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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF DIPOTASSIUM CLORAZEPATE AND ITS MAJOR METABOLITE NORDIAZEPAM IN PLASMA

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method is described for the quantitative analysis of dipotassium clorazepate (CZP) and its major metabolite nordiazepam (ND) in fresh human and dog plasma. The method consists of two separate selective ND extractions from a plasma sample without and with conversion of all the CZP to ND. For quantitation, diazepam (DZP) is used as the internal standard. The chromatographic phase utilized in a reversed-phase Hibar® EC-RT analytical column prepacked with LiChrosolv RP-18 with a solvent system consisting of acetonitrile—0.05 *M* sodium acetate buffer, pH 5.0 (45:55). The UV absorbance is monitored at 225 nm using a variable-wavelength detector. The mean assay coefficient of variation over a concentration range of 20—400 ng per ml of plasma is less than 3% for the within-day precision. Recoveries of ND, DZP and CZP (as ND) are essentially quantitative at all levels investigated. The calibration curves of ND are rectilinear (r^2 = 0.99) from the lower limit of sensitivity (2 ng/ml) to at least 2000 ng/ml in plasma. Applicability of the method to CZP and ND disposition studies in the anaesthetized mongrel dog is illustrated. When the two separate selective nordiazepam extractions from plasma cannot be performed immediately after blood sampling, an extrapolation kinetic method is suggested for the estimation of CZP concentration. In all previous in vivo studies, CZP has been determined only with gas—liquid chromatographic methods.

INTRODUCTION

Dipotassium clorazepate (CZP) is a 1,4-benzodiazepine antianxiety agent used in the management of many psychiatric disturbances [1]. The drug

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rapidly decarboxylates to nordiazepam (ND) in aqueous media, as can be found in the stomach [2]. Decarboxylation also occurs in the plasma but at a much lower rate. It is considered that CZP serves as a prodrug for active ND [3].

Different gas–liquid chromatographic (GLC) procedures, using electron-capture detection, have been suggested for the determination of CZP and/or ND in plasma with a reported sensitivity of 10 ng/ml after conversion of ND to a benzophenone [4] and 5 ng/ml without transformation of ND [5–7]. The plasma clorazepate levels reported from these GLC measurements were estimated from the difference between the total measured ND levels obtained by total acid conversion of CZP to ND, and the ND levels measured immediately after blood sampling and selective ND extraction [4–7].

Some papers have described reversed-phase liquid chromatography methods with UV detection at a fixed wavelength of 254 nm for the determination of ND in plasma with a sensitivity limit of 2, 20 and 50 ng/ml [8–10], respectively. The lowest sensitivity limit of 2 ng/ml was obtained for a 2-ml sample of plasma [8]. Brodie et al. [8] also report an interfering peak in control plasma having the same retention time of diazepam (DZP). In the method of Wallace et al. [10], a column temperature control system, which may not always be available, was used, and norchlordiazepoxide was reported to interfere with DZP under the column conditions described. With a variable-wavelength detector, a poor sensitivity limit of 30–80 ng/ml was reported at 230 nm [11] and 240 nm [12, 13].

In all previous in vivo studies [4, 5, 14], CZP has been determined only with GLC methods with a sensitivity limit of 5 ng/ml when expressed as ND [5–7]. In every case, the plasma sample had to be analyzed very shortly after blood sampling. On the other hand, it is to be noted that GLC methods are often associated with the problems of poor peak symmetry or tailing for ND. These latter difficulties are however eliminated with high-performance liquid chromatographic (HPLC) methods [10].

This paper describes a rapid, selective and more sensitive liquid chromatographic assay using a UV variable-wavelength detector set at 225 nm for analysis of ND in fresh human or dog plasma. It also shows its applicability for CZP determination in pharmacokinetic studies from measurement of total and actual ND plasma levels where the expression of total level is used for the combined levels of parent drug and metabolite measured as ND. In this procedure, the selective extraction of actual plasma ND concentration must be performed immediately after blood sampling. However, by considering the fact that this latter condition cannot always be met in clinical practice, an extrapolation kinetic method has been developed and is proposed here, in order to circumvent this difficulty. The technique is based on the equation for the first-order decarboxylation reaction kinetics of CZP [15].

