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DETERMINATION OF WATER SOLUBLE IMIDAZO-1,4-BENZODIAZEPINES IN BLOOD BY ELECTRON- CAPTURE GAS—LIQUID CHROMATOGRAPHY AND IN URINE BY DIFFERENTIAL PULSE POLAROGRAPHY

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SUMMARY

A sensitive and specific electron-capture gas—liquid chromatographic (GLC—ECD) assay was developed for the determination of 8-chloro-6-(2'-fluorophenyl)-1-methyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine (I) or 8-chloro-1,4-dimethyl-6-(2'-fluorophenyl)-4H-imidazo(1,5*a*)(1,4)benzodiazepine (II) in blood. The assay for both compounds involves extraction into benzene—methylene chloride (9:1) from blood buffered to pH 12.6. The overall recovery of I and II from blood is 86%±5.0 (S.D.) and the sensitivity limit of detection is of the order of 2 to 3 ng of I or II per millilitre of blood.

The major urinary metabolite of I is 8-chloro-6-(2'-fluorophenyl)-1-hydroxymethyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (IA) present as a glucuronide conjugate while 8-chloro-6-(2'-fluorophenyl)-4-hydroxyl-1-methyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (IB) and 8-chloro-6-(2'-fluorophenyl)-4-hydroxy-1-hydroxymethyl-4H-imidazo(1,5*a*)(1,4) benzodiazepine, (IC) are minor metabolites. The major metabolite IA is extracted into benzene—methylene chloride (9:1) from urine buffered to pH 11.0 (after incubation with glucuronidase—sulfatase at pH 5.0), and analyzed by differential pulse polarography (DPP) in 0.1 *M* phosphate buffer (pH 3). The overall recovery of IA is 84 ± 3.0% (S.D.) with a sensitivity limit of 50 ng per millilitre of urine. The metabolites of compound II have not as yet been elucidated. The GLC—ECD and DPP assays were applied to the determination of blood levels and urinary excretion in dogs following single 10 mg/kg intravenous and oral doses of I and following single 6 mg/kg intravenous and 10 mg/kg oral doses of II. Blood levels of compound I were also evaluated in man following intravenous infusion of single 10 mg doses.

INTRODUCTION

The imidazo-1,4-benzodiazepines, 8-chloro-6-(2'-fluorophenyl)-1-methyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (I)·maleate, and 8-chloro-1,4-dimethyl-6-(2'-fluorophenyl)-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (II)·hydrochloride are members of a series of the water soluble benzodiazepine analogs synthesized by Walser et al. [1]. They are of clinical interest as anti-anxiety agents of short duration of activity.

TABLE I

CHEMICAL NAMES AND PHYSICAL PROPERTIES OF THE COMPOUNDS

Compound	Chemical Name	MW	M.P.
I	8-chloro-6-(2'-fluorophenyl)-1-methyl-4H-imidazo [1,5*a*][1,4]benzodiazepine	325.77	152—154
IA	8-chloro-6-(2'-fluorophenyl)-1-hydroxymethyl-4H-imidazo[1,5*a*][1,4]benzodiazepine	341.78	258—260
IB	8-chloro-6-(2'-fluorophenyl)-4-hydroxy-1-methyl-4H-imidazo[1,5*a*][1,4]benzodiazepine	341.77	185—186
IC	8-chloro-6-(2'-fluorophenyl)-4-hydroxy-1-hydroxymethyl-4H-imidazo[1,5*a*][1,4]benzodiazepine	357.78	238—240
II	8-chloro-1,4-dimethyl-6-(2'-fluorophenyl)-4H-imadazo-[1,5*a*][1,4]benzodiazepine	339.80	247—250 (decomp.)
III*	7-chloro-5-(2'-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one	376.29	178—180
IV	5-Aminomethyl-1-[4-chloro-2-(2'-fluorobenzoyl)-phenyl]-2-methylimidazole dihydrochloride	416.74	300—302
V**	5-(1-Aminoethyl)-1-[4-chloro-2-(2'-fluorobenzoyl)-phenyl]-2-methylimidazole	430.77	

*Compound III is the reference standard in the GLC—ECD assay.
**Compound V has not as yet been synthesized.

The chemical names and physical properties of I and its major metabolites and II are given in Table I, and their chemical structures are given in Fig. 1. In vitro studies [2] on the biotransformation of I showed that the compound was mainly metabolized by hydroxylation, producing significant amounts of the 1-hydroxymethyl, (IA) and smaller amounts of the 4-hydroxy (IB) and the 1-hydroxymethyl-4-hydroxy (IC) analogs respectively. The metabolites of compound II have not as yet been elucidated. The parent compounds I and II are amenable to rapid and sensitive electron-capture gas—liquid chromatographic (GLC) analysis from blood as previously demonstrated for other benzodiazepines [3, 4]. The assay employs OV-1 as the liquid phase and a ^{63}Ni electron-capture detector (ECD) used in conjunction with an electron-capture linearizer for the determination of I or II with nanogram sensitivity. The reference standard in the assay is 7-chloro-5-(2'-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one, III.

The urinary excretion of I and its metabolites, IA, IB and IC was determined by GLC—ECD following enzymatic deconjugation and derivatization with bis-trimethylsilyl acetamide (BSA). The major urinary metabolite, IA, which is present in amounts far in excess of the minor metabolites, was also detemined by differential pulse polarography (DPP).

