Spectrofluorometric Determination of Alkoxy-Substituted Benzylimidazolidinones in Biological Fluids

J. ARTHUR F. de SILVA ˣ, NANCY MUNNO, LUCIUS D'ARCONTE †, and NORMAN STROJNY

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Abstract □ Sensitive and specific spectrofluorometric assays were developed for the determination of *d,l*-4-(3,4-dimethoxybenzyl)-2-imidazolidinone and *d,l*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone in blood and urine. Each compound is selectively extracted from buffered blood or urine and oxidized with alkaline permanganate to its respective fluorescent benzoic acid derivative, which is quantitated in acetic acid in ethanol (1:99). The method was applied to the determination of blood levels and urinary excretion in the dog following the administration of single intravenous and oral doses of each compound.

Keyphrases □ Imidazolidinones, substituted—spectrofluorometric analyses in biological fluids □ Spectrofluorometry—analyses, substituted imidazolidinones in biological fluids

The alkoxy-substituted benzylimidazolidinones *d,l*-4-(3,4-dimethoxybenzyl)-2-imidazolidinone (I) and *d,l*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (II) (Scheme I) were synthesized by Gruenman and Hoffer (1). Compound I is of clinical interest because of its cardiovascular inotropic effects as a vasodilator and for increased cardiac output (2, 3) and as a hypolipemic agent (4), while II has shown activity as a phosphodiesterase inhibitor (5–9).

Although both compounds are aromatic in nature, neither their UV absorption nor intrinsic fluorescence nor phosphorescence was sufficiently intense to enable their quantitation as the intact moiety. Investigation of possible derivatization reactions prompted the use of oxidation in alkaline permanganate, involving the cleavage of the methylene (CH_2) bridge in these compounds to form strongly fluorescent benzoic acid derivatives (III and IV, Scheme I). A similar reaction was reported in the analysis of the dihydrofolate reductase inhibitor trimethoprim (10) used as a potentiator in antibacterial combinations with sulfonamides. The chemistry of these reactions is also amenable to assay automation (11).

Both I and II were readily oxidized to yield their respective benzoic acids (III and IV), which were dissolved in acetic acid in ethanol (1:99) and quantitiated spectrofluorometrically at 345 nm with excitation at 295 nm. The sensitivity limit of the assay is 0.25 μg of I or 0.10 μg of II/ml of blood or urine using a 4-ml specimen/assay.

The method was applied to the determination of blood levels and urinary excretion in the dog following the administration of single doses of I or II by intravenous and oral routes.

EXPERIMENTAL

Standard Solutions—The analytical standards required were: I, $C_{12}H_{16}N_2O_3$, mol. wt. 236.3, mp 152–153°, and II, $C_{15}H_{22}N_2O_3$, mol. wt. 278.36, mp 126–127°, both of pharmaceutical grade purity (>99%). 3,4-Dimethoxybenzoic acid (III) (veratric acid[1]), $C_9H_{10}O_4$, mol. wt. 182.18, mp 180–181°; and 3-butoxy-4-methoxybenzoic acid (IV), $C_{12}H_{16}O_4$, mol. wt. 224, mp 143°, purity >99%.

Ten milligrams each of I–IV were accurately weighed and transferred into separate 10-ml volumetric flasks and dissolved in 10 ml of ethanol. These stock solutions (A-1, A-2, A-3, and A-4) contained 1 mg of the respective compounds/ml. Dilutions (1:10) of each were made in ethanol to prepare working solutions (B-1, B-2, B-3, and B-4) containing 100 μg/ml.

Separate serial dilutions of B-3 and B-4 were made with 1% acetic acid–ethanol (1:99 v/v) to yield standard solutions in the concentration range of 0.25–5.0 μg/ml. These solutions were used to obtain a calibration curve for the determination of the efficiency of the alkaline potassium permanganate oxidation of I and II to their respective benzoic acids.

Instruments—The spectrofluorometer[2] used was equipped with a 150-w zenon arc energy source[3] and photomultiplier[4]. It was used with the 10-nm slit arrangement in all four positions, with the instrument energy adjusted for maximum sensitivity using a standard solution of III and IV.

Reagents—All inorganic reagents were analytical reagent grade. They were used without further purification and were prepared in distilled deionized water. These reagents included 1 *M* KH_2PO_4–$K_2HPO_4 \cdot 3H_2O$ buffer, pH 7.0 (12); 1 *M* H_3BO_3–Na_2CO_3–KCl buffer, pH 9.0, prepared as described previously (13); alkaline potassium permanganate solution (0.1 *M* in 0.1 *N* NaOH); 0.1 *N* HCl; 0.5 *N* HCl; 1.0 *N* H_2SO_4; 0.1 *N* NaOH; and 0.5 *N* NaOH.

