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Spectrofluorometric Determination of Bufuralol in Blood and Urine

J. ARTHUR F. de SILVA x, JOHN C. MEYER, and CARL V. PUGLISI

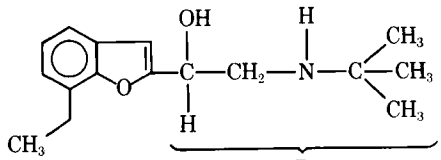
Abstract □ The benzofuran analog bufuralol, a β-adrenergic blocker, was determined in blood and urine by a specific and sensitive spectrofluorometric assay. The compound was extracted into ether from blood or urine adjusted to pH 10. The ether extract was separated by TLC to resolve the parent drug from any basic metabolites present, and the spots were eluted off the silica gel and quantitated fluorometrically in 0.1 *N* HCl. The overall recovery of the assay was 85 ± 3.0%; the sensitivity limit was 2–4 ng/ml of blood or urine, using a 2.5-ml specimen/analysis. The method was applied to the determination of blood levels in a dog following a single 10-mg/kg oral dose and in two human subjects administered a single 20-mg oral dose.

Keyphrases □ Bufuralol—spectrofluorometric analysis in blood and urine □ Spectrofluorometry—analysis, bufuralol in blood and urine □ β-Adrenergic blocking agents—bufuralol, spectrofluorometric analysis in blood and urine

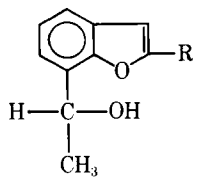
Several phenethanolamines, polycyclic aromatic alkanolamines, and naphthyloxyethanolamines, possessing β-adrenergic blocking activity in humans, have been investigated as therapeutic agents in cardiovascular disorders such as angina pectoris, tachyarrhythmias, and essential hypertension (1–3). All of these compounds contain an *N*-isopropyl side chain similar to that in isoproterenol and propranolol. A spectrofluorometric method for the determination of plasma propranolol levels (4) and a clinical evaluation of plasma propranolol levels and β-adrenergic blockade in humans (5) were reported.

β-Adrenergic activity was noted in a series of benzofuran derivatives, and the pharmacology of 6,7-dimethyl-α-[(isopropylamino)methyl]-2-benzofuranmethanol hydrochloride (I) was reported (6). The clinical evaluation of this compound against propranolol was also reported (7), and a spectrofluorometric assay for I was developed and used in pharmacokinetic studies in normal volunteers (8).

Another member of the benzofuran series, bufuralol [7-ethyl-α-[(*tert*-butylamino)methyl]-2-benzofuranmethanol hydrochloride] (II) (9), is under clinical investigation as a β-adrenergic blocking agent in the treatment of cardiac arrhythmias (10). A spectrofluorometric assay was developed for the determination of II in blood and urine by a modification of a procedure published for I (8). The modified procedure includes a



II



III

Scheme I

TLC separation step to resolve the parent drug (II) from its major metabolite (III) in blood and urine (Scheme I), thereby imparting specificity to the assay. Compounds II and III fluoresce in 0.1 *N* HCl with excitation at 250 nm and emission at 300–305 nm (Fig. 1). The sensitivity limit of the assay is 2 ng of compound/ml of blood, using a 2.5-ml specimen/analysis and specially selected instrumental conditions.

The method was used to determine blood levels of II in a dog given a 10-mg/kg oral dose and in two human subjects following administration of a single 20-mg oral dose.

EXPERIMENTAL

Standard Solutions—Compound II, $C_{16}H_{23}NO_2$·HCl (mol. wt. 297.82, mp 145–146°), of pharmaceutical grade purity (>99%) was used. Weigh out 11.40 mg of the hydrochloride salt equivalent to 10.0 mg of the free base. Transfer it into a 10-ml volumetric flask, dissolve in 5 ml of acetone, and dilute to volume with *n*-hexane. This stock solution (A) contains 1.0 mg of free base/ml. Prepare working solutions containing 0.25, 0.50, 0.75, or 1.0 μg/ml by making serial dilutions of Solution A with acetone–*n*-hexane (50:50).

