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DETERMINATION OF THE ANXIOLYTIC AGENT 8-CHLORO-6-(2-CHLOROPHENYL)-4H-IMIDAZO-[1,5-*a*] [1,4]-BENZODIAZEPINE-3-CARBOXAMIDE IN WHOLE BLOOD, PLASMA OR URINE 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 8-chloro-6-(2-chlorophenyl)-4H-imidazo-[1,5-*a*]-[1,4]-benzodiazepine-3-carboxamide [I] and its 4-hydroxy metabolite, 8-chloro-6-(2-chlorophenyl)-4-hydroxy-4H-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-carboxamide [II] in whole blood, plasma or urine. The assay for both compounds involves extraction into diethyl ether—methylene chloride (70:30) from blood, plasma, or urine buffered to pH 9.0. The overall recoveries of [I] and [II] are 92.0 ± 5.4% (S.D.) and 90.3 ± 4.9% (S.D.), respectively. The sensitivity limit of detection is 50 ng/ml of blood, plasma, or urine using a UV detector at 254 nm. The HPLC assay was used to monitor the blood concentration—time fall-off profiles, and urinary excretion profiles in the dog following single 1 mg/kg intravenous and 5 mg/kg oral doses, and following multiple oral doses of 100 mg/kg/day of compound [I].

INTRODUCTION

The compound 8-chloro-6-(2-chlorophenyl)-4H-imidazo-[1,5-*a*] [1,4]-benzodiazepine-3-carboxamide, [I] (Fig. 1), synthesized by Walser and co-workers [1,2], is a member of the imidazo-1,4-benzodiazepine class of compounds undergoing evaluation as anxiolytic agents [3]. It is structurally analogous to midazolam; a water soluble imidazo-1,4-benzodiazepine in clinical evaluation as a preoperative anesthesia inducing agent [4,5].

Studies on the biotransformation of ^{14}C-labeled [I] by the dog [6], indicated that the compound was metabolized by hydroxylation producing the 4-hydroxy compound, 8-chloro-6-(2-chlorophenyl)-4-hydroxy-4H-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-carboxamide, [II], as the major plasma metabolite

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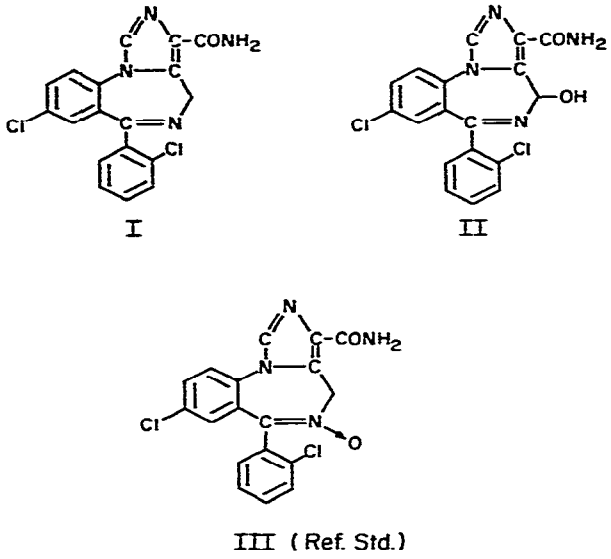

Fig. 1. Chemical structures of compounds [I], [II], and [III].

(Fig. 1). Electron-capture gas—liquid chromatography (EC—GLC) used in the determination of midazolam [7] was not applicable to the analysis of [I] at low concentration due to poor precision and reproducibility. The presence of the carboxamide group in the molecule adversely influences the chromatographic behavior of the compound by EC—GLC.

High-performance liquid chromatography (HPLC) was investigated and resulted in the development of a rapid, sensitive and specific assay for the determination of compounds [I] and [II] in blood, plasma or urine. The method presented herein determines compounds [I] and [II] by normal-phase HPLC using their ultraviolet (UV) absorbance at 254 nm for quantitation. The analogous compound, 8-chloro-6-(2-chlorophenyl)-4H-imidazo-[1,5-*a*] [1,4]-benzodiazepine-3-carboxamide-5-oxide, [III] (Fig. 1) is used as the internal standard. The HPLC assay was used to monitor the blood concentration—time fall-off profiles, and urinary excretion profiles in the dog following single 1 mg/kg intravenous and 5 mg/kg oral doses, and following multiple oral doses of 100 mg/kg/day of compound [I].