The Dragendorff reagent used was a modification[5] from a published procedure (14) and was prepared as follows. Solution A was 0.85 g of bismuth nitrate dissolved in 10 ml of acetic acid and diluted to 50 ml with distilled water. Solution B was 20 g of potassium iodide dissolved in 50 ml of water. Five milliliters each of Solutions A and B were mixed and diluted to 50 ml with 10% H_2SO_4, followed by the addition of 0.1 ml of 30% H_2O_2 to hasten the "aging" of the solution. The solution must be "aged" for at least 24 hr prior to use. This reagent is stable for 3–4 months but should be discarded when an appreciable precipitation of iodine becomes apparent.

The organic reagents used were ethyl acetate[6] (spectrograde), ether[6] (absolute) from a freshly opened can, formaldehyde[7] (37% solution), alcohol[8] (USP 200 proof), and acetic acid in ethanol (1:99 v/v).

Analysis of I in Blood—Whole blood, 1–4 ml, was pipetted into a 50-ml centrifuge tube for analysis. Along with the samples, a specimen of control blood and duplicate specimens of control blood containing internal standards of 2.5 and 5.0 μg of I, added by evaporation to dryness at 40° under nitrogen of 0.025 and 0.05 ml of Solution B-1, were processed. Six milliliters of 1.0 *M* phosphate buffer (pH 7) was added to all specimens, which were then extracted with 2 × 15 ml of ethyl acetate.

The samples were extracted by shaking for 10 min on a reciprocating shaker and centrifuging at 2000 rpm in a refrigerated centrifuge at 5° for 10 min. The supernatant ethyl acetate was transferred into another 50-ml tube, and the extracts were combined. The combined ethyl acetate extract was evaporated to dryness. The residue was dissolved in 3 ml of 0.5 *N* HCl and backwashed with 2 × 5-ml portions of ether by mixing vigorously for 1 min on a variable speed test tube mixer[9]. Then the samples were centrifuged, and the ether supernate was discarded.

All samples and standards were neutralized by the addition of 1.5 ml

[1] Aldrich Chemical Co.

[2] Model Mark I spectrofluorometer equipped with a specially selected high UV energy source and photomultiplier, Farrand Optical Co., Mount Vernon, N.Y.

[3] Hanovia lamp, Engelhardt Industries, Newark, N.J.

[4] RCA IP-28 (lumen output 10) photomultiplier.

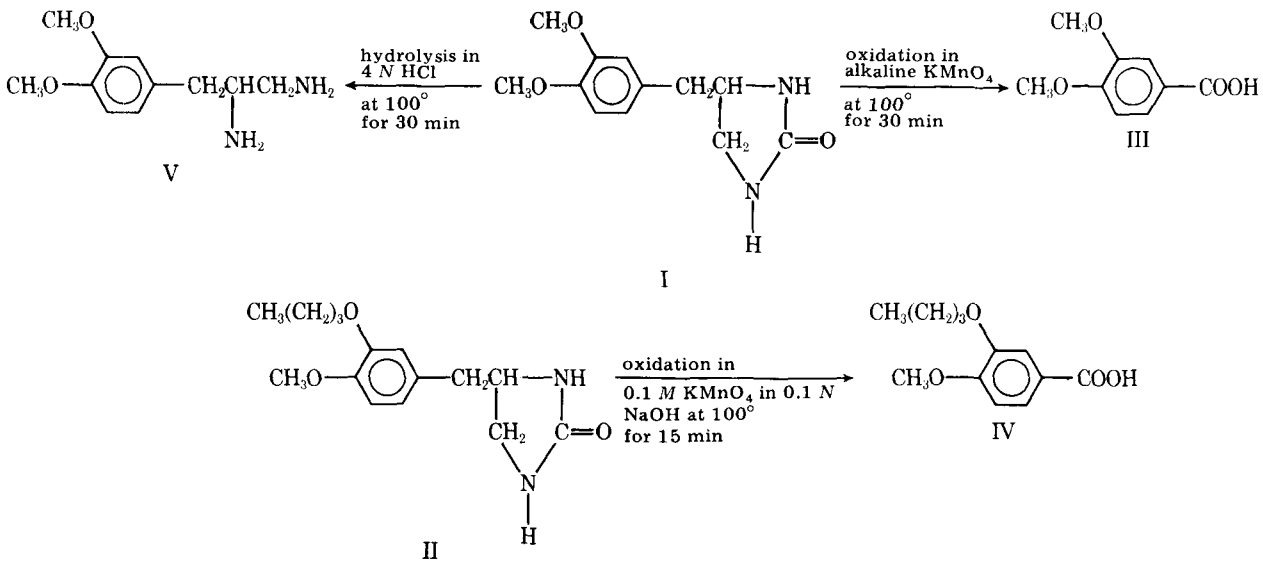
[5] R. Colarusso, E. Heylweil, and B. Z. Senkowski, Hoffmann-La Roche Inc., 1970, unpublished data on file.