EXPERIMENTAL

Reagents and chemicals

Diazepam, flurazepam, oxazepam and N-desalkylflurazepam were gifts of Dr. G. Caillé (Department of Pharmacology, University of Montreal). Nor-

diazepam, prazepam and pure dipotassium clorazepate were kindly donated by Hoffman-LaRoche (Vaudreuil, Canada), Parke Davis & Co. (Brockville, Canada) and J.J. McGilveray (Health Protection Branch, Ottawa, Canada), respectively. Tranxene® capsules were from Abbott Laboratories (Montréal, Canada). Pentobarbital and cimetidine were from C.D.M.V. (St-Hyacinthe, Canada) and Smith, Kline and French (Toronto, Canada). Acetonitrile, LiChrosolv grade, was obtained from BDH Chemicals (Toronto, Canada). Hexanes (mixture of isomers) and ethyl acetate, pesticide grade, were purchased from Fisher Scientific, Cat. No. H-300 and E-191, respectively (Montréal, Canada). Glacial acetic acid, analyzed reagent, aldehyde free, was purchased from J.T. Baker (Montréal, Canada). Glycine, reagent grade, was obtained from Fisher Scientific. All other reagents used were standard analytical grade.

The 0.05 *M* acetate buffer solution, pH 5.0, was prepared by adjusting the pH of an 800-ml aqueous acetic acid solution at 6.25×10^{-2} *M* by additon of a 1.0 *M* sodium hydroxide solution and of distilled water to complete the volume to 1 l. The 2.0 *M* glycine buffer solution, pH 9.0, was prepared by addition of a 1.0 *M* sodium hydroxide solution to an aqueous solution of glycine. The standard, DZP, was added to the extraction solvent mixture (60 ng/ml) to produce working standard plasma solutions with a concentration of 600 ng/ml.

Apparatus

The experiments were accomplished with a Varian liquid chromatograph Model 5010 (Varian, Palo Alto, CA, U.S.A.). The chromatograph was equipped with a Valco injector and a Varichrom variable-wavelength UV detector (Varian) set at 225 nm and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 56 recorder. The separations were performed on a reversed-phase Hibar® EC.RT column prepacked with LiChrosorb RP-18 (10 μm particle size, 250 × 4 mm I.D.; E. Merck, Darmstadt, G.F.R.).

Chromatographic conditions

The mobile phase, consisting of acetonitrile—0.05 *M* sodium acetate aqueous buffer, pH 5.0 (45:55, v/v) was delivered at a flow-rate of 1.5 ml/min. The resulting pressure was 50.6 bar. For the detector, the wavelength was set at 225 nm, the band width at 8 nm, the time constant at the slow position and the absorbance range at the 0.01—0.2 interval. The recorder was set at 1 mV full scale deflection and chart speed at 5 mm/min.

Glass tubes used in the extraction procedures were soaked for over 12 h in 70.4% nitric acid and rinsed in distilled water. All liquid volumes were delivered using Gilson automatic pipettes P20 to P1000.

Preparation of plasma standard solutions

A standard solution of ND was prepared in acetonitrile at a concentration of 5 mg/l. Aliquots of increasing volumes (10—300 μl) of this solution were evaporated to dryness at 38°C (Buchler rotary Evapo-mix; Buchler Instruments, Fort Lee, NJ, U.S.A.) in 15-ml screw-capped test tubes and 1 ml of fresh drug-free plasma (human or canine) was added in each to give samples for analysis in the range 50—1500 ng/ml.