EXPERIMENTAL

GLC—ECD of I or II in blood

Column conditions. The column packing was a pre-tested phase containing 3% OV-1 on 60—80 mesh Gas-chrom Q (Applied Science Labs., State College,

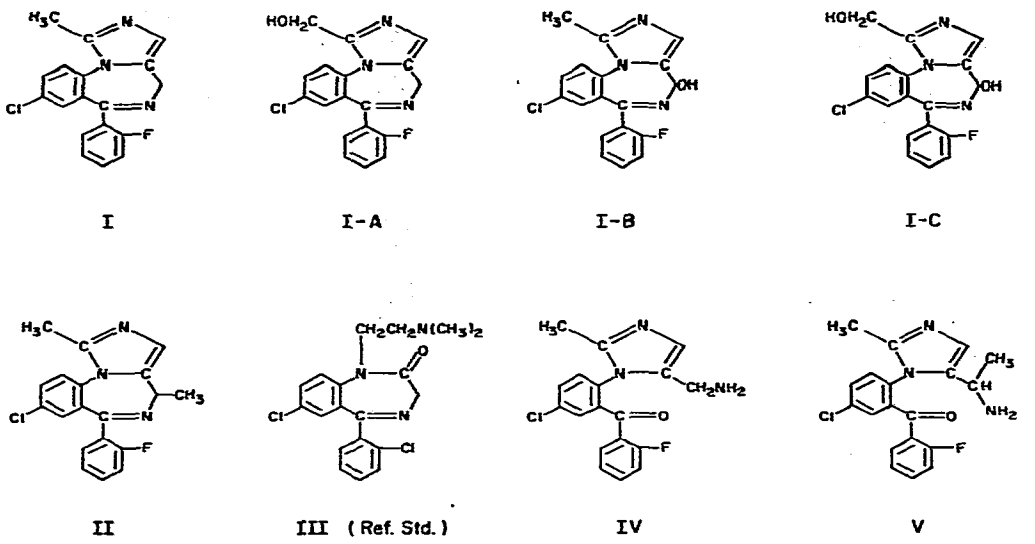

Fig. 1. Chemical structures of compounds referred to in Table I and in text.

Pa., (U.S.A.) packed in a U-shaped, 4 ft × 4 mm I.D. borosilicate glass column. The glass column was treated before packing with a 1% solution of Siliclad (Clay-Adams, New York, N.Y. U.S.A.) for 10 min, thoroughly rinsed with distilled water and dried for at least 1 h at 100°. The packed column was conditioned at 325° under "no flow" conditions for 4 h and then at 265° for at least 18 h with a nitrogen flow-rate of 40 ml/min.

Instrumental parameters. A Tracor Model 222 gas chromatograph, equipped with a ^{63}Ni ECD containing a 15 mCi ^{63}Ni β-ionization source was used. Argon—methane (9:1), (Matheson Gas Products, East Rutherford, N.J. U.S.A.) was used as the carrier gas and the column head pressure was pre-set at 40 p.s.i.g., with a column flow of 65 ml/min and a detector purge of 20 ml/min. The temperature settings were as follows: oven, 225°; injection port, 260°; detector, 325°. The conditions of flow-rate and column temperature must be adjusted to obtain a retention time of 5.5—6 min for I or II. Under these conditions, the reference standard III has a retention time of 9.5—10.5 min. Typical chromatograms for I and III recovered from dog blood and human blood are shown in Figs. 2 and 3, respectively. Chromatograms for II recovered from dog blood are similar to those shown in Fig. 2.

The ECD linearizer Model 114460 standing current was adjusted to $0.5 \cdot 10^{-9}$ A, the relative pulse width was adjusted to 0.15 which corresponds to 0.75 μsec and the attenuation was set at 16. The chart speed was 30 in. per hour and the time constant on the 1.0-mV Honeywell recorder (Model 194) was 1 sec (f.s.d.). Under these conditions 2.0 ng of I, 2.4 ng of II, and 1.0 ng of III give nearly full scale pen response on the 1.0-mV recorder. The minimum detectable amount of I or II is 2—3 ng/ml of blood.

Preparation of standard solutions. Weight out 10.00 mg each of the free base

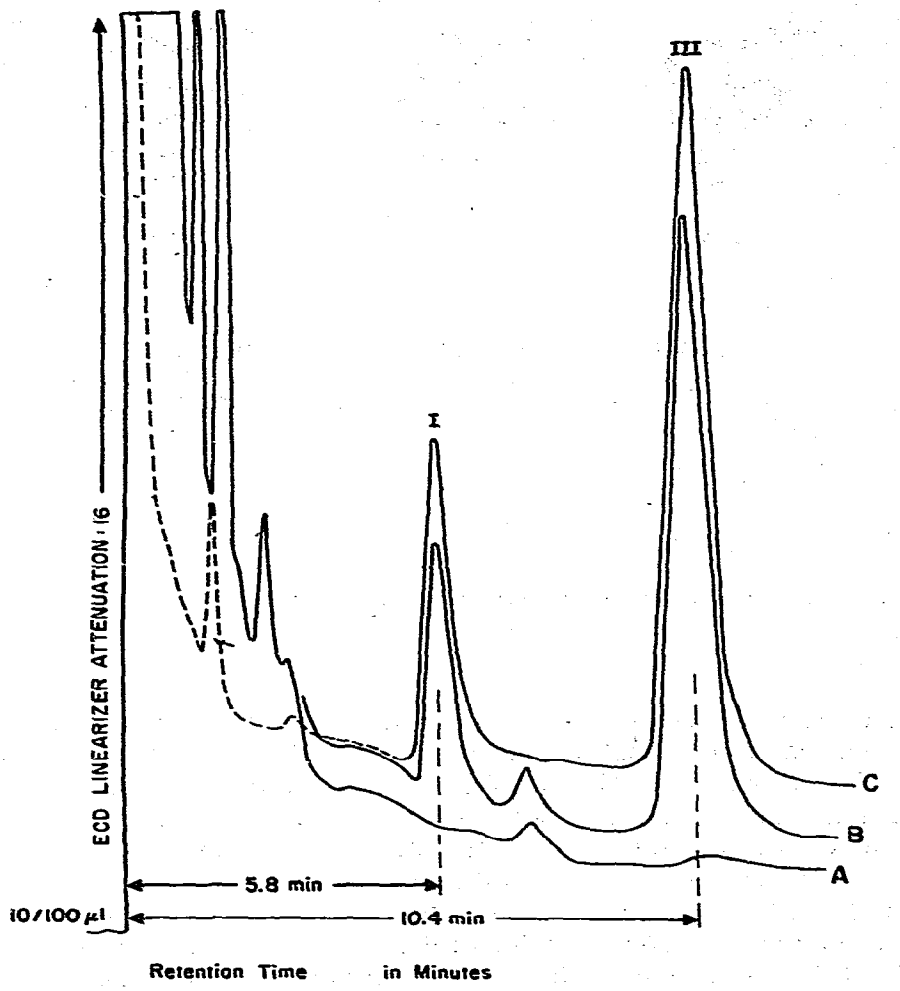

Fig. 2. Chromatograms of (A) control dog blood extract, (B) control blood extract containing added authentic standards, and (C) authentic standards of compounds I and III.