[6] Mallinckrodt Chemical Co.

[7] Allied Chemical Co.

[8] Pharmaco, Division of Publicker Industries.

[9] Vortex Supermixer, Lab-Line Instruments, Melrose Park, Ill.



Scheme I

of 0.1 *N* NaOH, and all residual ether was expelled by placing tubes in a 90° water bath for approximately 10 min and shaking the tubes every few minutes. At this point, standard solutions of I may be included for the determination of the percent conversion to III.

One milliliter of 0.1 *M* alkaline $KMnO_4$ solution was added to each tube, and the tube was placed in a boiling water bath for 5 min. A second 1-ml aliquot of 0.1 *M* alkaline $KMnO_4$ solution was then added to all tubes, which were replaced in the water bath for 5 min until equilibrated and then stoppered. The tubes were allowed to remain in the boiling water bath for 30 min for completion of the oxidation reaction and then were removed and cooled in an ice bath.

A 0.2-ml aliquot of formaldehyde (37%) was then added to each tube to reduce the excess permanganate to manganous dioxide, forming a brown precipitate while decolorizing the pink solution. The tubes were mixed well and allowed to stand for 5 min, and then sufficient 1 *N* H_2SO_4 (~1–2 ml) was added to dissolve the manganous dioxide precipitate to manganous sulfate. The tubes were then extracted twice with 10 ml of ether by shaking for 10 min on a reciprocating shaker. The samples were centrifuged, and the ether extracts were combined in a 15-ml centrifuge tube by evaporating the extracts sequentially to dryness.

The residue was dissolved in 3 ml of acetic acid in ethanol (1:99 v/v), and the fluorescence of this solution was determined at 345 nm with excitation at 295 nm. The fluorescence readings were corrected for control (blank) fluorescence, and the concentration of the drug in the unknowns was determined by direct comparison of their fluorescence to that of the internal standards processed along with the unknowns.

Analysis of II in Blood—Whole blood, 1–4 ml, was pipetted into a 50-ml centrifuge tube for analysis. Along with the samples, a specimen of control blood and duplicate specimens of control blood containing 2.5 and 5 μg of II as internal standards (suitable aliquots of Solution B-2) were processed. Six milliliters of pH 9.0, 1 *M* borate KCl–Na_2CO_3 buffer was added to all specimens, which were then extracted with 2 × 15 ml of ether.

The samples were extracted by shaking for 10 min on a reciprocating shaker and centrifuging at 2000 rpm at 5° for 10 min. The supernatant ether was transferred into another 50-ml tube, and the extracts were combined. The combined ether extracts were evaporated to dryness, and the residues were dissolved in 2 ml of 0.1 *N* HCl. At this point, standard solutions of II may be included for the determination of the percent conversion to IV.

Each solution was made alkaline by the addition of 1.0 ml of 0.5 *N* NaOH, followed by the addition of 2.5 ml of 0.1 *M* alkaline $KMnO_4$ solution to each tube. The tubes were then placed in a 100° water bath for exactly 15 min to effect the oxidation of II to IV. The tubes were removed and cooled immediately in an ice bath. A 0.2-ml aliquot of formaldehyde (37%) was added to each tube, which was mixed well and allowed to stand for 5 min (as in the assay for I). The rest of the procedure for II was identical to that for I.

Procedure for Urine Samples—*Directly Extractable Unconjugated Fraction*—The extraction procedure for I from urine was identical to that for blood. The specimens for analysis were processed along with a control specimen and duplicate control specimens containing 2.5 and 5.0 μg of added authentic standards. For I, the residue of the ethyl acetate extract of urine was dissolved in 100 μl of ethanol and transferred quantitatively onto a 20 × 20-cm silica gel chromatoplate[10]; the centrifuge tube was rinsed with an additional 50 μl of ethanol to effect a quantitative transfer.

The chromatoplate was developed for 11–15 cm, ascending in chloroform–ethyl acetate–methanol (90:10:10). The sample areas corresponding to I were identified by reference to authentic standards (25 μg) of I run alongside the sample extracts ($R_f \simeq 0.50$), eluted into 5 ml of ethanol, and evaporated to dryness. The residues were dissolved in 3 ml of 0.5 *N* HCl, and the samples were processed as in the procedure for I, beginning with the neutralization step prior to the addition of alkaline permanganate.