EXPERIMENTAL

HPLC analysis of compounds [I] and [II] in blood or plasma

Column. The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing 10-μm Partisil silica gel, generating 32,200 plates/m (Whatman, Clifton, NJ, U.S.A.).

Instrumental parameters. A Waters Model ALC/GPC-204 high-performance liquid chromatograph equipped with a Model 440 absorbance detector, operated at 254 nm, a Model M6000A solvent delivery system and a U6K injector was used. The isocratic mobile phase used was methylene chloride—methanol—ammonium hydroxide (96:3.85:0.15) at a pressure of 6.2 MPa and a constant

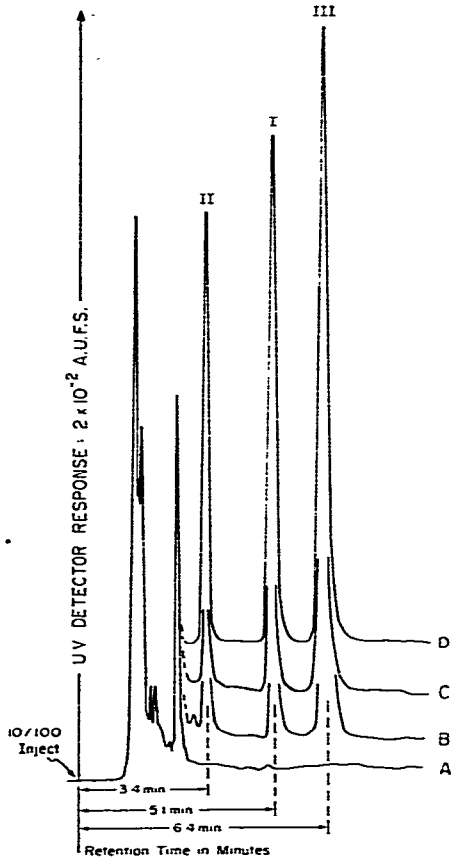

Fig. 2. Chromatograms of HPLC analysis of diethyl ether—methylene chloride extracts of (A) control dog blood, (B) dog blood following chronic oral dosing of a 100 mg/kg/day, (C) authentic standards recovered from control dog blood, and (D) authentic standards added to the residue of control dog blood.

flow-rate of 2.1 ml/min. Under these conditions, the retention times of compounds [I], [II] and [III] were 5.1, 3.4 and 6.4 min, respectively, with capacity factors (k') of 2.10, 1.05 and 2.85 for compounds [I], [II], and [III], respectively (Fig. 2). The UV detector sensitivity was $2 \cdot 10^{-2}$ a.u.f.s. and the chart speed on the 10-mV Hewlett-Packard recorder (Model No. 7132A) was 1.25 cm/min. Under these conditions 100 ng of [I], 60 ng of [II] and 140 ng of [III] per 10 μl injected gave nearly full scale pen response. The minimum detectable amount of [I] or [II] is 50 ng/ml of blood, plasma or urine.

Analytical standards. Compound [I] ($C_{18}H_{12}Cl_2N_4O$, M.W. = 371.23, m.p. = 300°C), compound [II] ($C_{18}H_{12}Cl_2N_4O_2$, M.W. = 387.24, m.p. = 304—307°C), and compound [III] ($C_{18}H_{12}Cl_2N_4O_2$, M.W. = 387.22, m.p. = 286—288°C) of pharmaceutical grade purity (> 99%) are used as analytical standards.

Prepare stock solutions of compounds [I], [II] and [III] in separate 10-ml volumetric flasks by dissolving 10 mg of each compound in 1 ml of methanol followed by 1 ml of mobile phase (see Reagents). Sonicate if necessary for 15—30 min for complete solubilization and dilute to volume with mobile phase.