Similarly, prepare standard solutions of Metabolite III, $C_{16}H_{23}O_3N(COOH)_2$ (mol. wt. 367.39, mp 115°), by weighing out 13.26 mg of the oxalate salt equivalent to 10 mg of the free base and proceeding as described for II. These solutions are used as external

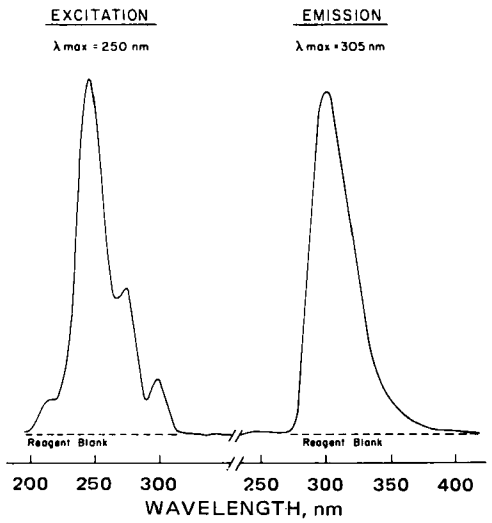


Figure 1—*Excitation–emission spectra of II in 0.1 N HCl.*

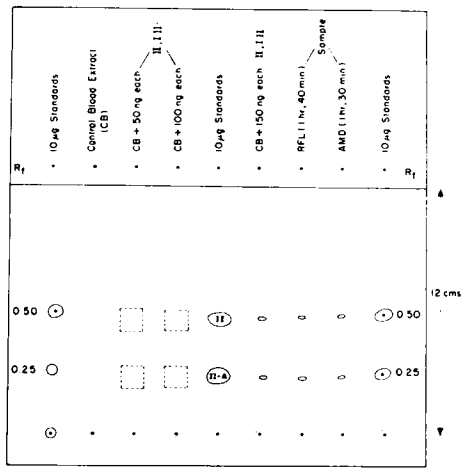


Figure 2—*One-dimensional thin-layer chromatogram of human blood extracts following oral administration of 20 mg of II in a tablet formulation. System 1 = methylene chloride–methanol–concentrated ammonium hydroxide (90:10:1).*

standards for preparing a calibration curve of the compound and are added to blood or urine as internal standards for the determination of percent recovery and of unknowns.

The spectrofluorometer[1] used was equipped with double grating monochromators, a specially selected xenon lamp[2] of high energy output in the UV region, and a selected photomultiplier tube[3] (lumen rating >10). A xenon–mercury vapor lamp[4] may also be used, because of its high UV energy output at 250 nm, but at the expense of higher control (blank) readings. The entrance windows to the excitation and emission monochromators had 5-nm slits in position, whereas the exit windows had 10-nm slits in position. The energy of the lamp was adjusted (focused through the excitation monochromator with the off-axis elipsoidal mirror) for maximum sensitivity, using a Pyrex rod having excitation–emission maxima at 310/360 nm in the sample chamber as the reference standard.

Reagents—All reagents[5] were of analytical reagent grade purity (>99%) and were used without further purification. All inorganic solutions were made up in distilled, deionized water. The inorganic reagents used in the assay were 1.0 *M* (pH 9.0) borate–sodium carbonate–potassium chloride buffer solution, prepared as previously described (11); hydrochloric acid (0.1 *N*), freshly prepared from ultrahigh purity acid[6]; 0.1 and 1.0 *N* NaOH; and a 0.1% solution of bromthymol blue indicator in ethanol–water (1:1). Ether[7] (absolute), of peroxide content <0.00005% and residue after evaporation <0.0005%, from a can opened fresh daily was used as the solvent for extraction.

Glassware Pretreatment—All glassware (volumetric flasks, centrifuge tubes, pipets, *etc.*) used in the assay were acid washed in a cleaning solution[8] (potassium dichromate in concentrated sulfuric acid) by soaking overnight in an acid bath. The glassware was rinsed with distilled water and oven dried. The inner surfaces of the glassware were then deactivated by soaking for 10 min in a freshly prepared 1% aqueous solution of a siliconizing agent[9]. The glassware was then rinsed thoroughly with distilled water and oven dried at 105° for 30 min prior to use.

Glassware so treated can be used five or six times with regular detergent washing in a commercial glassware washing machine before adsorption losses become appreciable and retreatment is necessary.

Assay in Blood or Urine—In a 50-ml glass-stoppered centrifuge tube, add either 2.5 ml of whole blood and 1.3 ml of 0.1 *N* NaOH or 2.5 ml of urine and 1.5 ml of 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0). Along with the unknowns, process a 2.5-ml specimen of control blood or urine and four separate 2.5-ml specimens of control blood or urine to which 25, 50, 75, or 100 ng of II and III are added as internal standards.