of I or II and the reference standard III into separate 10-ml volumetric flasks. Dissolve in 2.0 ml of acetone and make up to volume with *n*-hexane. These stock solutions contain 1.0 mg/ml and are used to prepare serial 1:10 dilutions in acetone—*n*-hexane (1:4) to yield solutions containing 1 µg/ml. Combine suitable aliquots of these solutions to prepare working standards containing the following concentrations per 100 µl of acetone—*n*-hexane.

(a) Four standard solutions for compound I, containing: 5.0, 10.0, 15.0 or 20.0 ng I, and 10.0 ng of III 100 µl of each solution (Std. 1, 2, 3 and 4, respectively).

(b) Four standard solutions for compound II, containing: 6.0, 12.0, 18.0 and 24.0 ng II and 10.0 ng of III 100 µl of each (Std. A, B, C and D, respectively).

Aliquots (100 µl) of each standard solution of either I or II are added

directly to blood (do not evaporate solvent) as internal standards for recovery determinations and for the construction of an internal standard curve from which the concentration in the unknowns is determined by interpolation.

Aliquots (10 μl) of each of the standard solutions of either I or II are directly analyzed by GLC—ECD to establish the external calibration curve.

Calibration of I, II and III by GLC—ECD. A calibration (external standard) curve of the peak area ratios of I or II to III versus concentration of I or II per 100 μl of acetone—*n*-hexane (1:4) is constructed. The external standard calibration curve is used to establish the parameters for GLC—ECD analysis while the recovered internal standards are used to prepare a calibration curve for the quantitation of the concentration of I or II in biological specimens. The internal standards must be run with each set of unknowns.

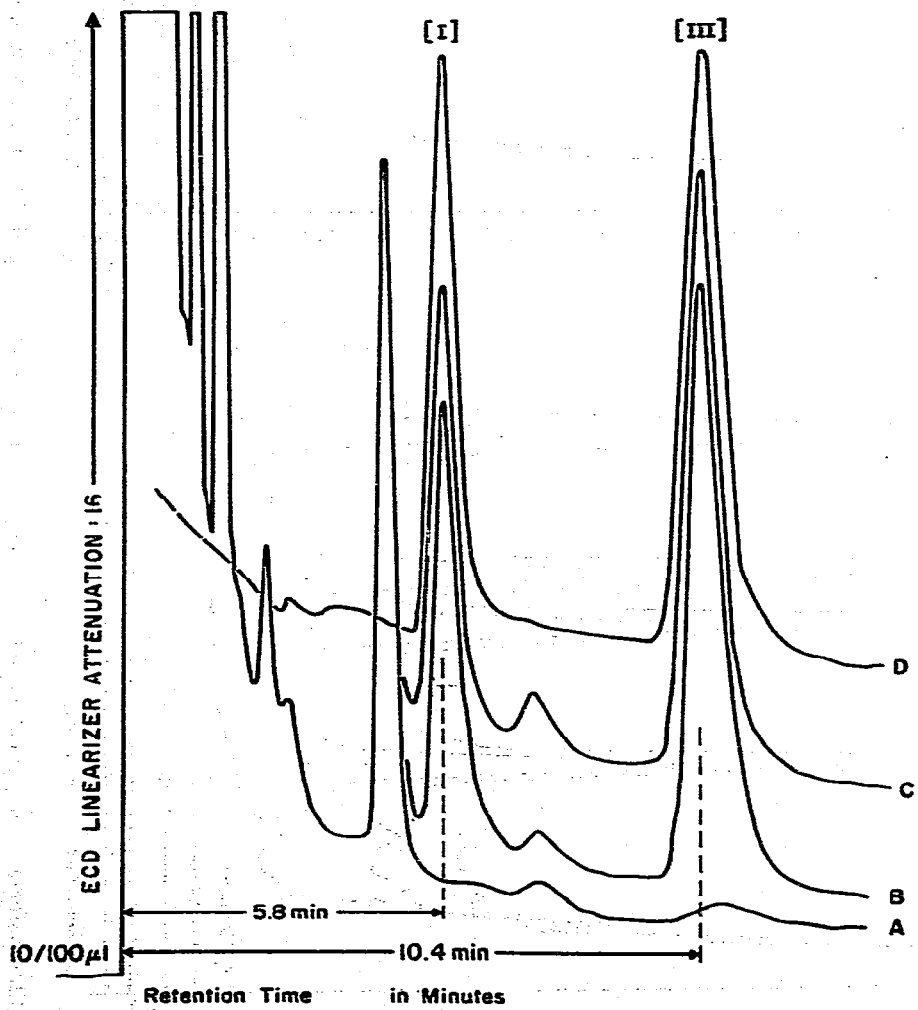

Fig. 3. Chromatograms of (A) control human blood extract, (B) control blood extract containing added authentic standards, (C) subject post-dose blood extract, and (D) authentic standards of compounds I and III.