These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in the mobile phase to contain the following amounts as indicated below:

Standard solution	Concentration (ng per 100 μl)		
	[I]	[II]	[III] (internal standard)
A	50	50	1400
B	100	100	1400
C	400	200	1400
D	800	400	1400
E	1000	600	1400
F	—	—	1400

Aliquots (10 μl) of solutions A—E are injected to establish the HPLC parameters using the UV detector at 254 nm. Aliquots (100 μl) of the same solutions are evaporated to dryness, the residue dissolved in 0.1 ml of methanol and reconstituted in 1 ml of control blood, plasma or urine as the processed standard calibration curve for the determination of the concentration in the unknowns. The determination of percent recovery requires that the processed standards be compared to authentic standards (100 μl of standards A—E) which have been added to the residue of extracted control blood, plasma or urine, to constitute the external standard curve. This is necessary due to a chromatographic enhancement effect which compounds [I], [II] and [III] exhibit; i.e. the peak height response is 10—20% greater when the compounds are chromatographed in the residue of biological extracts, than when chromatographed as pure standards.

A separate stock solution of the internal standard [III] containing 1 mg/ml of methanol is diluted in methanol to yield a working solution F containing 14 μg/ml, 100 μl of which are added only to the unknown blood or plasma samples. It is not added to the unknown urine samples due to an interfering peak whose retention time is close to that of compound [III]. Therefore, calculations in urine are carried out using the direct calibration technique which is discussed under Calculations.

Calibration of compounds [I], [II] and [III] by HPLC. Calibration (external standard) curves of the peak height ratio of [I] to [III], and [II] to [III] vs. concentration of [I] and [II] per 100 μl of mobile phase are constructed. Fresh calibration curves of the external standards and of the processed recovered standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Reagents. All reagents must be of analytical reagent grade (> 99% purity). Potassium phosphate buffer, 1.0 *M*, pH 9.0, is prepared by dissolving 174.18 g of K_2HPO_4 in 1 l of distilled deionized water and titrating to pH 9.0 with 1.0 *N* hydrochloric acid. Mix well and check final pH with a pH meter. Diethyl ether—methylene chloride (70:30) is the extraction solvent and a mixture of methylene chloride—methanol—ammonium hydroxide (96:3.85:0.15) is used as both the mobile phase for HPLC analysis and the solvent for preparing

calibration standard solutions of compounds [I], [II] and [III]. Diethyl ether (absolute), opened for no more than five days was purchased from Mallinckrodt (St. Louis, MO, U.S.A.); methylene chloride and methanol from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); hydrochloric acid and ammonium hydroxide from J.T. Baker (Phillipsburg, NJ, U.S.A.). A 1% aqueous solution of Prosil-28 (P.C.R. Research Chemicals, Gainesville, FL, U.S.A.) is used for siliconizing all the glassware used in the assay.

Analysis of blood or plasma

The flow diagram of the extraction procedure is shown in Fig. 3.

Into a 15-ml siliconized conical centrifuge tube (PTFE No. 13 stoppered), add 1400 ng of compound [III] (100 μl of solution F) as the internal standard, 0.2 ml of distilled deionized water, 1.0 ml oxalated whole blood or plasma, 2.5 ml of 1 *M* phosphate buffer, pH 9.0 (mix well), and extract with 8 ml of diethyl ether—methylene chloride (70:30) by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes/min. Along with the samples, run six 1.0-ml specimens of control blood or plasma (one used as a control blank and five to be used for the preparation of external standards) and five 1.0-ml specimens of control blood or plasma containing 0.1 ml of standard solutions A—E (equivalent to 50, 100, 400, 800, and 1000 ng of [I], 50, 100, 200, 400 and 600 ng of [II], and 1400 ng of [III] (internal standard) per 1.0 ml of blood or plasma). Centrifuge the samples at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min and transfer a 7.0-ml aliquot of the upper organic layer into another siliconized 15-ml conical centrifuge tube. Evaporate the organic layer to dryness at 45°C in a N-EVAP evapo-