The extraction of urine for II was carried out with ethyl acetate similarly to that for I. The residues were dissolved in 100 μl of ethanol and transferred to a silica gel chromatoplate as for I, and the plate was developed in benzene–methanol–acetic acid (90:10:10). After development, the sample areas for II were identified ($R_f \simeq 0.50$) and eluted with 3 ml of 0.1 *N* HCl, and a 2-ml aliquot was processed as described in the blood assay for II.

Conjugated Fraction—Following extraction of the unconjugated free fraction, the urine specimen was titrated to pH 5.3 and incubated at 37° for 2.5 hr with an enzyme preparation containing 150,000 units of glucuronidase and 50,000 units of sulfatase/ml[11] in a metabolic shaking incubator[12] to deconjugate any glucuronide–sulfate metabolites. The sample was extracted at pH 5.3 with the appropriate solvent and analyzed by TLC as described for I or II for acidic metabolites and then adjusted to pH 9.0, reextracted, and analyzed for basic metabolites.

An aliquot of the respective extracts was initially analyzed by TLC for the qualitative identification of metabolites using the procedure described below. A larger aliquot was then reanalyzed by TLC for the quantitation of metabolites (previously identified by R_f and their reaction to the Dragendorff treatment) by elution into methanol, followed by alkaline permanganate oxidation as previously described.

Qualitative Identification of I and II by TLC—Compounds I and II were difficult to detect on chromatoplates because of their poor UV absorption. For qualitative identification of I or II, the plate was sprayed with the modified Dragendorff reagent and heated in an oven at 100–104° for 30 min. The plate was then examined under short- and longwave UV light. Intact I and II and any metabolites containing the benzyl-2-imidazolidinone moiety were observed as bright blue-green to yellow fluorescent spots against a dark background. The visual sensitivity limit was about 0.5–1.0 μg of I or II.

This process is chemically destructive for II. However, when the addition product formed with I was eluted with ethanolic 0.5 *N* HCl (2:1) and examined fluorometrically, excitation and emission spectra similar

[10] E. Merck (F_{254}) silica gel G, 60-μm particle size, 250-μm bed thickness, Brinkmann Instruments, Westbury, N.Y.

[11] Glusulase, Endo Laboratories, Garden City, Long Island, N.Y.

[12] Dubnoff metabolic shaking incubator, GCA/Precision Scientific Instruments, Chicago, Ill.

Table I—Luminescence Properties of I and II and Their Benzoic Acid Derivatives

Compound	Solvent[a]	Fluorescence (25° C) Excitation/ Emission Maxima, nm	Fluorescence (25° C) Intensity, TM[b]/ μg/ml	Fluorescence (25° C) Sensitivity Limit, μg/ml	Phosphorescence (77° K) Excitation/ Emission Maxima, nm	Phosphorescence (77° K) Intensity, TM[b]/ μg/ml	Phosphorescence (77° K) Sensitivity Limit, μg/ml
I	1	280/315	80	1	285/455	1.2	20.0
	2	280/317	80	1	—	—	—
V	2	280/317	80	1	—	—	—
III	1	295/345	1500	0.1	290/435	550	0.050
II	1	280/315	80	1	285/440	3.3	10.0
	2	280/317	80	1	—	—	—
IV	1	295/345	1300	0.1	295/435	420	0.050

[a] Solvent 1 is acetic acid in ethanol (1:99), and Solvent 2 is ethanol–0.5 *N* HCl (2:1). [b] Fluorescence (TM) units = transmittance (T) × meter multiplier factor (M).

to those of authentic I were detected with about the same fluorescence quantum yield as I. Neither product was formed in solution with the Dragendorff reagent at either ambient or elevated temperature.

RESULTS AND DISCUSSION

The UV absorption spectra of I in 0.5 *N* HCl and 0.5 *N* NaOH showed a maximum of 228.5 nm ($A/\mu g/ml = 0.035$), a minimum at 253 nm, and a second maximum at 278.5 nm. The spectrum in ethanol showed a maximum at 231 nm ($A/\mu g/ml = 0.038$), a minimum at 253 nm, and a second maximum at 281 nm. The UV absorption spectra of II in ethanolic 0.5 *N* HCl and in 0.5 *N* NaOH showed a maximum at about 230 nm ($A/\mu g/ml = 0.026$ for ethanol) with a minimum at 250 nm and a second maximum at 280 nm. The weak UV absorption of these compounds, coupled with high blank values from biological samples, precluded its usefulness in an assay.