Add 10 ml of ether to all specimens, stopper the tubes with stoppers[10], and extract by shaking for 10 min on a reciprocating shaker. Centrifuge at 2000 rpm for 5 min (preferably at 0–4°) and transfer the ether layer to another 50-ml centrifuge tube. Repeat the extraction with another 10-ml portion of ether, combine the ether extracts, and add 5 ml of 0.1 *N* HCl. Shake the tubes for 10 min, centrifuge for 3 min, and remove the ether by aspiration.

Add 1 drop (0.1 ml) of bromthymol blue indicator to the acid extract and titrate to a permanent blue end-point with 1 *N* NaOH added dropwise. Extract this alkaline solution with 2 × 10 ml of ether by shaking and centrifuging as described and combine the ether extracts in a 15-ml centrifuge tube by serial evaporation at 45° in a rotary evaporator[11]. If the samples have to be left overnight, they must be vacuum dried for 15 min and left in a desiccator.

Add 100 μl of ether–methanol (90:10) to dissolve the residues and transfer quantitatively onto a 20 × 20-cm plate[12] of silica gel G of 250-μm bed thickness and 60-μm particle size. Rinse the tubes with 50 μl of ether–methanol (90:10) and combine with the original. Develop the plate for 12 cm ascending in a vapor-saturated chamber containing methylene chloride–methanol–concentrated ammonium hydroxide (90:10:1). Authentic standards of 10 μg of II and III are run alongside the sample extracts as reference standards (Fig. 2).

Scrape silica gel areas (1.5 cm^2) corresponding to II ($R_f \simeq 0.50$) and III ($R_f \simeq 0.25$) off the TLC plate into separate 15-ml centrifuge tubes. Add 1 ml of borate–sodium carbonate–potassium chloride buffer (pH 9.0) to the tubes and slurry the silica gel on a mixer[13] for 30 sec. Extract the slurry with 2 × 4 ml of ether by shaking and centrifuging as before.

Combine the ether extracts in a 15-ml centrifuge tube and extract into 2.5 ml of 0.1 *N* HCl[6]. Remove the ether layer by aspiration and determine the fluorescence of the parent drug (II) at 305 nm in a 1-cm path quartz cell[14], with excitation at 250 nm. Determine the fluorescence of the metabolite (III) at 300 nm with excitation at 250 nm.

Calculations—The fluorescence readings[15] of the unknowns and the internal standards are corrected for the control (blank) reading. The concentration of II in the unknowns is determined by interpolation from a calibration curve of the internal standards processed along with the samples. The concentration of III is determined similarly.

Determination of Percent Recovery—The overall percent recovery of the added internal standards of II and III is determined by

[1] Model Mark I, Farrand Optical Co., Valhalla, N.Y.
[2] Hanovia lamp, Englehardt Industries, Newark, N.J.
[3] RCA 1-P-28 photomultiplier tube.
[4] Hanovia or Osram.
[5] ACS certified reagents.
[6] Ultrex, J. T. Baker and Co.
[7] Mallinckrodt Chemical Co., St. Louis, Mo.
[8] Chromerge, Monostat, New York, N.Y.
[9] Siliclad, Clay Adams Co., New York, N.Y.
[10] Teflon (du Pont).
[11] Buchler Evapomix, Buchler Instruments, Fort Lee, N.J.
[12] E. Merck F_{254}, Brinkmann Instruments, Westbury, N.Y.
[13] Vortex supermixer, Labline Industries, N.Y.
[14] Quarasil, Precision Cells Inc., New York, N.Y.
[15] [*TM*] = transmission (*T*) × meter multiplier factor (*M*).