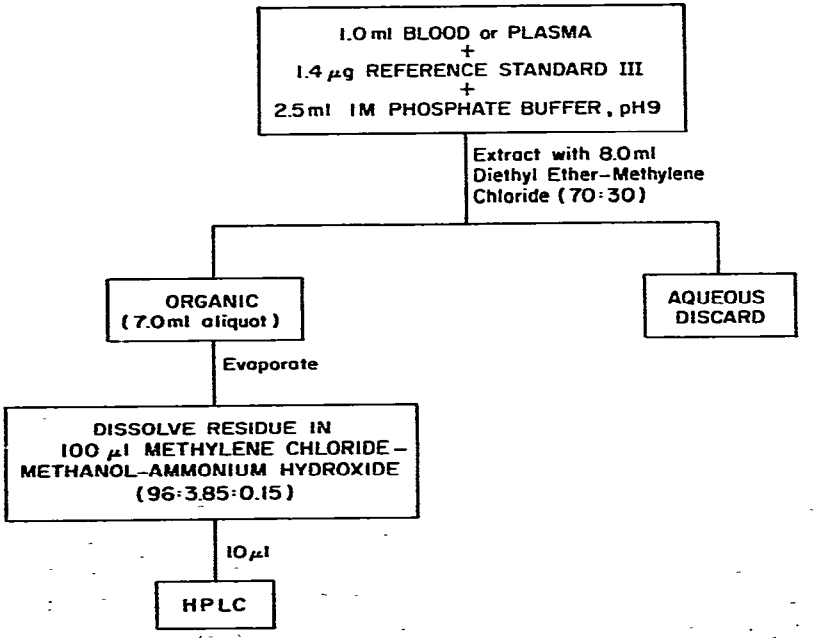

Fig. 3. Flow diagram of the extraction procedure for compounds [I], [II], and [III] from blood or plasma.

rator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residue in 100 μl of mobile phase [methylene chloride—methanol—ammonium hydroxide (96:3.85:0.15)] and inject a 10-μl aliquot into the liquid chromatograph. Typical chromatograms of blood extracts are shown in Fig. 2. The analysis of urine is identical to that for blood or plasma except that the addition of the internal standard [III] to the unknowns is omitted.

Calculations

The peak height ratio of [I] or [II] to [III] of the respective processed standards recovered from blood or plasma is determined and plotted graphically vs. concentration (ng/ml of specimen) to establish the calibration curve. Similarly, the peak height ratio of [I] or [II] to [III] in the aliquots of the respective blood or plasma unknowns injected is also determined. The concentrations of [I] and [II] in the unknowns represented by their peak height ratios are interpolated directly from their respective standard curves, because the recovery factors, and therefore the peak height ratio of [I] or [II] to [III] remains constant, irrespective of the sample aliquot injected. Thus, concentration (ng) in the unknowns interpolated from the processed standard curve = ng [I] or [II] per 1.0 ml of blood or plasma.

If, however, the peak due to the internal standard [III] is either diluted out, due to high concentration ($>$ 10 μg) of [I] or [II] in blood or plasma, or in urine where it is omitted, then the direct calibration technique must be used whereby a calibration curve of peak height of the recovered standards of [I] or [II] vs. concentration (ng/ml of specimen) is plotted and used for the quantitation of the unknowns. The amount of [I] or [II] per aliquot of the unknown sample injected has to be corrected for the total volume of the sample and the recovery factor for each compound.

RESULTS AND DISCUSSION

A sensitive and specific HPLC assay was developed for the determination of compound [I] and [II] from 1 ml of blood, plasma or urine using UV detection at 254 nm for quantitation. This method enabled the rapid and accurate quantitation of compounds [I] and [II] for routine analysis of the large number of samples obtained during pre-clinical pharmacokinetic and toxicological studies. The major UV absorption bands of compounds [I], [II], and [III] occur at 215—220 nm and 255—260 nm, and are shown in Fig. 4. The Waters Model 440 absorbance detector used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp, allowed for quantitation of [I], [II], and [III] in the nanogram range.