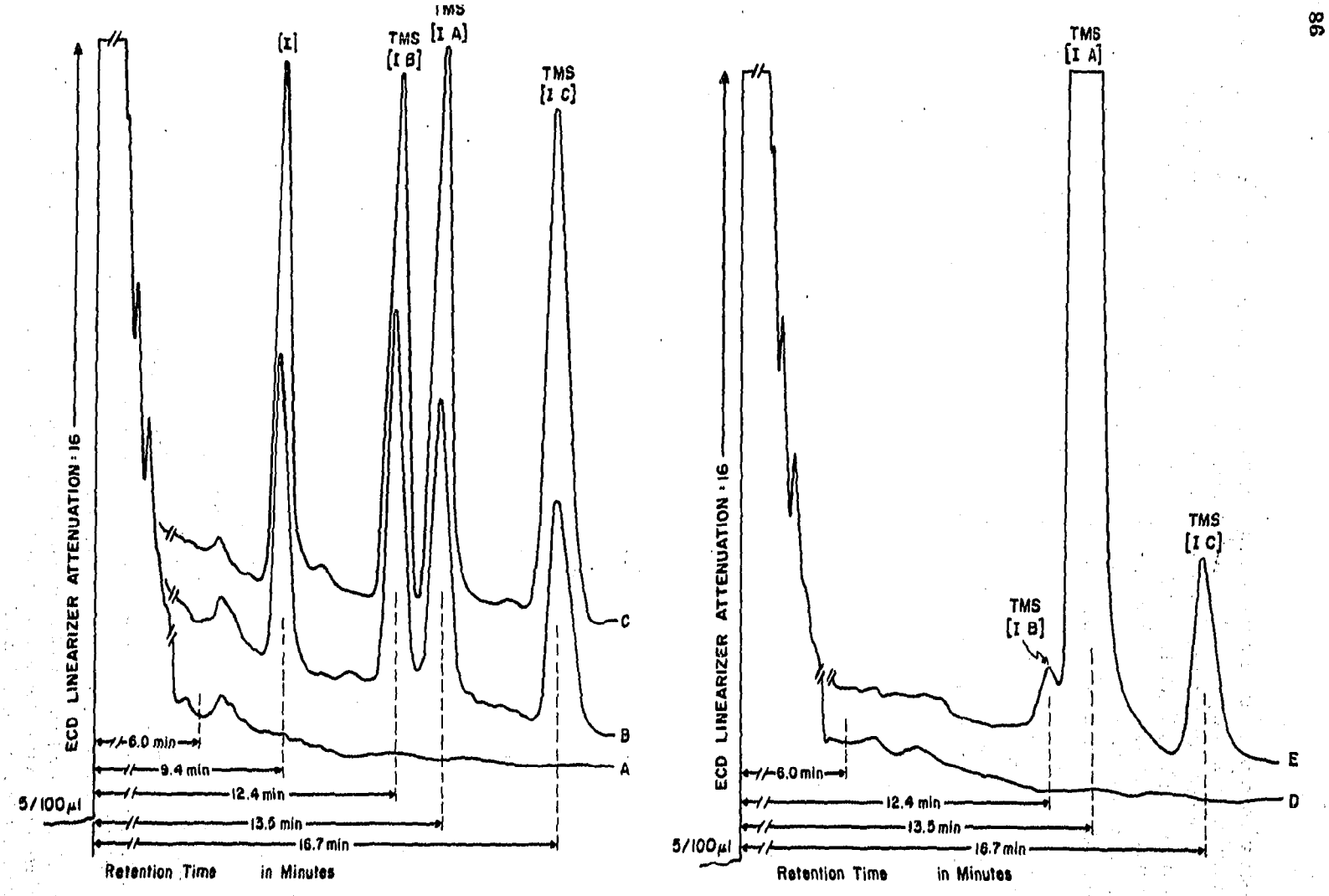

Fig. 4. Chromatograms of (A) patient 0-h urine, (B) patient 0-h urine plus added authentic standards, (C) authentic Standards, (D) directly extractable fraction in 0—12 h urine, and (E) post-glusulase extractable fraction in 0—12 h urine.

Reagents. All reagents were of analytical reagent grade (> 99% purity), and all inorganic reagents were prepared in distilled, deionized water.

Saturated solution of Na_3PO_4, (pH 12.6). Add 200 g of $Na_3PO_4 \cdot 12H_2O$ to 500 ml of distilled water and stir vigorously for 30 min. Allow the crystals to settle to the bottom of the container and remove the supernatant reagent as needed.

The organic reagents used are mixtures of the following: benzene—methylene chloride (9:1), the extraction solvent, benzene—acetone—methanol (17:2:1) and acetone—*n*-hexane (1:4), the solvents for GLC—ECD analysis which are stored over anhydrous sodium sulphate. Benzene and methylene chloride are nanograde (Mallinkrodt, St. Louis, Ma., U.S.A.) and *n*-hexane is 99 mol.% pure, "H-301" from Fisher (Pittsburgh, Pa., U.S.A.).

Extraction of blood for the determination of I or II by GLC—ECD. Into a 15-ml centrifuge tube (PTFE stoppered), add 1.0 ml of whole blood, 2.0 ml of pH 12.6 saturated Na_3PO_4 buffer (mix well), and extract with 8 ml of benzene—methylene chloride by shaking at 80—100 strokes per min for 15 min on a reciprocating shaker (Eberbach Corp., Ann Arbor, Mich., U.S.A.). Along with the samples, run a specimen of control blood (taken preferably from the subject prior to medication) and four specimens of control blood containing either 100 μl of standard solutions 1, 2, 3, or 4 for compound I, or standard solutions A, B, C, or D for compound II added as the respective internal standards. Centrifuge the samples for 10 min at 1500 *g* in a refrigerated centrifuge (Damon/IEC Model PR-J, Needham, Mass., U.S.A. rotor No. 253, 2400 rpm) at 5°, and transfer a 7.5-ml aliquot of the upper organic layer into a clean 15-ml conical centrifuge tube. Evaporate to dryness at 55° in a N-Evap Model N-07 evaporator (Organomation Assoc., Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen. Vacuum dry the residues over Drierite pellets in a vacuum desiccator for 15 min to remove all traces of moisture and dissolve the residues in 100 μl of benzene—acetone—methanol (17:2:1). Inject a 10-μl aliquot for GLC—ECD analysis. The peaks due to I or II and the reference standard III are identified by their respective retention times (Figs. 2 and 3).

Calculations. The concentration of I or II in the unknows represented by their respective peak area ratios is interpolated directly from the blood recovered internal standard curves in which the peak area ratios of either I or II to III are plotted graphically versus total concentration added per millilitre of blood. The absolute recovery of I or II is determined from the ratio of the slope value [peak area/ng] of the internal to that of the external standard curves.