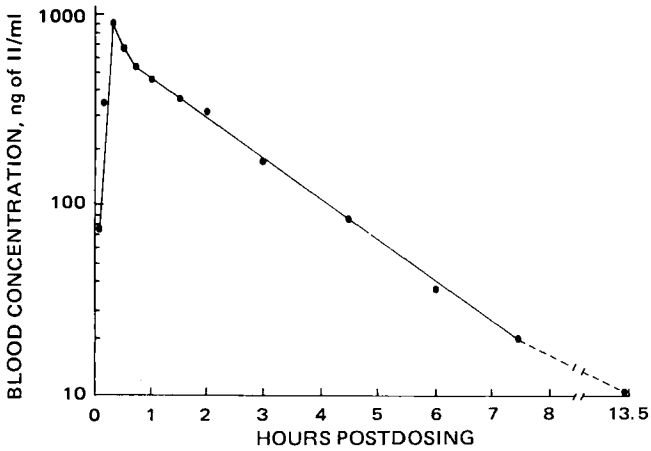


Figure 3—*Blood level fall-off curve of II in a dog following oral administration of a 10-mg/kg dose of the clinically used tablet formulation.*

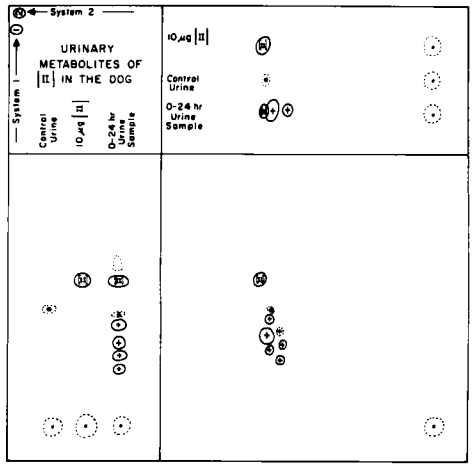


Figure 4—*Two-dimensional thin-layer chromatogram of an extract of dog urine postdosing. System 1 = methylene chloride–methanol–concentrated ammonium hydroxide (90:10:1). System 2 = acetone–methyl ethyl ketone–concentrated ammonium hydroxide (89:10:1). Key: *, longwave UV fluorescence; and +, urinary metabolites of II.*

direct comparison of the slope of the internal standard curve to that of the external standard curve, prepared with 25, 50, 75, or 100 ng of II/2.5 ml of 0.1 *N* HCl[6], prewashed with 4 ml of ether, by shaking for 5 min before the fluorescence is determined. Overall percent recovery should be determined routinely as a check on precision and reproducibility.

RESULTS AND DISCUSSION

The benzofuran analogs I and II exhibited analytically usable UV absorption and fluorescence in acidic media. Both compounds exhibited weak phosphorescence in ether–isopentane–ethanol (5:5:2) at 77° K (liquid nitrogen), with excitation at 255 and emission at 445 nm, with a decay half-life of 2.8 sec. Their potential sensitivity, however, was not sufficient for quantitation in the nanogram range.

Although II and III exhibited weak UV absorbance at 250 nm in 0.1 *N* HCl [$A^{1\%}_{1cm} = 650$], their fluorescence behavior in the same medium was sufficiently intense for their quantitation with nanogram sensitivity. Both compounds exhibited the same excitation maximum at 250 nm. Their emission spectra differed in their maxima by only 5 nm, with II at 305 nm and III at 300 nm. The relative fluorescence intensity of the two compounds measured at their respective excitation–emission maxima was similar.

The linear range of fluorescence covered a sufficiently wide concentration range, from 10 to 3000 ng/ml of 0.1 *N* HCl. The ability to measure less than 10 ng recovered from blood required extensive sample cleanup, which was effected by back-extraction into acid, reextraction at pH 9.0, and a TLC separation step, which also imparted specificity to the assay.

Compounds II and III were quantitatively extracted into ether from blood or urine buffered to pH 10.0 and were also back-extracted quantitatively (>95%) into 0.1 *N* HCl. This fact was used as an effective cleanup step to reduce the amount of endogenous impurities carried through the procedure. Following TLC separation, the area of silica gel corresponding in R_f to that of 10 μg of authentic standards of II and III was carefully scraped off and extracted into ether after slurrying with pH 9.0 buffer. Concentrations less than 100 ng of compound could not be visualized on the TLC plate; however, the areas on the plate of similar R_f were located mainly by comparison to the R_f of the authentic reference standards run alongside the sample extracts.

The overall recovery of internal standards of 25, 50, 75, and 100 ng of II added per milliliter of blood or urine taken through the entire assay was about 86 ± 3.0%, while that of III was about 88 ± 7.4%. The limit of detection was approximately 2–4 ng of each compound/2.5 ml of 0.1 *N* HCl[6] or 2.5 ml of blood or urine extracted. These low sensitivity limits can be realized if certain essential precautions are meticulously observed. These precautions include the pretreatment of all glassware used with a siliconizing agent[9] to minimize adsorption losses and the use of spectrofluorometer[1] equipped with a specially selected high UV energy (250 nm) xenon energy source[2] and a specially selected high lumen output (>10) photomultiplier tube[3].