Normal-phase (adsorption) HPLC analysis is the method of choice, since it is a simple three-step operation which involves selective extraction, sample concentration, and direct analysis by HPLC which ensures optimum resolution, peak symmetry and sensitivity of compounds [I], [II] and [III]. The shorter retention time of compound [II] with respect to that of compound [I] is probably due to intramolecular hydrogen bonding between the amide and hydroxyl group in [II] making it apparently less polar than [I]. Reversed-phase

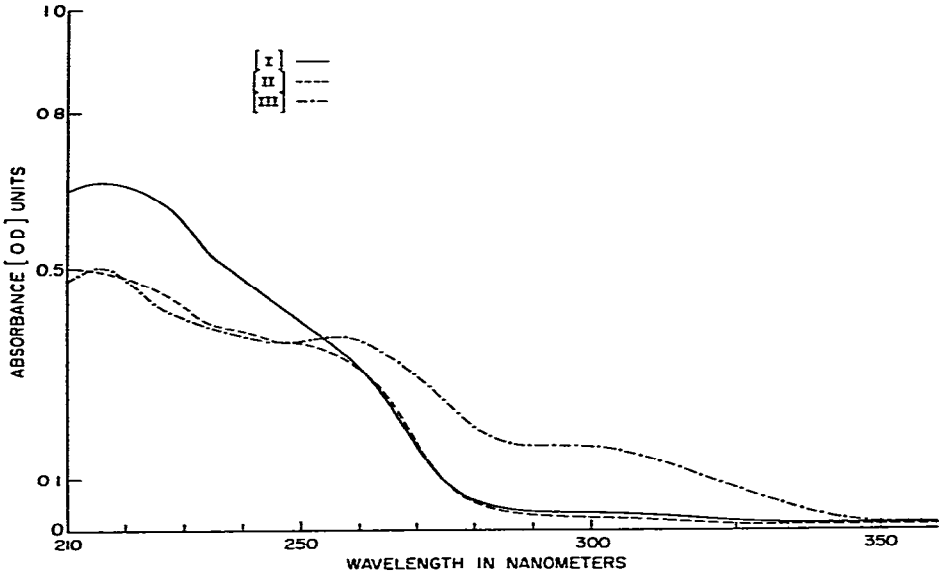

Fig. 4. UV absorption spectra of 5 μg/ml solutions of compounds [I], [II], and [III] in methanol.

HPLC was not practicable due to poor resolution between [I] and [II], and poor peak shape which adversely affected the sensitivity and quantitation of both compounds.

Compound [III] which is the N-oxide of [I], was chosen as the internal standard in the assay, due to its similar extraction and chromatographic behavior as compounds [I] and [II]. Compound [III] has not been identified as a metabolite of [I].

Recovery, sensitivity limits and statistical validation of the HPLC assay

The overall recoveries of compounds [I] and [II] from blood, plasma or urine are of the order of 92.0 ± 5.4% (S.D.) and 90.3 ± 4.9% (S.D.), respectively. The sensitivity limit of detection of [I] is 50 ± 5.3 ng (S.D.) and for [II] is 50 ± 1.7 ng (S.D.) per ml of blood, plasma or urine, using UV detection at 254 nm.

The intra-assay variability of [I] over the concentration range of 100—1000 ng/ml of blood showed a mean coefficient of variation of 1.5% while that for [II] over the concentration range of 100—600 ng/ml of blood was 2.3% (Table IA). The inter-assay variabilities of [I] and [II] showed mean coefficients of variation of 2.6 and 2.3%, respectively (Table IB).

Application of the method to biological specimens

The HPLC method was used to determine the blood concentration—time fall-off profiles and urinary excretion profiles in the dog following single 1 mg/kg intravenous and 5 mg/kg oral doses of compound [I]. The blood concentration of compound [I] following the intravenous administration ranged from 0.99 μg/ml at 1 min to 0.11 μg/ml at 30 h (Table II). Following oral administration (5 mg/kg) a peak concentration of 0.37 μg [I] per ml was observed at

TABLE I

STATISTICAL EVALUATION OF THE HPLC ASSAY

For A, $n = 4$; for B, $n = 3$.