Analysis of urinary metabolites of I

Intact I is not excreted in the urine. The major urinary metabolite of I is the 1-hydroxymethyl analog (IA) (Fig. 1) which is excreted as a glucuronide conjugate. Two other minor metabolites, the 4-hydroxy analog, (IB) and the 1-hydroxymethyl-4-hydroxy analog, (IC) are also excreted as glucuronide conjugates [2]. None of the above compounds are excreted in the free or unconjugated form. All three metabolites are extracted into benzene—meth-

ylene chloride (9:1) from urine buffered to pH 11.0 (after incubation with glucuronidase-sulfatase at pH 5.3), the residue of which is silylated with BSA in acetonitrile and analyzed by GLC—ECD. Since the 1-hydroxymethyl metabolite (IA) is present in concentrations far in excess of the other two minor metabolites, it can be more conveniently analyzed by DPP in 0.1 *M* pH 3.0 phosphate buffer as the supporting electrolyte, using the reduction peak of the azomethine group for quantitation.

GLC—ECD analysis. The parameters used in the assay in blood were used with the following modifications: column, 6 ft. × 4 mm I.D.; borosilicate glass column containing 5% OV-1 on 100—120 mesh Gas-Chrom Q; oven temperature, 250°: column flow, 75 ml/min. The conditions of flow-rate and column temperature must be adjusted to obtain a retention time of 9.4 min for compound I and 12.4, 13.5 and 16.7 min for the TMS derivatives of compounds IA, IB, and IC, respectively. No reference standard is used in this assay.

Standard solutions. The analytical standards required are given in Table I.

Dissolve a weight equivalent to 10.0 mg (free base) of compounds I, IA, IB, and IC separately in 10 ml of methanol to yield stock solutions containing 1 mg/ml. Make serial 1:10 dilutions of these stock solutions to yield working solutions in the following concentrations: Std. 5, containing: 7 ng I, 7 ng IA, 4 ng IB, 5 ng IC; Std. 6, containing: 14 ng I, 14 ng IA, 8 ng IB, 10 ng IC; Std. 7, containing: 21 ng I, 21 ng IA, 12 ng ng IB, 15 ng IC: and Std. 8, containing: 28 ng I, 28 ng IA, 16 ng IB, 20 ng IC per 100 μl of methanol.

Aliquots (100 μl) of each standard solution are added directly to urine as internal standards for recovery determinations and for the construction of an internal standard curve as their TMS derivatives, from which the concentration in the unknowns are determined by interpolation.

Aliquots (100 μl) of each standard solution are also directly silylated and 5 μl aliquots are analyzed by GLC—ECD as the respective TMS derivatives to establish the external calibration curve.

Extraction of urine. Into a 15-ml conical centrifuge tube add 0.2 ml of urine, 0.8 ml of 0.2 *M* (pH 5.3), sodium acetate buffer, and 1% (v/v) (10 μl) of Glusulase enzyme preparation containing 100,000 units of β-glucuronidase and 50,000 units of sulfatase per ml (Endo Labs., Garden City, N.Y., U.S.A.). The tubes are gently agitated to mix the reagents homogeneously, then stoppered loosely and placed in a Dubnoff incubation shaker (Precision Scientific, Chicago, Ill. U.S.A.) at 37° for 2 h to effect enzymatic deconjugation. Along with the unknowns, process a 0.2 ml specimen of control urine and four 0.2 ml specimens of control urine containing 100 μl of standard solutions 5, 6, 7, or 8. Cool the samples to room temperature, add 2 ml of 1.0 *M* (pH 11.0) phosphate buffer, and extract with 8 ml of benzene—methylene chloride (9:1). Centrifuge the samples as in the blood assay and transfer a 7.5-ml aliquot of the supernatant into another 15-ml tube, evaporate to dryness and vacuum dry the residue for 15 min in a vacuum dessicator. Dissolve the dry residue in 100 μl of BSA (Pierce, Rockford, Ill., U.S.A.) in acetonitrile (1:4) to form the TMS derivatives of compounds IA, IB, and IC. Compound I does not derivatize and is determined as its intact moiety. Inject a 5-μl aliquot for GLC—ECD

analysis. The peaks due to I and the TMS derivatives of IA, IB, and IC are identified by their respective retention times (Fig. 4).

Calculations. The concentration of compound I and the TMS derivatives of compounds IA, IB and IC in the unknown is determined by interpolation from the calibration curve of the internal standards processed along with the unknowns, using the direct calibration (peak area versus concentration) technique. The percent recovery of the internal standards is determined by comparing the slope value [peak area (cm^2) per ng of compound] of the internal standards to that of the external standard curve.

DPP analysis

Conditions for polarographic analysis. A Model 174 polarographic analyzer with a Model 172A drop timer (Princeton Applied Research Corp., Princeton, N.J., U.S.A.) were used in the differential pulse mode in conjunction with a three-electrode semi-micro polarographic cell consisting of a dropping mercury electrode (DME), a saturated calomel electrode (SCE) and a platinum wire as the auxiliary electrode as previously described [5]. The pulse amplitude was —50 mV, the drop time was 2.0 sec, and the drop-rate was 2.42 mg/sec. ($m^{2/3} \cdot t^{1/6} = 1.803$). The current range was between 0.5 and 5.0 μA for a peak response of full scale deflection, the scan range was 1.5 V, and the scan-rate was 1 mV/sec. The samples were scanned between —0.450 V and —0.750 V versus SCE, and the polarograms were recorded on a Houston Omnigraph Model 2200-3-3 (X—Y) recorder (Houston Instruments, Bellaire, Texas, U.S.A.). The analytical peak due to the reduction of the azomethine group ($>C_5 = N_4^-$) of IA occurs at —0.640 V versus SCE in a supporting electrolyte consisting of 0.1 *M* (pH 3) phosphate buffer containing 0.005% methoxypolyethyleneglycol-550 as the maximum suppressor. Typical polarograms for the analysis of IA in urine are shown in Fig. 5.