Since the excitation–emission wavelengths (250/305 nm) used are in the UV spectral range, monochromator gratings of high spectral resolution (<15 nm) in the UV are necessary. The spectrofluorometer[1] used in this study met these specifications. The use of a high purity 1-cm path quartz cell[14] of very low fluorescence background and high UV transmission characteristics (>90% at 200 nm) is also essential. The use of high purity 0.1 *N* HCl made from high purity hydrochloric acid[6], using distilled deionized water as the solvent, for fluorescence measurement further ensured very low background in the determination of II and its metabolite in biological specimens.

Application to Biological Specimens—*Dog Studies*—Blood levels of II were determined in a dog (in a pilot study) following a single 10-mg/kg oral dose of the clinically used 20-mg tablet formulation. Blood specimens were taken at suitable time intervals up to 24 hr (Fig. 3). The data indicate rapid absorption of the drug, with a measurable level seen at 15 min and a peak blood level of 940 ng/ml seen at 30 min postdosing. The blood level declined thereafter to a nonmeasurable amount at 24 hr, with an "apparent" half-life of elimination of about 80 min.

The urinary excretion of II in the 0–24-hr excretion period accounted for only 1.0% of the dose as intact drug in both the unconjugated and conjugated forms. The urine extract was analyzed by two-dimensional TLC (Fig. 4) and showed the presence of one major and three minor metabolites in the extract, indicating extensive biotransformation of the drug. In the blood assay, Solvent System I, consisting of methylene chloride–methanol–concentrated ammonium hydroxide (90:10:1), was capable of resolving the parent drug (II) and four basic metabolites from each other, thereby ensuring the specificity of the fluorometric assay.

The characterization of the major metabolites of II in the dog and in humans using GLC–mass fragmentography will be reported elsewhere[16]. The major basic metabolite in humans and in the dog results from the oxidation of the 7-ethyl group in the molecule, wherein the methylene group is oxidized to a secondary alcohol (Metabolite III). Several phenolic metabolites formed due to hydroxylation of the phenyl ring were also identified as minor urinary metabolites. The *tert*-butylaminomethyl side chain is apparently unaltered.

Human Studies—In a pilot study, blood levels were determined in two healthy normal volunteers to test the clinical utility of the assay. Two subjects (AMD and RFL) were each given a single 20-mg oral dose (tablet), and blood samples were taken at the time intervals shown in Table I. Blood levels of the intact drug (II) and its major metabolite, the alcohol (III), are summarized in Table I and plotted semilogarithmically *versus* time in Fig. 5. In both subjects, the intact drug was measurable up to 9 hr postdosing.

[16] R. J. Francis and R. F. Long, Roche Products Ltd., Welwyn Garden City, Hertfordshire, England, unpublished data on file.