Compound	Amount added (ng)	Amount found (ng ± S.D.)	Coefficient of variation (%)
A. Intra-assay variability			
[I]	100	94.6 ± 2.8	2.9
	400	403.3 ± 4.0	0.9
	800	814.5 ± 7.1	0.9
	1000	987.6 ± 11.1	1.1
			Average = 1.5
[II]	100	96.8 ± 3.0	3.1
	200	197.7 ± 2.0	1.0
	400	412.6 ± 14.1	3.4
	600	592.9 ± 10.1	1.7
			Average = 2.3
B. Inter-assay variability			
[I]	100	101.8 ± 6.4	6.2
	400	393.4 ± 10.4	2.6
	800	811.7 ± 8.1	0.9
	1000	993.1 ± 5.7	0.6
			Average = 2.6
[II]	100	104.2 ± 4.5	4.3
	200	191.7 ± 6.7	3.5
	400	402.7 ± 4.2	1.0
	600	600.0 ± 1.4	0.2
			Average = 2.3

0.5 h (Table II), with drug concentrations measurable through 10 h. Compound [II] was non-measurable following either single-dose administration of compound [I].

The urine concentration of directly extractable (unconjugated) [I] was non-measurable in the two dogs following the 1 mg/kg intravenous administration (0—48 h), whereas following the 5 mg/kg oral dose, urinary excretion of directly extractable parent drug [I] accounted for 16.3% of the dose in one dog, and 2.1% of the dose in the second dog (Table III). The metabolite [II] was non-measurable in all cases. Attempts at measuring the conjugated fraction of [I] and [II] were hampered by extracted endogenous impurities.

The assay was also used to monitor the blood concentrations of compounds [I] and [II] following multiple oral dosing in the dog (Table IV). Blood specimens were obtained from six dogs at 1, 3 and 6 h on day 15, following consecutive daily doses of 100 mg/kg of compound [I]. The maximum concentrations of compound [I] occurred at 1 h and ranged between 0.10 and 0.25 μg/ml of blood. Compound [II] was measurable at every time point with maximum concentrations occurring at 3 h, except for dog A, whose maxima occurred at 1 h. Maximum concentrations of compound [II] were consistently

TABLE II

CONCENTRATIONS OF COMPOUND [I] IN DOG BLOOD FOLLOWING THE ADMINISTRATION OF INTRAVENOUS AND ORAL DOSES OF COMPOUND [I]

Time	Concn. (μg [I] per ml of blood)	
	Intravenous Dose 1 mg/kg	Oral Dose 5 mg/kg
1 min	0.99	N.S.T.*
2.5 min	0.87	N.S.T.
5 min	0.84	N.S.T.
10 min	0.83	0.13
15 min	0.74	N.S.T.
20 min	0.69	0.31
30 min	0.73	0.37
45 min	0.59	0.35
1 h	0.47	0.33
1.5 h	0.57	0.32
2 h	0.58	0.31
3 h	0.55	0.26
4 h	0.50	0.24
6 h	0.32	0.16
8 h	0.31	0.12
10 h	0.27	0.10
24 h	0.15	N.M.**
30 h	0.11	N.M.
48 h	N.M.	N.M.

*N.S.T. = No sample taken.
**N.M. = Non-measurable (<50 ng/ml).

TABLE III

URINARY EXCRETION OF DIRECTLY EXTRACTABLE (UNCONJUGATED) [I] IN TWO DOGS FOLLOWING INTRAVENOUS AND ORAL DOSES OF COMPOUND [I]

Excretion period (h)	Percentage of dose excreted as directly extractable [I]			
	Dog A		Dog B	
	Intravenous Dose 1 mg/kg	Oral Dose 5 mg/kg	Intravenous Dose 1 mg/kg	Oral Dose 5 mg/kg
0—24	N.M.*	16.11	N.M.	2.05
24—48	N.M.	0.16	N.M.	N.M.
Total	N.M.	16.27	N.M.	2.05

*N.M. = Non-measurable (<50 ng/ml).

higher than those of compound [I], and ranged between 0.12 and 0.35 μg/ml of blood.

