined (Fig. 2) using a Farrand spectrofluorometer equipped with a 150-W xenon lamp. The corrected excitation maxima coincide with the UV absorption maxima, and the corrected emission maximum is at 475 nm. The choice of a fluorescence detector in HPLC depends upon the sensitivity required. The total fluorescence yield is proportional to the energy output of the excitation source. Although the xenon lamps in most spectrofluorometers yield a relatively uniform spectral continuum, they lack high stability due to arc wander and tend to fall sharply in energy output below 300 nm. Deuterium lamps are less intense sources but are more stable and have their maximum intensity between 200 and 300 nm. Both types of illumination sources were investigated for use in the HPLC assay of I.

The Schoeffel Model FS970 fluorescence detector utilizes a 5-μl flow cell and a deuterium lamp and gave the greatest response with the excitation monochromator set at 230 nm, and a broad-band pass filter > 418 nm for fluorescence detection. How-

ever, the excitation band at 280 nm was preferred (Fig. 2) since the fluorescence quenching effects due to extracted endogenous impurities was less than at 230 nm.

The Hitachi spectrofluorometer utilizes a xenon lamp and gave the greatest response with excitation at 360 nm and fluorescence emission at 475 nm. It also yielded more sensitive detection for compound I in biological extracts, notwithstanding the use of a 100-μl flow cell (which tended to broaden the peaks), due to an even further reduction in fluorescence quenching effects and, also, to a higher intensity of excitation energy at this wavelength.

Extraction

Compound I was completely extracted into ether at pH 2.7 from aqueous buffer. However, direct ether extracts of blood at pH 2.7 were too contaminated with lipids, hence, a protein precipitation step was necessary prior to extraction. The acetone precipitation procedure reported for 2-hydroxynicotinic acid[17] yielded cleaner sample residues (for HPLC) when extracted at pH 2.7 with diethyl ether.

The extraction of I from plasma and urine was successful in samples buffered directly to pH 2.7. However, urine extracts contained considerable amounts of UV absorbing endogenous impurities which limited the sensitivity to 1 μg of I for a 1-ml specimen when fluorescence detection was used.

Recovery and sensitivity limits of the HPLC assay

The overall recovery of I was 69 $\pm$ 10% (S.D.) from 1.0 ml of oxalated whole blood and 84 $\pm$ 6% (S.D.) from 1.0 ml of plasma. The sensitivity limit of the HPLC assay was about 100 ng of I by UV absorption at 365 nm and 25 ng by fluorometric detection using a Schoeffel Model FS970 fluorescence detector or 5 ng using a Hitachi Model 204 spectrofluorometer, using either 1 ml of blood or plasma per assay. Typical chromatograms of dog blood extracts are shown in Figs. 3 and 4.

Where a choice of biological sample for analysis may be specified, then plasma is preferred, due to simpler sample preparation, higher overall recovery, and shorter analysis time for expeditious sample throughout.

The recovery of I from urine was 90 $\pm$ 5% (S.D.) and the sensitivity limit was 1.0 μg/ml using the Hitachi spectrofluorometer as the detector. The primary limitation of the sensitivity of the urine assay is due to the endogenous UV absorbing organic acids extracted at pH 2.7, which tend to quench the fluorescence of I. These interferences cannot be removed by back washing with dilute base without incurring significant losses of I.

APPLICATION OF THE METHOD TO BIOLOGICAL SPECIMENS

Blood levels of compound I in the Rhesus monkey

Blood levels were determined in a Rhesus monkey following the intravenous administration of a 10 mg/kg dose of the sodium salt dissolved in isotonic saline. Blood concentrations above 0.5 μg/ml were determined by UV and those below that were determined by fluorescence detection.

The blood level data (Table II) indicated that intact I levels decline in an exponential fashion and would probably be detectable throughout a 48-h experimental interval.

TABLE II

BLOOD LEVELS OF COMPOUND I IN THE RHESUS MONKEY

Dose, 10 mg (free acid)/kg (102.48 mg of sodium salt = 100 mg of free acid); route, intravenous bolus; weight: 9.45 kg.

Sampling time	*Concentration (μg/ml)*
Predrug (no heparin)	—*
Predrug (+heparin)	—*
2 min post i.v.	200
5 min	140
10 min	135
25 min	123
40 min	90
55 min	60
70 min	40
2 h	11.5
4 h	6.0
23 h	0.6
30.5 h	0.5

* No interfering peaks due to heparin or endogenous impurities extracted.