Extraction procedure

To each of the above 1-ml plasma standard solutions (or samples to be assayed), 0.5 ml of 2 *M* glycine buffer, pH 9.0 was added with vortex mixing after each addition. A 10-ml aliquot of the solvent mixture of extraction (hexane—ethyl acetate, 70:30, v/v) containing 60 ng of DZP per ml were added and again each tube was mixed briefly. All tubes were then mixed in an Eberbach multi-tube reciprocal shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 10 min. All tubes were then centrifuged at 320 *g* for 5 min. A portion (approximately 8 ml) of the upper layer (organic phase) was transferred to a 15-ml centrifuge tube and the solvents evaporated to dryness at 38°C in the Buchler rotary Evapo-mix. To the dry residue, 500 µl of ethyl acetate were added, vortexed for 20 sec and evaporated to dryness. The latter step is done to make sure that all the ND and DZP possibly deposited on the upper inner walls of the tubes would be dissolved and drained in the bottom, allowing complete recaptation with mobile phase prior to injection in the liquid chromatograph. This residue was thus reconstituted in 100 µl of column eluent solution (acetonitrile—0.05 *M* sodium acetate buffer, pH 5.0, 45:55, v/v) and 50 µl were injected into the liquid chromatograph for quantitation of ND.

Determination of actual and total ND in dog plasma

Venous blood was withdrawn at different time intervals, in heparinised Vacutainer® tubes, from an anaesthetized mongrel dog after having received a single intravenous dose of 0.5 mg/kg of CZP. Blood samples were immediately centrifuged (within 1 min) at 560 *g* in a refrigerated centrifuge set at 5°C for 9 min. Here, a delay for blood centrifugation would increase the amount of in vitro CZP hydrolysis to ND. Four 1-ml plasma samples were immediately transferred into four different screw-capped test tubes where two of them contained 0.5 ml of cold 2 *M* glycine buffer, pH 9.

The two 1-ml plasma samples added to cold glycine buffer are promptly analyzed for actual ND. To each of these test tubes, the extraction solvent mixture (hexane—ethyl acetate) is added as described above for the quantitation of actual ND.

The other two 1-ml plasma samples are then analyzed for total ND (actual ND and ND from CZP hydrolysis). For this, 150 µl of 1 *N* hydrochloric acid were added to both tubes which were vortexed for 15 sec and then incubated at 37°C for 1 h to allow complete hydrolysis of unchanged CZP. After cooling the test tubes to room temperature, 150 µl of 1 *N* sodium hydroxide and 0.5 ml of 2 *M* glycine buffer, pH 9 were added with vortex mixing. The total ND is then extracted as described above and quantitated.

Determination of CZP in plasma

Plasma CZP concentrations were obtained from the difference between total and actual ND plasma concentrations. This difference must be multiplied by 1.51 to correct for difference in the molecular weight of CZP and ND.

Calculations for quantitation of ND

Standard curves were drawn using the measured peak height ratios of ND

to DZP against the known concentrations of ND in the standard plasma samples. The equations of the standard lines were obtained by least-squares regression analysis and were used to determine ND concentrations in unknown plasma samples.

Conversion of CZP to ND

A solution of pure CZP in bicarbonate buffer, pH 9.5 (7.5 mg per 100 ml) was prepared and diluted in fresh human plasma to give plasma concentrations of 75, 150, 300, 450 and 750 ng/ml of CZP that are equivalent to 50, 100, 200, 300 and 500 ng/ml of ND. These plasma samples were analyzed in duplicate for total ND after acid conversion of CZP to ND as described above.

Intravenous solution of CZP

The intravenous solution of dipotassium clorazepate was prepared, just before use, by dissolving the content of two 15-mg capsules of Tranxene® in 30 ml of a 0.005 *M* isotonic phosphate buffer, pH 7.0. The resulting solution was then immediately filtered on a HA type Millipore filter (Millipore, Mississauga, Canada) with pore sizes of 0.45 μm. This solution was found to be stable enough for in vivo work as no formation of ND could be measured within 10 min after its preparation.