Sample preparation. Into a 50 ml centrifuge tube add 2.0 ml of urine and 3 ml of 0.2 *M* (pH 5.3) sodium acetate buffer. Adjust to pH 5.0 with glacial acetic acid and mix well on a Vortex super mixer. Add 1% by volume of Glusulase enzyme preparation and shake gently to produce a homogeneous mixture. Along with the samples process a 2.0 ml specimen of control urine and separate 2.0 ml specimens of control urine containing 0.4, 0.8, 1.2 or 1.6 μg of IA as the internal standards. Stopper the tubes and place in a Dubnoff metabolic shaking incubator at 37° for 2 h. Cool the samples to room temperature and adjust the pH to 11.0 (using a pH meter) by titrating the samples dropwise with 2.5 *M* NaOH and adding 1 ml of 1 *M* (pH 11) phosphate buffer. Extract the samples with 16 ml of benzene—methylene chloride (9:1) by shaking for 10 min at a moderate speed on a reciprocating shaker. Centrifuge the samples in a refrigerated centrifuge at 5° for 5 min at 1500 *g*, and transfer a 14-ml aliquot of the upper organic layer into a 15-ml conical centrifuge tube. Evaporate to dryness at 55° under a stream of clean, dry nitrogen. Dissolve the residues in 100 μl of methanol and add 2 ml of 0.1 *M* (pH 3) phosphate buffer containing 0.005% methoxypolyethyleneglycol-550 as the maximum suppressor. Mix well in a Vortex action mixer and deoxygenate the samples for 1 to 2 min with nitrogen bubbled through a microporosity filter stick (No. JD-5385-01) coarse porosity (SGA Scientific Inc., Bloomfield, N.J.,

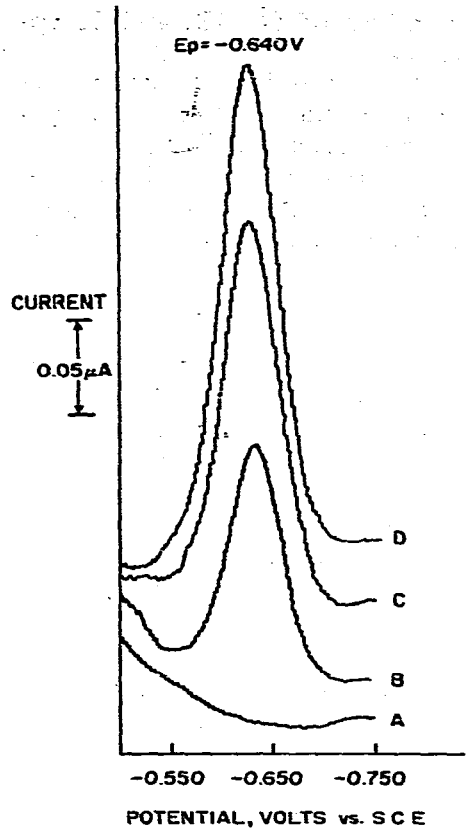

Fig. 5. DPP of de-conjugated compound IA. (A) Control Urine, (B) authentic standard recovered from urine, (C) authentic standard, (D) 0—12 h post-dose urine.

U.S.A.). Transfer the deoxygenated sample into the polarographic cell containing the three operational electrodes and analyze the samples for IA by scanning between —0.450 and —0.750 V versus SCE (Fig. 5).

Calculations. The current (μA) resulting from IA and its overall recovery is determined as described previously [5]. The concentration of IA in the unknowns is determined by interpolation from its respective internal standard curves, making the necessary corrections for the aliquots taken.

RESULTS AND DISCUSSION

A sensitive and specific GLC—ECD assay was developed for the determination of compounds I or II from 1 ml or less of blood. Compound I undergoes biotransformation in the dog and in man yielding three urinary metabolites.

The parent drug I is equally well extracted from blood at pH 9, 11, or 12.6, into benzene—methylene chloride (9:1). Extraction at pH 12.6 yielded chromatograms devoid of interfering peaks in the retention areas of interest and was therefore chosen as the pH of choice for extraction from blood. The urinary

metabolites IA, IB, and IC are quantitatively extracted at pH 11. The metabolites of compound II have not as yet been characterized.

The GLC—ECD behaviour of compounds I or II manifested adsorption losses upon chromatographic analysis. These losses were corrected by deactivating the glass column with a 1% solution of Siliclad in water. Compound III was chosen as the reference standard for the blood-assay due to its similar GLC—ECD and extraction behaviour to compounds I and II. Under the above operational conditions, the life span of the column is usually 5—6 months of continuous use.

Recovery and sensitivity limits of the GLC—ECD assay

The overall recovery of I and II from blood is 86 ± 5.0% (S.D.), and the sensitivity limit is of the order of 2 to 3 ng of I or II per millilitre of blood. The direct extraction procedure is limited to 1 ml or less of blood.

The overall recovery of compound I and the TMS derivatives of IA, IB and IC from urine is 92 ± 5.3% (S.D.) with sensitivity limits of 10—15 ng of each compound per millilitre of urine analyzed.

DPP analysis

The analysis of 1,4-benzodiazepines by DPP is well documented [5, 6, 7]. Compounds I and II exhibited two polarographic peaks in 0.1 *M* sulphuric acid but only one peak in buffers ranging from pH 3 to pH 4. This phenomenon is due to the hydrolysis of the [$>C_5 = N_4^-$] azomethine bond to yield "open ketones", compounds IV and V (Fig. 1). DPP was used to determine the rate of hydrolysis of compounds I and II in acidic solutions, its reversibility and stability in solutions of varying pH.

It was found that an equilibrium exists between the parent compounds I and II and their respective open ketones, IV and V, that this equilibrium is strongly pH dependent and that from pH 3.0 through pH 11.0 (pH 11 is used for extraction) only the intact compounds are present. Similar phenomena of pH dependence on chemical stability were reported for flurazepam [6].

The overall recovery of metabolite IA from urine determined by DPP is 84.3 ± 3% (S.D.). The sensitivity limit is 50 ng of IA per millilitre of urine using a 2 ml specimen per analysis.