The luminescence properties of I and II and their respective benzoic acid derivatives were determined using the spectrofluorometer[2] modified to accommodate a Lewis–Kasha phosphoroscope as described previously (15) (Table I). Compounds I and II had weak intrinsic fluorescence and phosphorescence. The intrinsic fluorescence of I in ethanol–0.5 *N* HCl (2:1) (excitation 280/emission 317 nm) was linear with concentration in the range of 2.5–50.0 μg of I/ml of final solution. The sensitivity limit for blood or urine was 2.5–3.0 μg of I/ml using a 4-ml sample/assay and was not deemed sufficiently sensitive for pharmacokinetic studies. A similar direct assay of II was not feasible because the necessary "cleanup" by backwashing acid solutions of II results in losses of II and the unwashed samples have a high and variable background fluorescence.

Investigation of possible derivatives that could be used to measure therapeutic levels of I led to acid hydrolysis of I in 4 *N* HCl to 3-(3,4-dimethoxyphenyl)-2-aminopropylamine (V, Scheme I). This compound exhibited weak fluorescence properties similar to intact I and, therefore, was not investigated further. Alkaline hydrolysis did not yield any usable derivatives.

Oxidation of the methylene group of I with alkaline permanganate at 100° for 30 min, using a modification of a similar reaction in the assay of trimethoprim (10), produced >65% yield of III. The reaction product was characterized by two-dimensional thin-layer cochromatography with authentic III, using benzene–methanol–acetic acid (90:10:5) in the first dimension and ethyl acetate–methanol–concentrated ammonium hydroxide (90:10:5) in the second dimension. The alkaline permanganate oxidation reaction also was applicable to II.

The optimum yield of IV, about 65 ± 5.0%, was obtained by oxidation at 100° for exactly 15 min. Prolonged oxidation caused an apparent breakdown of IV and a decrease in the fluorescence. The reaction products were characterized by two-dimensional TLC, utilizing benzene–methanol–acetic acid (90:10:10) in the first dimension and ethyl acetate–ethanol–ammonium hydroxide (90:10:5) in the second dimension. One major product was detected with R_f values of 0.48 and 0.04 in the respective systems. This product ran as one spot when cochromatographed with authentic IV.

Two minor products were also seen on the chromatoplate[10]. Component 1, R_f 0.48 and 0.00, exhibited weak fluorescence with spectra similar to those of IV; Component 2, R_f 0.26 and 0.05, did not exhibit any significant fluorescence. Their chemical structures have not been elucidated. No residual unconverted parent compound was detectable on the plate at the R_f of II—*viz.*, 0.44 and 0.47 in the respective systems, indicating complete oxidation of the compound.

The benzoic acid derivatives III and IV exhibited strong fluorescence and phosphorescence in acetic acid in ethanol (1:99) (Table I). In each case, the fluorescence yield was linear with concentration in the range of 0.10–25 μg/3 ml of final solution. The intrinsic fluorescence was used as the basis for developing a sensitive assay in biological fluids.

Extraction of I from blood or urine at different pH values using different solvents yielded complete recovery at pH 7.0 in ethyl acetate but not in ether. When 0.5 *N* HCl was used to back-extract I into the acid as a cleanup step, I was completely recovered into the acid phase from ether but not from ethyl acetate. The overall recovery of 0.5–5.0 μg of I was about 92 ± 5% from 1 or 4 ml of blood when the blood was extracted into ethyl acetate at pH 7.0 and evaporated to a residue, the residue was dissolved in 0.5 *N* HCl, and the acid was backwashed with ether as a cleanup step; that from 1 or 4 ml of urine was about 86 ± 5%. However, the overall recovery of I taken through the entire assay, including oxidation in alkaline permanganate and measurement as III, was 60 ± 5% and reflects the limitation of the yield of this step. The complete evaporation of ether from the aqueous phase prior to oxidation was essential because the presence of ether or its peroxides reduced the permanganate with precipitation of manganous dioxide and resulted in incomplete and/or erratic

