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DETERMINATION OF CHLORDIAZEPOXIDE, DIAZEPAM, AND THEIR MAJOR METABOLITES IN BLOOD OR PLASMA BY SPECTROPHOTODENSITOMETRY*

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SUMMARY

An analytical procedure was developed for the determination of chlordiazepoxide, diazepam and their major metabolites in blood or plasma. Demoxepam, a metabolite of chlordiazepoxide, is determined by spectrofluorometry after selective extraction. The remaining compounds are determined by spectrophotodensitometry after thin-layer chromatographic separation.

The sensitivity limit of the spectrofluorometric determination of demoxepam is 0.1 to 0.2 μg while that of the spectrophotodensitometric determination of chlordiazepoxide, diazepam and their N-desmethyl metabolites is 0.05 to 0.2 μg. The sensitivity and specificity of the assay renders it suitable for monitoring plasma levels of chlordiazepoxide and its major metabolites following single or chronic oral administration of chlordiazepoxide hydrochloride. The sensitivity limit for diazepam and nordiazepam, its major metabolite, renders the assay useful only for the determination of plasma concentrations resulting from high dosage of diazepam. The assay was used to determine chlordiazepoxide and its metabolites following oral administration of Librium. The data showed a significant correlation to those obtained on the same specimens by differential pulse polarography and by radioimmunoassay.

INTRODUCTION

Chlordiazepoxide hydrochloride, 7-chloro-2-methylamino-5-phenyl-3H-1, 4-benzodiazepine-4-oxide [I] hydrochloride, is the active ingredient in Librium which is marketed as an antianxiety agent. Metabolic studies in man [1—5] have shown that the compound is biotransformed to form three major metabolites which are present in the blood or plasma; N-desmethylchlordiazepoxide [II], demoxepam [III], and nordiazepam [V] (Fig. 1).

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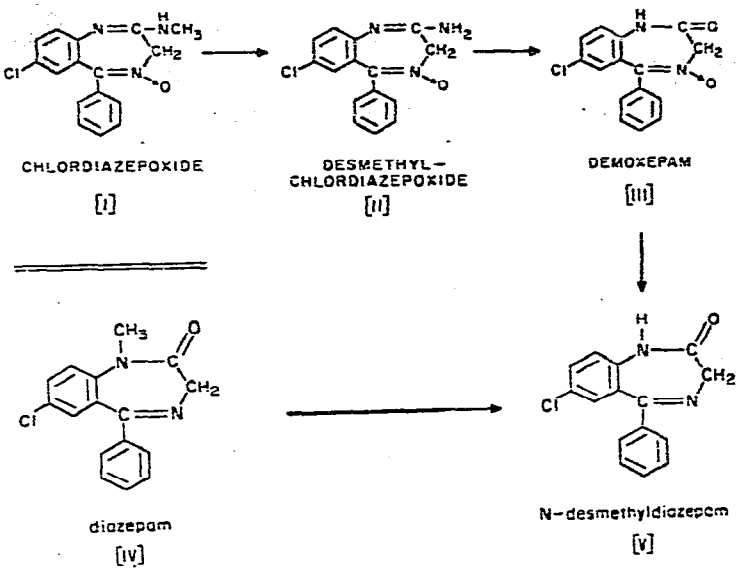

Fig. 1. Chemical structures of compounds I—V.

Spectrofluorometric methods employing selective extraction procedures followed by mild acid hydrolysis and photochemical rearrangement to yield fluorescent derivatives have been used for the specific determination of therapeutic levels of chlordiazepoxide, N-desmethylchlordiazepoxide and demoxepam [1,3]. An electron-capture gas—liquid chromatographic (GLC—ECD) method was reported [6] for the specific determination of intact chlordiazepoxide; however, it does not measure the major metabolites. A differential pulse polarographic (DPP) method was also reported [7] which is capable of measuring virtue of thin-layer chromatographic (TLC) separation. Each compound is eluted separately and quantitated by the reduction of the azomethine [$>C_5N_4$-] peak. Recently a radioimmunoassay (RIA) was developed for the specific determination of chlordiazepoxide per se. This assay has been applied in bioavailability studies and to monitor patients undergoing chronic therapy [8]; however, it does not measure any metabolites.

Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4 benzodiazepin-2-one [IV], is the active ingredient in Valium, which is marketed as a psychotropic agent and muscle relaxant. The compound undergoes biotransformation in man by N-demethylation to nordiazepam, the major blood metabolite [9] (Fig. 1).

GLC—ECD [10,11] is the method of choice for measuring blood concentrations of diazepam and nordiazepam following either single or chronic oral dosing, while DPP [12,13] and RIA [14] have proved useful in toxicological analysis.

Densitometric techniques for measuring compounds in situ after TLC [15, 16] either by intrinsic UV absorption [17] or by fluorescence emission [18, 19] have been shown to be rapid and sensitive methods of analysis. An analytical method was developed for chlordiazepoxide, diazepam and their major

metabolites in plasma utilizing reflectance spectrophotodensitometry. The assay involves selective extraction of the compounds into diethyl ether from plasma buffered to pH 9.0 with 1 *M* phosphate buffer. Demoxepam is determined by selective extraction into 0.1 *N* NaOH and photolytic reaction to a quinazoline derivative which is determined by spectrofluorometry. The other benzodiazepines are separated by TLC and then analyzed in situ by spectrophotodensitometry at 260 nm. The assay was used to determine chlordiazepoxide and its metabolites following oral administration of Librium. The TLC assay data showed a significant correlation to those obtained by DPP [7] and to results for chlordiazepoxide obtained by RIA [8]. The assay was also used to determine diazepam and nordiazepam following suspected ingestion of overdoses of Valium. The results were compared to those obtained by DPP and/or GLC—ECD.

EXPERIMENTAL

The reagents used in this assay are the same as those described for DPP analysis [7].

Standard solutions

Weigh out 25.0 mg each of chlordiazepoxide [7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{16}H_{14}N_3OCl$ (mol.wt. = 299.71; m.p. = 236—236.5°)]; N-desmethylchlordiazepoxide [2-amino-7-chloro-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{15}H_{12}N_3OCl$ (mol.wt. = 285.73, m.p. = 255—256°)]; demoxepam [7-chloro-1,3-dihydro-2H-1,4-benzodiazepin-2-one-4-oxide, $C_{15}H_{11}N_2O_2Cl$ (mol.wt. = 286.72, m.p. = 235—236°)]; diazepam [7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one; $C_{16}H_{13}ON_2Cl$ (mol. wt. = 284.74; m.p. = 131—135°)]; and nordiazepam [7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one; $C_{15}H_{11}ON_2Cl$ and dissolve in 25 ml of methanol to give stock solutions of 1000 μg/ml. Prepare a working solution containing all five compounds by transferring 1 ml of each stock solution into a 100-ml volumetric flask and diluting to volume with acetone to give a working solution containing 10.0 μg of each compound per ml. A fresh working solution should be prepared every two weeks.

TLC separation is performed on E. Merck silica gel F_{254} G-60 TLC plates (manufactured by E. Merck, Darmstadt, G.F.R., and marketed by Brinkmann, Westbury, N.Y., U.S.A.).

Instrumentation

Fluorescence measurements were performed on a Farrand Mark I Spectrofluorometer (Farrand, Valhalla, N.Y, U.S.A.). A TLC spectrophotodensitometer (Model 137190 visual—UV chromatogram analyzer (Farrand) equipped with a 150W d.c. xenon arc energy source was used for in situ spectrophotometric analysis in the 45° reflectance mode [20].

used for in situ spectrophotometric analysis in the 45° reflectance mode [20].

Light source for conversion of demoxepam to fluorescent product. The light source used is a Pyro-Lux R-57 lamp (Luxor, New York, N.Y., U.S.A.), with the samples placed 12 in. from the lamp inside an aluminum foil lined reflector box. UV protective glasses should be worn when working with this lamp.

Instrumental parameters for the chromatogram analyser

Absorption (Vis—UV) Mode. The excitation monochromator was set at 260 nm when assaying for chlordiazepoxide and its metabolites and at 250 nm when assaying for diazepam and its metabolite. The energy is incident upon the plate through the beam condenser lens (200—375 nm) in the exciter leg containing a 0.625-in. reducer (spacer) and no optical filters. The analyzer leg was used with the 200—300 nm lens, in conjunction with a 0.125-in. reducer without an optical filter. A 10-nm entrance slit was used with an RCA 1P-28 photomultiplier. The single beam mode of scanning was used. The chromatoplate was scanned spatially at a rate of 4 in./min in the single-beam recording mode, with the recorder chart-speed also set at 4 in./min.