Extrapolation kinetic method

A solution of pure CZP in bicarbonate buffer, pH 9.5 (20 mg per 200 ml) was prepared and 45—225 μl of this stable solution were immediately added to 30 ml of fresh drug-free human plasma to give plasma concentrations of 150, 300, 500 and 750 ng/ml of CZP. To three of these mixtures, a known amount of ND was added (100 ng/ml). The time t was noted and measured after CZP addition. The synthetic mixtures were kept at refrigerative temperature of 4°C and 1-ml plasma samples in duplicate were taken at times corresponding to 1, 3 and 5 h after their preparation. These specimens were then subjected to total and actual ND analysis as described above, in order to calculate the residual CZP concentration. The initial plasma concentration of CZP, C^0_{CZP}, was estimated from a linear regression analysis of the logarithm of the residual plasma concentration of CZP, C_{CZP}, versus time t, followed by an extrapolation of C_{CZP} to zero time:

$$\log C_{CZP} = \log C^0_{CZP} - k\ t/2.3$$

where k represents the first-order rate constant of transformation of CZP to ND.

RESULTS AND DISCUSSION

HPLC of some benzodiazepines has been performed on a reversed-phase column. A mobile phase was chosen by taking into consideraiton the following criteria: simplicity, retention times of the studied compounds, intracolumn pressure produced and solvent toxicity, polarity, viscosity and analysis sample solubility. A mixture of a 0.05 *M* sodium acetate buffer solution (pH 5)—acetonitrile (55:45, v/v) demonstrated the best resolution between

ND and DZP peaks with a total analysis time of less than 10 min (Fig. 1). The mobile phase was relatively insensitive to small variations of pH and in the acetate buffer concentration. The retention times of the drugs tested in this study are listed in Table I. Flurazepam, which has a retention time of 3.6 min here, was not eluted from the column or was found to have a retention time of 41 min under the conditions used by Brodie et al. [8] and MacKichan et al. [9], respectively. None of the drugs tested (Table I) represents a risk of interference with the analysis of ND or DZP. The absorbance of ND at 225 nm was found to be at a maximum under the conditions chosen here. At 254 nm, the wavelength commonly used in fixed-wavelength detectors, the absorbance represents 1/3 of the absorbance observed at 225 nm. For fifteen drug-free fresh human and canine blood samples processed, it was found that normal plasma components in the extract did not interfere with the peaks of ND and DZP. Although chromatographic background is negligible with fresh plasma, this was not always the case with frozen specimens. The buffers and solvent mixture of extraction chosen here were however found to lead to a minimum of interference in the analysis. On the other hand, benzene, when tested as a solvent of extraction, led to a significant interference in the assay