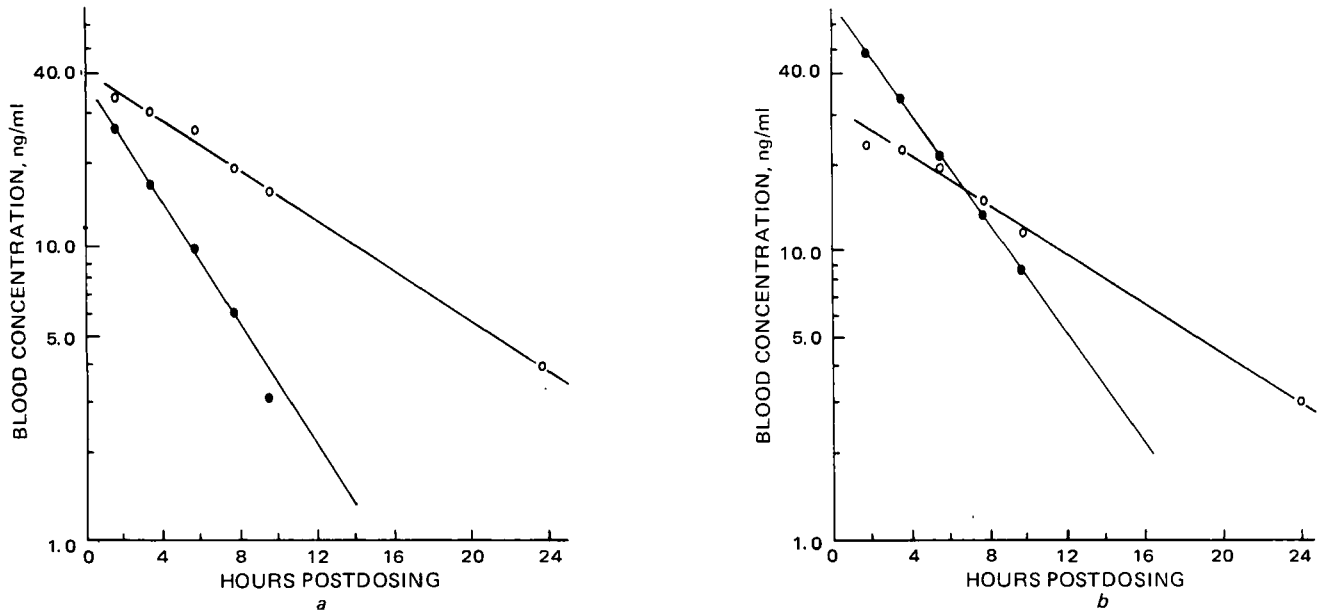


Figure 5—*Blood level fall-off curves of II (●) and its major metabolite, III (○), in humans following a single 20-mg oral dose of the drug. Key:* a, *Patient AMD, weight of 84.1 kg, dose of 20 mg = 0.24 mg/kg; and* b, *Patient RFL, weight of 87.3 kg, dose of 20 mg = 0.23 mg/kg.*

These data were further verified by GLC–mass fragmentography and showed excellent correlation in most cases (Table I), attesting to the accuracy and specificity of the spectrofluorometric assay. The blood levels of II declined monoexponentially, with an apparent half-life of elimination of about 3 hr in both subjects. The levels of III were measurable up to 24 hr postdosing and declined monoexponentially, with an apparent half-life of elimination of about 7 hr in both subjects.

Specificity of Blood Assay—The chromatographic purity and homogeneity of the bands having the same R_f as authentic II and III from the human blood extracts (Fig. 2) were further verified following elution. They were characterized by the similarities of their respective luminescence spectra compared to the authentic standards and also by cochromatography with the respective authentic standards by TLC analysis in a different solvent system, in which no separation of the cochromatographed compounds was evident. Electron-capture GLC analysis of II and III as either the *N*-heptafluorobutyryl or *N*-heptafluorobutyryl-*O*-trimethylsilyl ether derivative was inconclusive due to the formation of mixed products with multiple peaks, especially for the alcohol (III). Nevertheless, electron-capture GLC analysis of these derivatives showed excellent sensitivity and has worthwhile potential as an alternative means of analysis.

These data substantiate the clinical utility of the spectrofluorometric assay for the determination of the parent drug, II, and its major metabolite, III, in blood.

Table I—Blood Levels of II in Humans following a Single 20-mg Oral Dose Determined by a TLC–Spectrofluorometric Assay

Sample	Hours Postdosing	II[a], ng/ml of Blood		Metabolite[b] III, ng/ml of Blood
		Patient AMD[c]		
1	0 hr	—		—
2	1 hr, 30 min	26	(25.9)[d]	34
3	3 hr, 20 min	17	(14.1)	30
4	5 hr, 40 min	10	(10.1)	26
5	7 hr, 40 min	6	(5.4)	19
6	9 hr, 35 min	3	(4.2)	16
7	23 hr, 45 min	N.M.[e]	(1.1)	4
		Patient RFL[f]		
1	0 hr	—		—
2	1 hr, 40 min	48	(48.7)[d]	23
3	3 hr, 30 min	34	(28.3)	22
4	5 hr, 35 min	21	(21.7)	19
5	7 hr, 45 min	13	(14.7)	15
6	9 hr, 40 min	9	(12.1)	12
7	23 hr, 55 min	N.M.	(2.5)	3

[a] Calculated as free base equivalent of hydrochloride salt. [b] Calculated as free base equivalent of hemioxalate salt. [c] Weight of 84.1 kg; dose of 20 mg = 0.24 mg/kg. [d] Determined by GLC–mass fragmentography. [e] N.M. = not measurable, <2 ng/ml of blood. [f] Weight of 87.3 kg; dose of 20 mg = 0.23 mg/kg.

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[x] To whom inquiries should be directed.