Calibration. Aliquots of the working solution were applied to each TLC plate to provide known concentrations of each benzodiazepine to calibrate the response of the instrument according to the expected range of the unknowns and to provide a standard for each plate to compensate for inter-plate variability.

Assay in plasma for chlordiazepoxide, diazepam and their metabolites

Procedure. Prior to each analysis pre-develop the necessary number of chromatoplates for 15 to 20 cm ascending in a vapor-saturated chamber using chloroform as the developing solvent to clean the surface of hydrocarbon ("octoils") impurities.

Into a 50-ml glass-stoppered centrifuge tube, add 1 (or 2) ml of blood or plasma sample, 5 ml of 1 *M* potassium phosphate buffer (pH 9.0), and 7 ml of fresh diethyl ether. Along with the samples, process three separate specimens of control blood or plasma containing 0.5, 1.0, and 2.0 μg each of chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, diazepam, and nordiazepam as internal standards, prepared by adding 0.05, 0.1, and 0.2 ml of the working standards into 50-ml centrifuge tubes. The solutions are then evaporated to dryness in a 65° water-bath under a stream of nitrogen, and 1 ml of control blood or plasma specimen is added to the residues.

Stopper the tube with a PTFE stopper, shake on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.) for 10 min at a moderate speed, and then centrifuge for 5 min at 2300 rpm (1300 *g*) at 0—5° in a refrigerated centrifuge (Model PR-J with a No. 253 rotor; Damon/IEC Division, Needham, Mass., U.S.A.). Carefully remove the diethyl ether layer using a 10 ml serological pipet and transfer into a 50-ml glass-stoppered centrifuge tube. Re-extract the sample with a second 7-ml aliquot of diethyl ether as described above. Combine the second extract with the residue of the first extract in the 50-ml tube.

Assay for demoxepam. Add 4.0 ml of 0.1 *N* NaOH into the 50-ml tube containing the combined ether extracts and extract demoxepam into the alkaline phase. Centrifuge the samples and transfer the ether supernatant into a fresh 15-ml centrifuge tube and evaporate to dryness in a 40° water-bath (Evapomix; Buchler, Fort Lee, N.J., U.S.A.). This residue is used for the analysis of chlordiazepoxide, N-desmethylchlordiazepoxide, diazepam and nordiazepam. While this step is being completed, the NaOH fraction containing demoxepam is processed as follows. The 0.1 *N* NaOH phase is washed once with 10 ml of

ether, centrifuged, the ether removed by aspiration, and the tubes warmed in a 50° bath to expel any residual ether. The tubes are stoppered and exposed to UV light from a Pyro-Lux R-57 lamp contained in an aluminum foil lined reflector box placed in a single row approximately 12 in. from the light source for 20—30 min. Read the fluorescence in a 1-cm path quartz cell in a spectrofluorometer (Farrand Mark I or equivalent) at 450 nm, exciting at 380 nm and determine the demoxepam concentration as described previously [1,3].

Assay for chlordiazepoxide, diazepam and their respective N-desmethyl metabolites. Dissolve the residue of the combined extract containing chlordiazepoxide, diazepam and their respective N-desmethyl metabolites in 100 μl of diethyl ether and transfer quantitatively on to a pre-developed 20 × 20 cm Merck silica gel F_{254} G-60 plate, applying the samples not less than 2.5 cm apart (7 samples per 20-cm plate). Rinse the tube with two successive 50-μl aliquots of ether and transfer on to the chromatoplate. Apply mixtures (0.50 μg and 2.0 μg) of authentic standards of the benzodiazepines to each chromatoplate as reference standards for calibrating the instrument and as markers for locating the benzodiazepines in the biological extracts. Develop the plate twice in a vapor-saturated chamber using chloroform until the solvent front has ascended 15 cm. Air-dry the plate after each development. Then develop the plate using chloroform—acetone (1 : 1) until the solvent front has ascended 15 cm. Air-dry the plate and examine under short-wave UV, and identify the areas on the silica gel corresponding to chlordiazepoxide ($R_F \approx 0.3$), N-desmethylchlordiazepoxide ($R_F \approx 0.15$), diazepam ($R_F \approx 0.6$), and nordiazepam ($R_F \approx 0.5$) by comparison to the R_F of the authentic standards run alongside the sample extracts (Fig. 2).