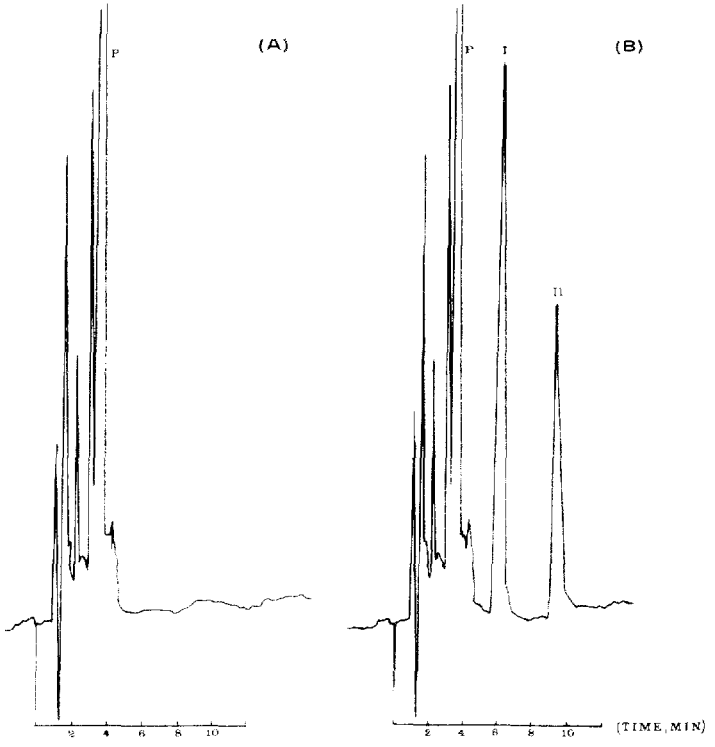

Fig. 1. Chromatograms of blank plasma extract and of a plasma extract of nordiazepam and diazepam. (A) Chromatogram of an extract of diazepam- and nordiazepam-free plasma; (B) chromatogram of an extract of the same plasma sample containing 600 ng/ml of nordiazepam and 600 ng/ml of diazepam. Peaks: P = pentobarbital; I = nordiazepam; II = diazepam.

TABLE I

RETENTION TIMES FOR SOME DRUGS

Drugs	Retention time (min)
Dipotassium clorazepate	2.1
Flurazepam	3.6
N-Desalkylflurazepam	3.8
Pentobarbital	4.0
Cimetidine	4.8
Oxazepam	5.0
Nordiazepam	6.2
2-Amino-5-chlorobenzophenone	7.5
Diazepam	9.2
Prazepam	14.5

on frozen plasma samples. Finally, it is to be noted that, in general, interference in frozen plasma samples was reduced by thawing them rapidly.

The precision of assay for ND was assessed by repeated analyses on fresh drug-free human plasma specimens spiked with known concentrations of ND. As shown in Table II, within-day and day-to-day precision of the method, as measured by the coefficient of variation, ranged from 1.65 to 2.84% and 2.94 to 9.82%, respectively.

TABLE II

REPRODUCIBILITY OF PLASMA NORDIAZEPAM ASSAY

	Concentration (ng/ml)	n^*	C.V. (%)
Within-day	20	10	2.84
	200	10	1.65
	400	10	2.42
Day-to-day	100	5	9.82
	300	5	2.94
	1200	5	4.26

*n = number of samples.

The minimum measurable concentration of ND for this procedure is 2 ng/ml of original plasma sample, allowing a signal-to-noise ratio of 5. The limits of detection of ND, setting the detector sensitivity at 0.005 full scale and allowing a signal-to-noise ratio of 2, is 1 ng/ml. The sensitivity of this HPLC procedure is thus found to be much higher than those obtained previously (30—80 ng/ml), when using a variable-wavelength detector [11—13]. With a fixed-wavelength detector, the lowest sensitivity reported (2 ng/ml), was obtained with a 2-ml plasma sample [8] instead of a 1-ml plasma sample.

The accuracy of the assay for ND was evaluated from three series of five

spiked fresh drug-free human plasma with concentrations of 85, 700 and 1250 ng/ml. The mean relative errors between the observed and the theoretical true concentration, were found to be 1.82, 2.34 and 5.34%, respectively.

For determination of analytical recovery of ND and DZP, the drugs were added to a solvent mixture of the mobile phase and to fresh drug-free human plasma to achieve concentrations of 50 and 500 ng/ml for ND and 500 ng/ml for DZP. The recovery for ND and DZP was then estimated by comparing peak heights of ND or DZP after the extraction step, where peak heights are corrected for the carefully measured aliquot taken for assay, with peak heights obtained after injection of the standard solutions containing known concentrations of ND or DZP. Recovery for ND and DZP ranged from 101.2 to 101.6%.

The calibration curves were found to be linear for a concentration of 2—2000 ng of ND per ml of fresh drug-free human or canine plasma. The cor-