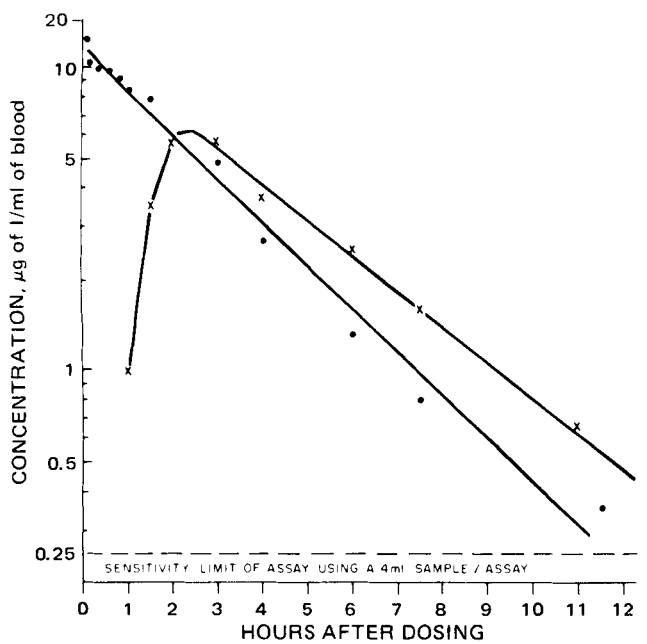


Figure 1—*Blood level fall-off curves of I in a dog following single intravenous (●) and oral (×) administration of a 100-mg dose.*

Table II—Urinary Excretion of I following the Administration of Single 100-mg Doses by Intravenous and Oral Routes

Route	Hours	Micrograms per Milliliter	Total, mg	Cumulative Percent of Dose Recovered
Intravenous	0–24	74.0	28.11	28.1
	24–26	16.0	2.62	30.7
	26–47	0.8	0.38	31.1
Oral	0–24	83.0	28.21	28.2
	24–48	1.1	0.42	28.6

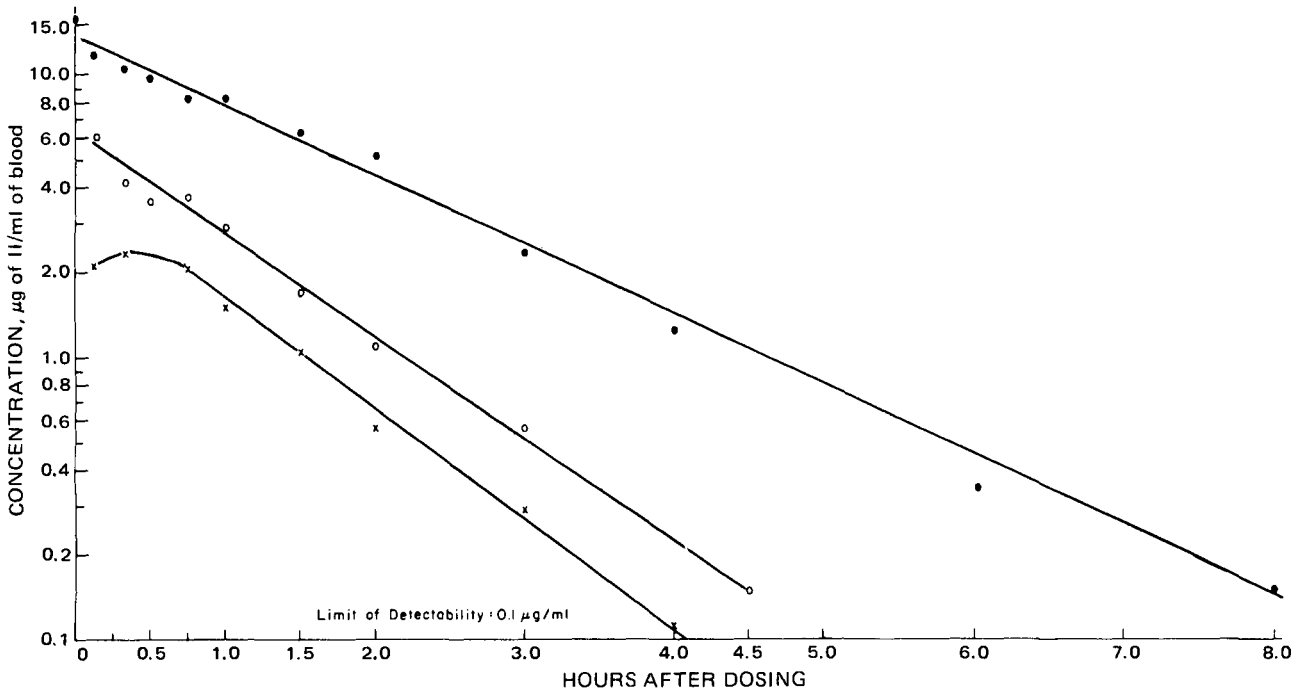


Figure 2—*Blood level fall-off curves of II in a dog following intravenous and oral administration. Key: ●, 10 mg/kg iv; O, 5 mg/kg iv; and ×, 5-mg/kg oral suspension in polysorbate 80.*

oxidation of I to III. The sensitivity limit of the assay was 0.25 μg of I/ml of blood or urine using a 4-ml specimen/assay and a sample to blank fluorescence ratio of 2:1 as the limit of detectability.