If the determination of diazepam and nordiazepam is of primary importance then use benzene—*n*-propanol—conc. ammonium hydroxide (80 : 20 : 1) as the developing solvent to achieve optimal resolution of diazepam ($R_F \approx 0.58$) and nordiazepam ($R_F \approx 0.47$) from extracted lipids. Under these conditions the resolution of chlordiazepoxide ($R_F \approx 0.38$), N-desmethylchlordiazepoxide ($R_F \approx 0.24$) and demoxepam ($R_F \approx 0.28$) is unsatisfactory for densitometric analysis.

Spectrodensitometry. Set the absorption range to give 90% full-scale negative deflection on the recorder pen for the 2-μg standard of chlordiazepoxide, with the baseline set at 90—95% full scale on the recorder. Scan the chromatoplate in the absorption mode using the parameters described and record the UV absorption of each compound as symmetrical peaks. The peak area [peak height (centimeters) × width (centimeters) at half-height], which is determined using either the slope baseline technique or electronic digital integration, is proportional to concentration of the benzodiazepine. In order to obtain greater sensitivity, set the absorption range to give 90% full-scale negative deflection for the 0.5 μg standard. Re-scan the chromatoplate as before.

Construct a calibration curve of peak area versus concentration of each of the respective added authentic standards of chlordiazepoxide, diazepam and their respective N-desmethyl metabolites. Determine the concentration of these compounds in the unknowns by interpolation. Determine the overall recovery of the added authentic standards of these compounds by comparison of the absorption (as peak area) of the respective benzodiazepines recovered from

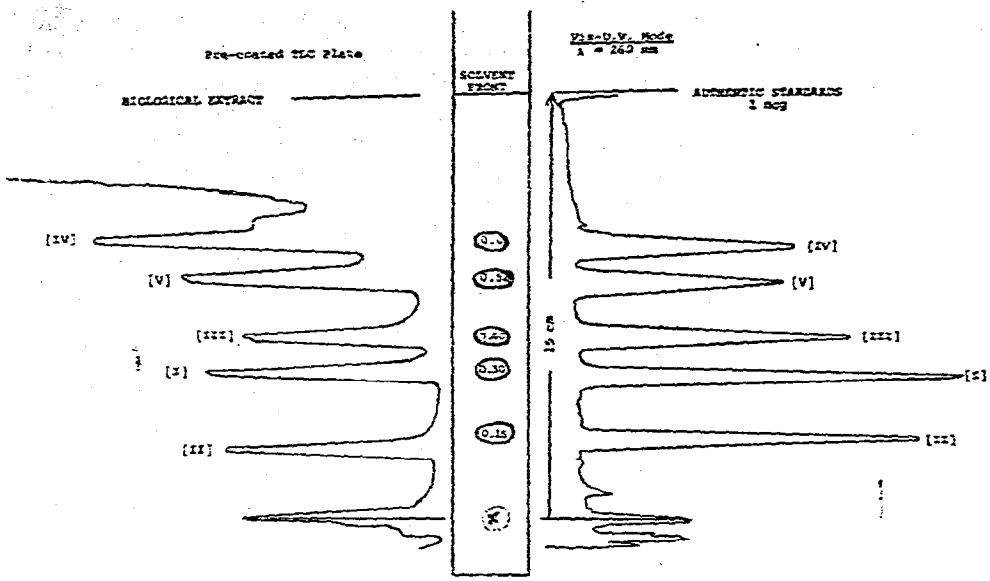

Fig. 2. TLC separation and spectrophotodensitometric analysis of compounds I—V. The chromatoplate was developed twice in chloroform to move endogenous lipids up to the solvent front, followed by a final development in chloroform—acetone (1 : 1) to move the compounds.

plasma against that of the respective authentic standards scanned on the chromatoplate. Percent recovery should be determined routinely as a check on the reproducibility of the assay. After spectrodensitometry, the sample areas may be scraped, eluted and analyzed by other techniques such as either spectrofluorometry [3] or DPP [7].