Gas chromatographic behavior of [I]

Although the sensitive and specific gas chromatographic analysis of the 1,4-

TABLE IV

CONCENTRATIONS OF COMPOUNDS [I] AND [II] IN DOG BLOOD FOLLOWING MULTIPLE ORAL DOSING FOR 15 DAYS

Dose: 100 mg [I]/kg/day.

Dog	Time (h)	Concn. (μg/ml of blood)	
		[I]	[II]
A	1	0.15	0.23
	3	0.07	0.22
	6	N.M.*	0.13
B	1	0.10	0.08
	3	0.07	0.12
	6	0.05	0.10
C	1	0.13	0.16
	3	0.07	0.18
	6	N.M.	0.11
D	1	0.13	0.11
	3	0.08	0.16
	6	N.M.	0.08
E	1	0.25	0.27
	3	0.18	0.35
	6	0.05	0.27
F	1	0.12	0.10
	3	0.06	0.15
	6	N.M.	0.08

*N.M. = Non-measurable (<50 ng/ml).

benzodiazepines using electron-capture detection is well documented [8], it was not applicable per se to the determination of [I] at low concentrations. The EC—GLC assay developed for midazolam [7] was initially applied to the determination of [I] which showed tailing on both OV-1 and OV-17 liquid phases, albeit with intrinsic high sensitivity to electron-capture detection. The chromatograms also indicated ghosting or memory effects on GLC analysis probably due to adsorption in the system resulting in poor precision and reproducibility especially at low concentration. The compound also showed adsorption losses on glassware which was circumvented by treatment with Prosil-28®, a siliconizing agent. It was apparent that both derivatization of the compound and clean up were necessary to improve its chromatographic behavior. Since back extraction of benzodiazepines into acid from a biological extract is an effective means of sample clean up, this step was investigated. It was noted that back washing the compound with dilute acid (0.1 *N* hydrochloric acid) resulted in a sharp symmetrical Gaussian shaped peak with enhanced electron-capture detector sensitivity (500 pg for full scale response at $0.5 \cdot 10^{-9}$ A), on a 1.22 m × 4 mm I.D., 3% OV-17 column at 245°C, indicating some form of rearrangement to a less polar moiety (Fig. 5). The authentic compound gave a tailing peak with a 4.9-min retention time (Fig. 5, trace A) whereas the acid-washed compound showed a sharper (Gaussian shaped) peak with a shorter