Application of the GLC—ECD and DPP assays in biological specimens

Studies in the dog. Blood level profiles of compound I were determined in a dog following the intravenous and oral administration of a 10 mg/kg dose of compound I, whereas those of compound II were determined in a dog following a 6 mg/kg intravenous dose, and a 10 mg/kg oral dose, respectively. The blood levels of compounds I and II following the intravenous administration were measurable through 12 and 30 h, respectively. After a 10 mg/kg oral dose, peak levels of 0.99 μg/ml were observed at 30 min for compound I whereas peak levels of 2.87 μg/ml were observed at 1.5 h for compound II, see Figs. 6 and 7, respectively.

The urinary excretion of compound I and IA, IB and IC was determined by DPP following the 10 mg/kg intravenous and oral doses of I. Direct extraction

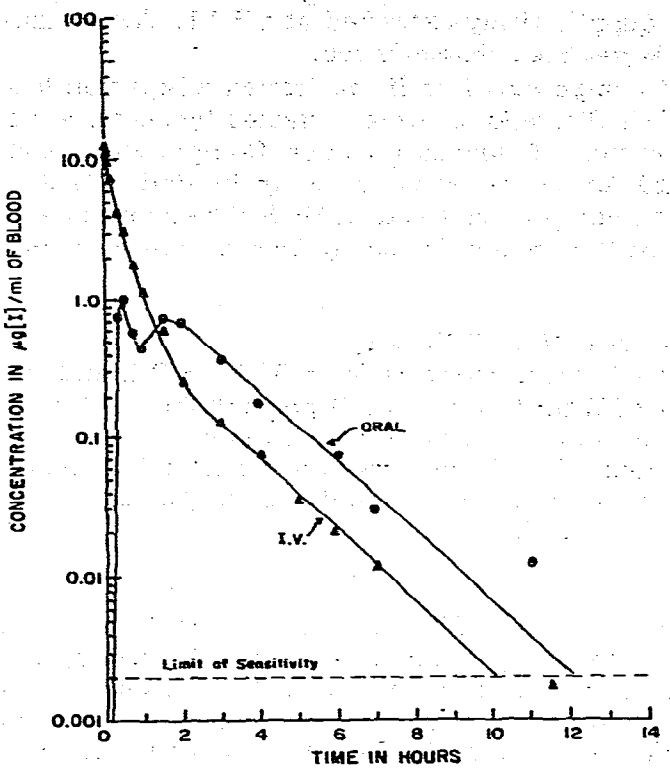

Fig. 6. Blood level fall-off curves of I in the dog following a 10 mg/kg dose of the maleate salt of I by intravenous and oral routes.

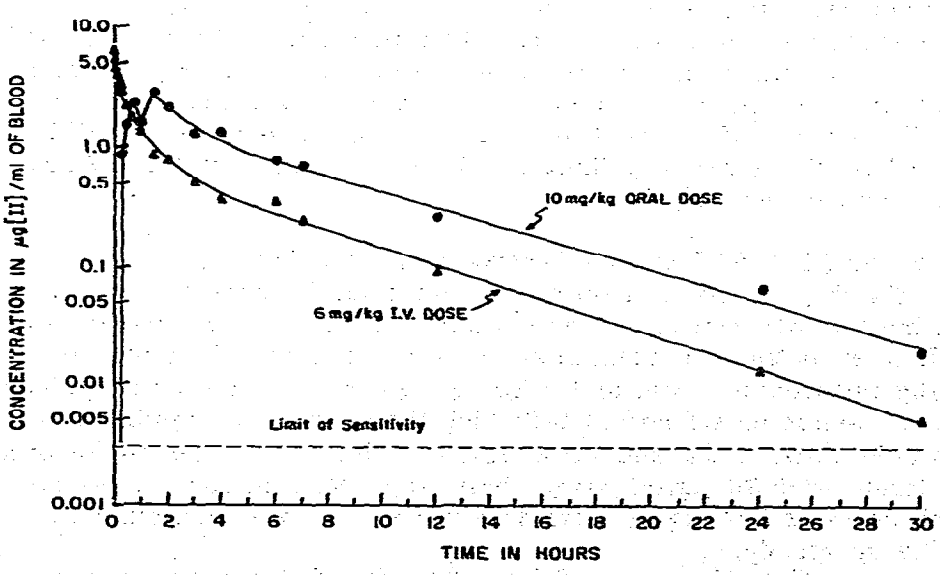

Fig. 7. Blood level fall-off curves of II following intravenous and oral administration of II·HCl in a dog.

at pH 11.0 showed that less than 0.3% of the administered dose was excreted as I, IA, IB, and IC in the unconjugated form.

The major urinary metabolite of I, IA, is present as a glucuronide or sulfate conjugate and accounts for 4.82% and 5.49% of the 10 mg/kg intravenous and oral doses respectively (Table II). The metabolites of compound II have not as yet been characterized.

Studies in man. A pilot study was conducted in which five healthy volunteers were administered a single 10 mg intravenous infusion of I as the maleate salt at a rate of 10 mg/min for one minute (total dose 10 mg free base). Blood samples were collected from the five subjects and pooled urine specimens were collected from subjects 3, 4, and 5.

TABLE II

URINARY EXCRETION OF IA IN THE DOG FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION OF A 10 MG/KG DOSE OF I-MALEATE

Determined by DPP after enzymatic deconjugation. n.m. = not measureable (<50 ng/ml of urine).

Excretion period (h)	% of dose excreted as IA	
	Intravenous	Oral (Capsule)
0—24	3.2	4.76
24—48	1.36	0.73
48—72	0.26	n.m.
Total	4.82	5.49

(A) Blood level profile. The blood concentration data for subjects 1—5 are plotted semilogarithmically versus time in Fig. 8.

The blood level profile indicates an initial rapid disposition of I, with blood levels declining 5 to 10 fold within the first hour following administration of the dose. The drug is also rapidly eliminated from the body, with "apparent" half-lives ranging from 1.3 to 2.2 h in the five subjects studied.