Compound II differed significantly from I in its partitioning behavior. Extraction of II from blood or urine at different pH values using different solvents indicated that II was completely recovered at pH 9.0 in ether or ethyl acetate. However, II could not be back-extracted into 0.1 *N* HCl as a cleanup step, nor could an acid solution be backwashed without loss of II. The ether extracts of blood at pH 9.0 were relatively free of impurities, and the oxidation step reduced blood blank values significantly. Thus, evaporation of ether extracts and dissolution of the residues in 0.1 *N* HCl, followed by alkaline permanganate oxidation, resulted in sufficiently low blanks for the analysis of up to 4 ml of blood. Extraction of urine was accomplished with ethyl acetate. Although the recovery of II in ether was 89 ± 6% from blood and 80 ± 5% from urine, the overall recovery of II, including the alkaline permanganate oxidation step and measurement as IV, was 60 ± 5%. The sensitivity limit of the assay was 0.10–0.20 μg of II/ml of blood or urine using a 4-ml specimen/assay.

Application of Assay for I in Biological Specimens—To demonstrate the utility of the assay, blood I concentrations and urinary excretion of I were determined following the intravenous and oral administration of single 100-mg doses given to the same dog 7 days apart. The intravenous dose was administered in propylene glycol solution, while the oral dose was the pure drug hand packed in a gelatin capsule.

Following both intravenous and oral dosing, blood levels were measurable for up to 11 hr and were below the sensitivity limit of the assay thereafter. The blood level data were plotted semilogarithmically with time in Fig. 1. Following the intravenous administration of I, the least-squares fit of a monoexponential equation to the blood concentration–time curve resulted in an estimated half-life of about 2 hr. The lag in the oral blood level curve probably reflects slow absorption. TLC examination of the extracts of the dog blood specimens showed the presence of only intact I.

The urinary excretion data following intravenous and oral administration of I are given in Table II. The amount of intact I recovered in the urine in 0–48 hr was 31% following intravenous administration and 28.6% following oral administration of I. TLC examination of the dog urine extracts showed small amounts of an unidentified metabolite in addition to I. This unconjugated metabolite yielded III upon oxidation in alkaline permanganate.

Small amounts of I and a metabolite were detected following enzymatic hydrolysis, two-dimensional TLC using ethyl acetate–acetone–methanol (85:15:15) in the first dimension and benzene–methanol (66:35) in the second dimension, and the Dragendorff visualization treatment previously described. The respective R_f values for I were 0.38 and 0.62; and for the metabolite, they were 0.08 and 0.27. This metabolite did not form a fluorescent derivative similar to III by alkaline permanganate oxidation. Its structure has not been elucidated.

Application of Assay for II in Biological Specimens—To demonstrate the utility of the assay for II, blood concentrations and urinary excretion of II were determined in a dog following intravenous and oral administration. Intravenous doses of 5 and 10 mg of II/kg were administered in propylene glycol solution to the same dog 7 days apart. A single 5-mg/kg suspension in polysorbate 80[13] was administered orally to a second dog. The blood level data were plotted semilogarithmically with time in Fig. 2.

Following intravenous administration of II, the least-squares fit of a monoexponential equation to the blood level–time curves resulted in estimated half-lives of approximately 1.2 hr for the 10-mg/kg dose and 0.9 hr for the 5-mg/kg dose. Following oral administration of a 5-mg/kg dose as a suspension in polysorbate 80[13], early blood levels indicated rapid absorption. TLC examination of the ether extracts of the dog blood specimens for II, using the solvent system described for the urine assay, showed the presence of only the intact drug.

The urinary excretion of II is listed in Table III. The intact drug accounted for about 1.9, 1.0, and 1.2% of the 10- and 5-mg/kg iv doses and the 5-mg/kg po dose, respectively, in the directly extractable unconjugated fraction. Chromatographic examination of the ethyl acetate extracts of the unconjugated fraction in a 0–24-hr dog urine specimen showed the presence of intact II and considerable amounts of UV-absorbing components. Dragendorff treatment did not produce any fluorescent area other than that for II.

Following extraction of the free fraction and enzymatic hydrolysis, the ethyl acetate extract (pH 5.3) of the deconjugated fraction was analyzed by one-dimensional TLC, using benzene–methanol–acetic acid (90:10:10),

Table III—Urinary Excretion of II in the Dog following the Intravenous and Oral Administration of the Drug

Dose, mg/kg	Route	Total Milligrams Excreted[a]	Dose Excreted as Intact II, %
10 (118 mg)	Intravenous	2.23	1.89
5 (53 mg)	Intravenous	0.499	0.95
5 (63 mg)	Oral	0.736	1.17

[a] The 0–24-hr postdosing urine pool.