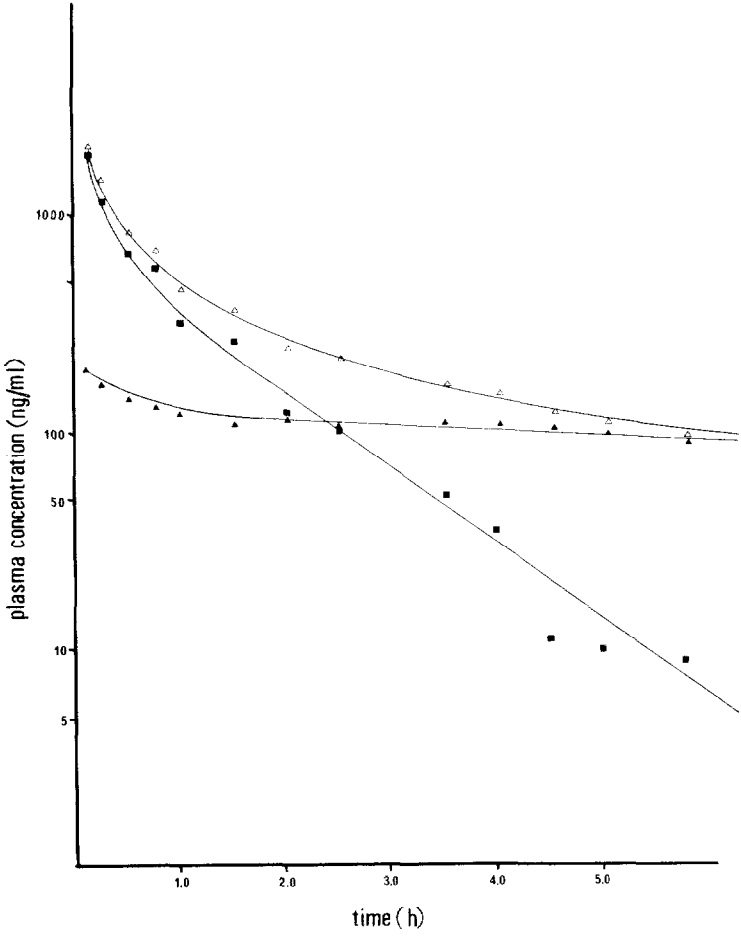

Fig. 2. Plasma concentration of dipotassium clorazepate, actual and total nordiazepam after a single intravenous dose of 0.5 mg/kg of dipotassium clorazepate in an anaesthetized mongrel dog. ■, CZP; ▲, actual ND; △, total ND.

relation coefficient obtained from the linear regression was always greater than 0.99. In a representative calibration curve, the linear equation, $Y = 0.0031 X + 0.0004$ (X = 50—1200 ng/ml, $n = 6$, $r = 0.9993$) was found. The 95% confidence intervals for the slope and the ordinate intercept were 0.0001 and 0.1091, respectively.

The lifetime of the column (which is 5 cm shorter and less expensive than those used in refs. 8—10) appears to be very good, as it is still in an excellent condition after a 1-year use in an applied research project.

The degree of acid conversion of CZP to ND was evaluated from fresh drug-free human plasma spiked with pure CZP to achieve concentrations of 75, 150, 300, 450 and 750 ng/ml. These samples were then analyzed in duplicate for ND according to the procedure of acid conversion of CZP to ND. Conversion to ND ranged from 100.5 to 102.4%. A detectable amount of the 2-amino-5-chlorobenzophenone, as a possible acid conversion product of ND, was not observed. Injection of 50 µl of a fresh solution of pure CZP (0.5 mg %) dissolved in a bicarbonate buffer pH 9.5 showed that the amount of ND was less than 1% when the solution was injected at 0, 5 and 10 min after solution preparation. These data indicate that the drug sample used was relatively pure and that CZP was relatively stable under these conditions of the HPLC method.

When applied to the collected plasma samples of an anaesthetized mongrel dog after receiving a single intravenous dose of CZP, it was found that the distribution and elimination of CZP could be described by an open two-compartment pharmacokinetic model (Fig. 2). The terminal elimination half-life of CZP, actual ND and total ND were estimated to be 0.84, 5.02 and 3.55 h, respectively.