Studies in the dog

The blood concentrations of I were determined in a dog following the intravenous and oral administration of the sodium salt of I at a dose equivalent to 1.0 mg/kg of the free acid.

Specimens were drawn at appropriate times following intravenous and oral administration of an aqueous solution of the sodium salt and the concentration of I determined, using both UV and fluorescence detectors in tandem to accommodate the wide concentration range measured over the 48 h experimental period.

Following intravenous administration an exponential elimination profile was observed (Fig. 5) with an initial blood concentration of 4.35 μg/ml measured at 1 min declining to non-measurable amounts by 48 h. Following oral administration rapid absorption was indicated by a peak blood concentration of 0.27 μg/ml at 20 min with concentrations declining to non-measurable amounts at 48 h. Comparison of areas under the curve following oral and intravenous administrations, suggests virtually complete absorption.

The urinary excretion data (Table III) indicated that the free acid was rapidly eliminated mainly in the 0–24 h excretion period accounting for 44% of the dose following intravenous, and 37% of the dose following oral, administration. The total amount of the dose recovered in the 0–72 h collection period was 48.6 and 44.4% following the intravenous and oral administrations, respectively. The drug recovered was in the directly extractable unconjugated form. The extracted urines were then adjusted to pH 5.5 and subjected to enzymatic deconjugation with 2% Glusulase (Endo Labs., Garden City, N.Y., U.S.A.) for 2 h to hydrolyze any conjugates present. The post-Glusulase incubation fraction did not yield any significant amounts of either parent drug or the phenolic analog I-A, an *in vitro* metabolite isolated from a phenobarbital pretreated rat liver (9000 *g*) microsomal supernatant[16].

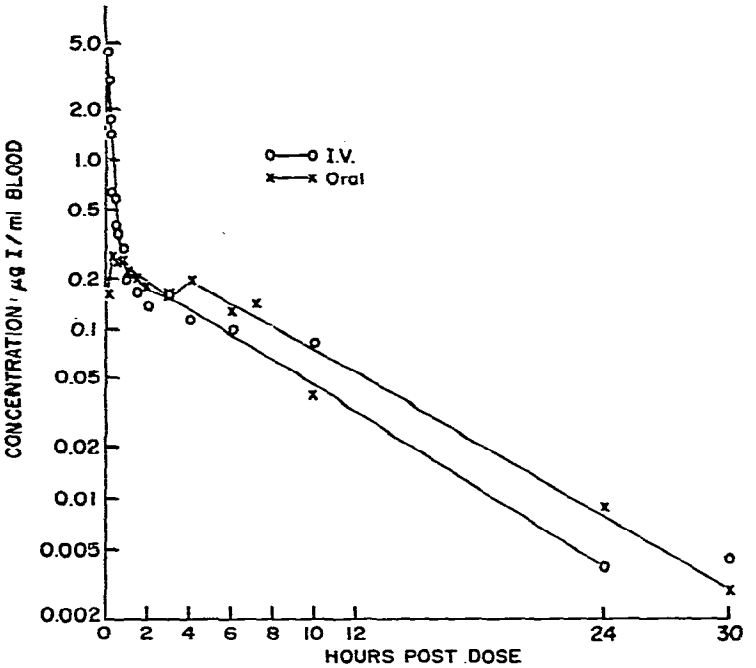


Fig. 5. Blood concentration-time curves of compound I (free acid) in a dog following single intravenous and oral administartion of the sodium of I at a dose equivalent to 1.0 mg/kg of the free acid.

TABLE III

URINARY EXCRETION PROFILE OF COMPOUND I IN THE DOG FOLLOWING THE INTRAVENOUS AND ORAL ADMINISTRATION OF A DOSE EQUIVALENT TO 1.0 MG/KG

Route	*Total dose administered*	*Excretion interval (h)*	*Total mg I (acid) recovered**	*Recovery of dose (%)*
i.v.	15.84 mg sodium salt (13.81 mg acid)	0–24	6.02	43.6
		24–48	0.65	4.7
		48–72	0.044	0.3
				48.6
Oral	15.53 mg sodium salt (13.52 mg acid)	0–24	5.06	37.4
		24–48	0.82	6.1
		48–72	0.12	0.9
				44.4

* As unconjugated directly extractable compound.

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