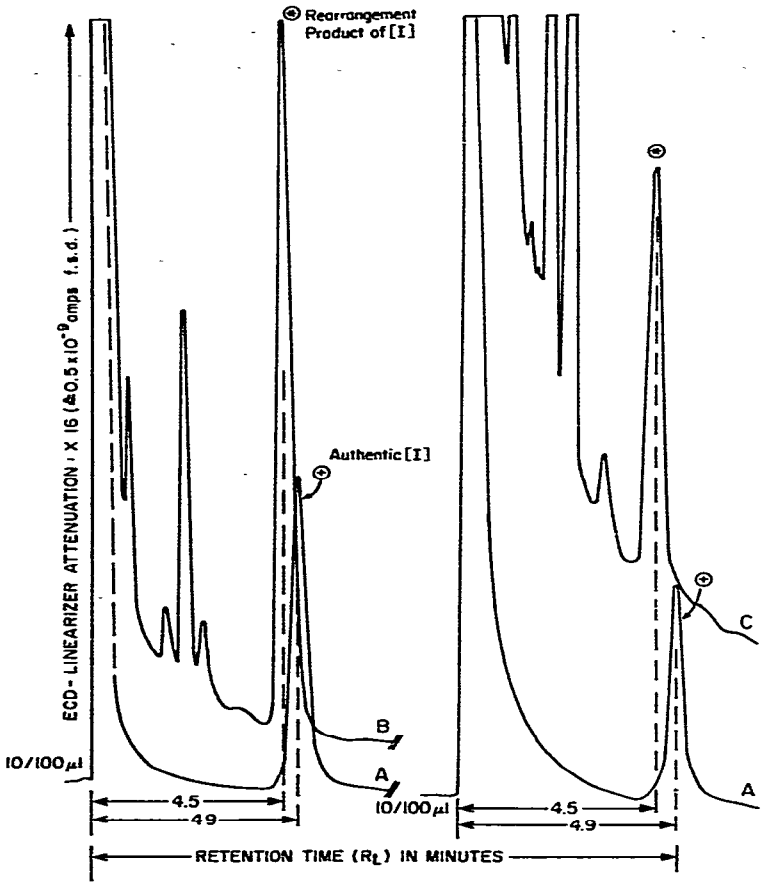

Fig. 5. Chromatograms of the EC—GLC analysis of (A) authentic [I]; (B) authentic [I] after treatment with 0.1 *N* hydrochloric acid indicating a rearrangement product with a shorter retention time; (C) authentic [I] extracted from blood, after dilute acid treatment, indicating a similar rearrangement product.

retention time of 4.5 min (Fig. 5, trace B), suggesting a rearrangement product. Similar behavior was observed for the compound recovered from blood after back extraction into acid (Fig. 5, trace C).

The partial ring opening of 1,4-benzodiazepines in dilute acids (e.g. 0.1 *N* hydrochloric acid) due to hydrolysis of the 4,5-azomethine group to yield the open ketone was demonstrated by differential pulse polarographic (DPP) analysis [7,9]. Similarly the DPP analysis of [I] indicated that 50% hydrolysis of [I] in 0.1 *N* hydrochloric acid was achieved in approximately 30 min, based on the polarographic reduction of the 4,5-azomethine group [10]. At equilibrium approximately 70% of [I] is in the open ketone form. Although this reaction is reversible upon alkalination for most benzodiazepines [7,9], compound [I], however, after standing in acid, and then alkalinized (pH 7.0 or 13.0) (which should undergo cyclization back to the expected parent compound), apparently undergoes a rearrangement to a new product, as indicated by a greatly diminished polarographic reduction peak for the 4,5-azomethine group.

These leads are under investigation for the development of a more sensitive EC—GLC assay (potential sensitivity of 250—300 pg/ml) for future clinical pharmacokinetic evaluation.

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REFERENCES

1 A. Walser, T. Flynn and R.J. Fryer, J. Heterocycl. Chem., 15 (1978) 577.
2 A. Walser, R.I. Fryer and L. Benjamin, U.S. Patent No. 4,166,185, August 28, 1979.
3 J. Sepinwall and A. Davidson, Hoffmann-La Roche, The Pharmacology of Imidazo-1,4-benzodiazepines, 1979, unpublished data on file.
4 J.G. Reves, R. Vinik, A.M. Hirschfield, C. Holcomb and S. Strong, Can. Anaesth. Soc. J., 26 (1979) 42.
5 C.R. Brown, F.H. Sarnquist, C.A. Canup and T.A. Pedley, Anaesthesiol., 50 (1979) 467.
6 S.J. Kolis, E.J. Postma, T.H. Williams, G.J. Sasso and M.A. Schwartz, Hoffmann-La Roche, 1980, unpublished data on file.
7 C.V. Puglisi, J.C. Meyer, L. D'Arconte, M.A. Brooks and J.A.F. de Silva, J. Chromatogr., 145 (1978) 81.
8 J.A.F. de Silva, in K. Tsuji (Editor), GLC and HPLC Determination of Therapeutic Agents, Chromatographic Science Series, Vol. 9, Part 2, Marcel Dekker, New York, 1978, pp. 581—636.
9 J.A.F. de Silva, C.V. Puglisi, M.A. Brooks and M.R. Hackman, J. Chromatogr., 99 (1974) 461.
10 M.A. Brooks, J.C. Meyer and M.R. Hackman, Hoffmann-La Roche, 1977, unpublished data on file.