The evaluation of the extrapolation kinetic method, for cases when the two separate selective ND extractions from plasma cannot be performed immediately, is illustrated in Fig. 3. Fig. 3 reveals that the kinetics of CZP

TABLE III

ACCURACY AND PRECISION OF THE EXTRAPOLATION KINETIC METHOD

Theoretical concentration* (ng/ml)			Percent of concentration found	
CZP	ND		Total ND**	CZP
750	0		108.45	95.46
500	100		101.76	94.43
300	100		107.10	93.82
150	100		111.66	86.42
		$\bar{X}$***	107.24	92.53
		S.D.	4.13	4.13
		C.V.	3.85	4.46

*CZP concentrations of 750, 500, 300 and 150 ng/ml are equivalent to 469.69, 331.13, 198.68 and 99.34 ng of ND per ml.

**Average of the values obtained at 1, 3 and 5 h.

***$\bar{X}$ = arithmetic mean; S.D. = standard deviation; C.V. = coefficient of variation (%).

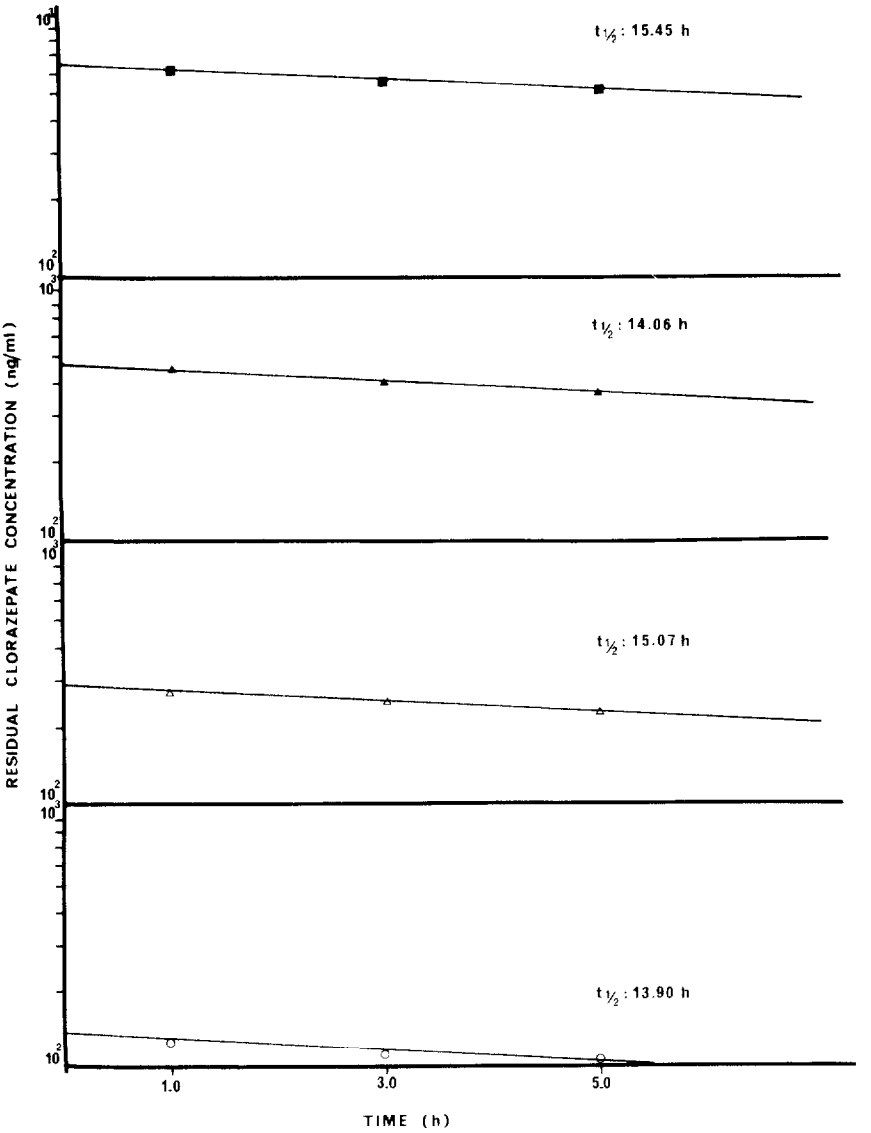

Fig. 3. First-order CZP decarboxylation plot for the estimation of CZP plasma concentration at time of blood sampling. Theoretical initial concentrations: ■, 750 ng CZP/ml; ▲, 500 ng CZP/ml and 100 ng ND/ml; △, 300 ng CZP/ml and 100 ng ND/ml; ○, 150 ng CZP/ml and 100 ng ND/ml.