(B) Urinary excretion profile. Urine specimens from subjects 3, 4, and 5 were analyzed for intact I, IA, IB and IC in the directly extractable unconjugated form using the GLC—ECD assay. No measurable levels of either the intact drug or any of the above metabolites were seen in these subjects (Fig. 4D). The levels (if any) were below the sensitivity limit of the GLC—ECD assay (less than 10 to 15 ng/ml of urine). The samples were re-analyzed by GLC—ECD following glucuronidase-sulfatase incubation.

Trace amounts of IB and approximately 1% of the dose as IC were seen in the conjugated fraction. The major metabolite was IA (Fig. 4E) which accounted for approximately 50% of the administered dose, of which up to 96% was excreted during the first twelve hours. Since this compound was excreted in amounts far in excess of the other two minor metabolites, the urinary excretion profile of IA was determined by DPP and the data summarized in Table

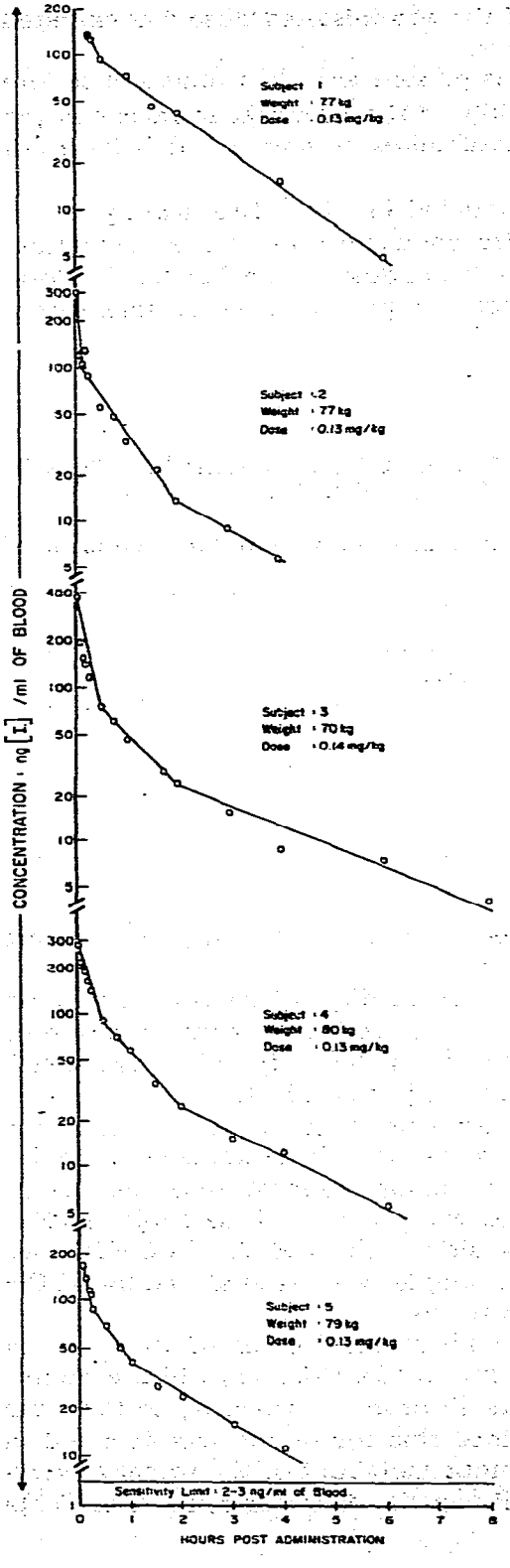

Fig. 8. Blood level fall-off curves in five subjects following the intravenous infusion of a single 10 mg dose of I-maleate.

III. Extensive elimination occurs in the 0—12 h period, paralleling the blood level fall off profile, with substantially smaller amounts being eliminated in the 12—24 h period and thereafter. The overall recovery of the administered dose in the 0—72 h period as this metabolite ranged from 45 to 57% of the dose.

The urine levels of IA obtained by GLC—ECD were statistically evaluated against those obtained by DPP using linear regression analysis. The resulting least square line ($r = 0.99$) indicated that a slope of 1 and a intercept of 0 fell within the 95% confidence limits (Table IV) showing that the two assays were equivalent.

TABLE III

URINARY EXCRETION OF IA IN MAN FOLLOWING A 10-MG INTRAVENOUS INFUSION OF I-MALEATE

Determined by DPP after enzymatic deconjugation. n.m. = not measureable (<50 ng/ml of urine).

Excretion period (h)	% Dose excreted		
	Subject 3	Subject 4	Subject 5
0—12	41.0	43.2	27.1
12—24	11.4	1.7	14.0
24—48	3.8	n.m.	3.8
48—72	1.0	n.m.	n.m.
Total	57.2	44.9	44.9

TABLE IV

CORRELATION OF GLC—ECD AND DPP IN THE ANALYSIS OF IA IN URINE

Correlation coefficient $r = 0.99$

Subject	Excretion period (h)	Concentrations μg/ml or urine	
		GLC—ECD	DPP
3	0—12	1.20	1.20
3	12—24	0.98	0.95
4	0—12	1.37	1.44
4	12—24	0.15	0.14
5	0—12	1.35	1.23
5	12—24	1.17	1.17

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REFERENCES

1 A. Walser and R.I. Fryer, U.S. Patent Application 504924, May 6, 1975, and Belgium Patent 839365, March 9, 1976 to Hoffmann-La Roche, Basle, Switzerland.
2 G. Woo, S. Kolis and M.A. Schwartz, Pharmacologist, 19 (1977) 164.
3 J.A.F. de Silva and C.V. Puglisi, Anal. Chem., 42 (1970) 1725.
4 J.A.F. de Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfeld, Anal. Chem., 48 (1976) 10.
5 M.R. Hackman, M.A. Brooks, J.A.F. de Silva and T.S. Ma, Anal. Chem., 46 (1974) 1075.
6 J.A.F. de Silva, C.V. Puglisi, M.A. Brooks, and M.R. Hackman, J. Chromatogr., 99 (1974) 461.
7 M.A. Brooks and J.A.F. de Silva, Talanta, 22 (1975) 849.