[13] Tween 80, Sigma Chemical Co., St. Louis, Mo.

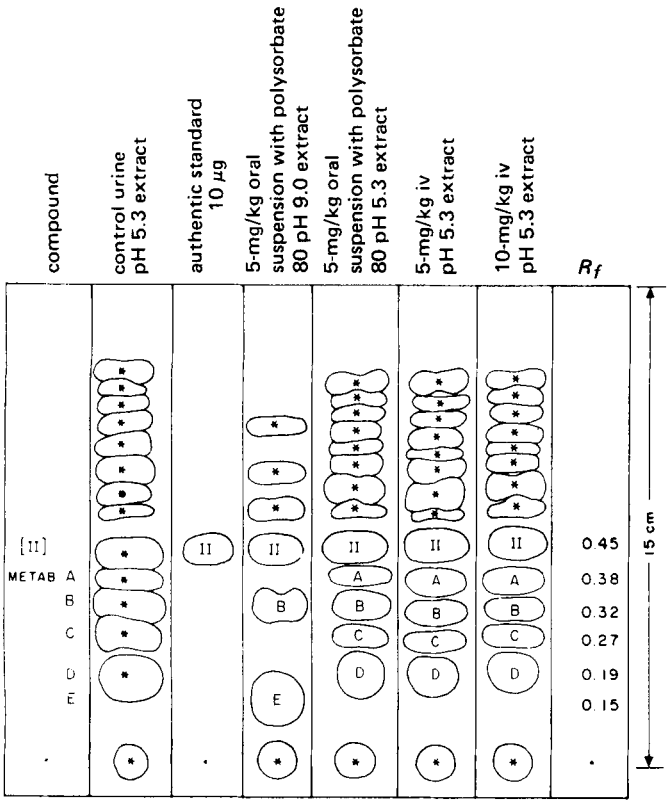


Figure 3—*Thin-layer chromatogram of ethyl acetate extracts of a 0–24-hr pool of urine (after enzyme incubation) from dogs treated with II by intravenous and oral routes of administration. (* = UV-absorbing components in the sample extract that do not fluoresce on either Dragendorff treatment or alkaline permanganate oxidation.)*

and showed the presence of intact II (R_f 0.45) and four metabolites (A–D) at R_f values of 0.38, 0.32, 0.27, and 0.19 (Fig. 3). These metabolites appeared as UV-absorbing bands and could be distinguished from coextracted endogenous urinary components by their intense yellow to blue-green fluorescence produced after Dragendorff treatment.

The urine was then titrated to pH 9.0 and extracted with two volumes (15 ml) of ethyl acetate. The residue of this extract, when analyzed by TLC, showed another metabolite (II-E) which migrated with R_f 0.15. This compound did not form a fluorescent product with the Dragendorff reagent and was detected as a pale-white area against a charred background after heating. The compound also did not form a fluorescent derivative upon alkaline permanganate oxidation.

Since urinary Metabolites A–D produced fluorescent products similar to IV by the alkaline permanganate oxidation reaction, it was assumed that the 3-butoxy-4-methoxybenzyl portion of the molecule was intact and that metabolic alteration was confined to the imidazolidinone portion of the molecule for these metabolites.

When Metabolites A–D were eluted, oxidized, and measured fluorometrically, their concentrations corresponded to 1.3, 1.4, and 0.7% of the doses, respectively, assuming that these compounds behaved identically to II in the assay procedure. The conjugated metabolite (II-E) appeared to be the major urinary metabolite by UV absorption on the TLC plate but it did not form a fluorescent benzoic acid and, therefore, could not be quantitated. It is conceivable that either the 3-butoxy and/or the 4-methoxy group was dealkylated to yield a phenolic metabolite and that the corresponding benzoic acid derivative formed did not fluoresce under the acidic solvent conditions used. The excretion data in the dog showed that the intact drug was not excreted in the urine in significant amounts. The analysis of feces was hampered by high blank values resulting from interfering materials in the extract. Nevertheless, the analysis of feces did show that fecal excretion of intact II accounted for less than 10% of the dose.

Assay Specificity—The assay for I or II in blood was deemed to be specific for the respective parent compounds by virtue of selective extraction and the absence of interfering metabolites. The analysis of urine for the free or directly extractable and the conjugated fractions required chromatographic separation to effect specificity prior to quantitation.

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