decarboxylation is first-order at all CZP plasma concentrations tested here with or without addition of ND. The average half-life for the decarboxylation process in plasma at 4°C was found to be 14.62 h (S.D. = 0.76, C.V. = 5.18%). As shown in Table III, this study indicated that plasma concentrations of CZP and total ND can be determined with a relatively good accuracy and precision. The average deviations from theoretical plasma concentrations were found to be 7.47 and 7.24% for CZP and ND measurements, respectively.

In clinical practice, the application of this extrapolation kinetic method would imply that after blood sampling, the time would have to be recorded, the blood kept and centrifuged at 4°C, and the plasma separated and stored at 4°C until completion of the analysis on plasma specimens sampled at convenient and appropriate time intervals.

CONCLUSION

The HPLC procedure proposed here is a rapid, sensitive and reproducible technique for the determination of ND and CZP. The sensitivity of the method is particularly better than that reported previously with GLC for ND and CZP, and with HPLC for ND when using a variable-wavelength UV detector. The selectivity of the method, for benzodiazepines, may also represent an alternative to other HPLC procedures when an interaction with another compound is noted and found to be undesirable. The extrapolation kinetic technique appears to be a new valuable tool for estimating CZP when it is not possible to determine that compound immediately after blood sampling. Preliminary studies indicate that the method can be used in pharmacokinetic studies and can be adapted to analysis of other benzodiazepine compounds. The chromatograms are interference-free from normal components of fresh human and canine plasma as well as from some drug metabolites and commonly used drugs.

REFERENCES

1 D.J. Greenblatt and R.I. Shader, N. Engl. J. Med., 291 (1974) 1011.
2 C.W. Abruzzo, M.A. Brooks, S. Cotler and S.A. Kaplan, J. Pharmacokinet. Biopharm., 4 (1976) 29.
3 D.J. Greenblatt, R.I. Shader, M. Divoll and J.S. Harmatz, Brit. J. Clin. Pharmacol., 11 (1981) 11S.
4 D.J. Hoffman and A.H.C. Chun, J. Pharm. Sci., 64 (1975) 1668.
5 A.H.C. Chun, P.J. Carrigan, D.J. Hoffman, R.P. Kershner and J.D. Stuart, Clin. Pharmacol. Ther., 22 (1977) 329.
6 D.J. Greenblatt, J. Pharm. Sci., 67 (1978) 427.
7 E. Rey, J.M. Turquais and G. Olive, Ann. Biol. Clin., 36 (1978) 355.
8 R.R. Brodie, L.F. Chasseaud and T. Taylor, J. Chromatogr., 150 (1978) 361.
9 J.J. MacKichan, W.J. Jusko, P.K. Duffner and M.E. Cohen, Clin. Chem., 25 (1979) 856.
10 J.E. Wallace, H.A. Schwertner and E.L. Shimek, Jr., Clin. Chem., 25 (1979) 1296.
11 T.B. Vree, A.M. Baars, Y.A. Hekster and E. van der Kleijn, J. Chromatogr., 162 (1979) 605.
12 P.M. Kabra, G.L. Stevens and L.J. Marton, J. Chromatogr., 150 (1978) 355.
13 J.M. Foreman, W.C. Griffiths, P.G. Dextraze and I. Diamond, Clin. Biochem., 13 (1980) 122.
14 E. Rey, P. Giraux, D. de Lauture, J.M. Turquais, J. Chavinie and G. Olive, Eur. J. Clin. Pharmacol., 15 (1979) 175.
15 R. Raveux and M. Briot, Bull. Chim. Ther., 4 (1969) 303.