RESULTS AND DISCUSSION

Analytical parameters

The Farrand spectrophotodensitometer used can be operated only in the reflectance mode [20] and not in the transmission mode, which is utilized in the Schoeffel and Zeiss spectrophotodensitometers. The most significant advantages of spectrophotodensitometry in the reflectance mode are the minimization of energy losses due to internal absorption or self-quenching due to the thickness of the silica gel bed and transmission losses through a glass plate [15,16].

Commercially available TLC plates of 250-μm bed thickness and 60-μm particle-size silica gel G gave reproducible data with respect to peak area measurement of varying concentrations of the benzodiazepines on the same chromatoplate and between chromatoplates. In addition, the background was more uniform than that found on soft gel plates (such as the Quantum TLC plates previously used [7]) resulting in less background noise and baseline drift.

The response of the TLC spectrophotodensitometer to each benzodiazepine was determined at 5-nm intervals from 210 nm to 350 nm, and is plotted in

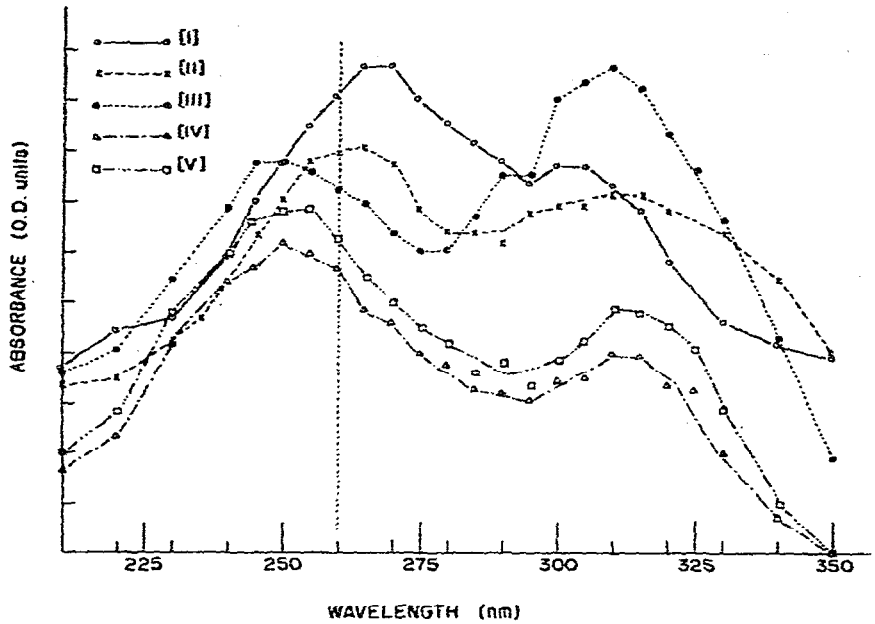

Fig. 3. UV absorption spectra of compounds I—V adsorbed on silica gel containing fluorescent indicator.

not be determined by automatic scanning because the reflected energy increased rapidly between 200 nm and 300 nm. This required continual re-setting of the baseline offset in both the single beam mode and in the double beam ratio mode. The optimal wavelength for the quantitation of the benzodiazepines was chosen to be 260 nm.

Although demoxepam is resolved from the other benzodiazepines using chloroform—acetone (1 : 1) as the developing solvent system ($R_F \approx 0.40$) (Fig. 3), a UV-absorbing impurity present in certain plasma samples gave erroneously high values for demoxepam by spectrophotodensitometry as compared to DPP analysis following elution of the same spot. Consequently, demoxepam is determined prior to TLC by selective extraction into 0.1 *N* NaOH, conversion to a quinazolinone derivative and quantitation by spectrofluorometry as described previously [1,3].

The recovery of chlordiazepoxide, N-desmethylchlordiazepoxide, diazepam and nordiazepam is 75 ± 10%, 72 ± 6%, 92 ± 8%, and 95 ± 5%, respectively with sensitivity limits in the range of 0.05 to 0.20 μg/ml by spectrophotodensitometry using a 1-ml plasma specimen per assay. The recovery of demoxepam is 95 ± 5% with a sensitivity limit by spectrofluorometry of 0.1 to 0.2 μg/ml using a 1-ml plasma specimen per assay.

The sensitivity of the spectrophotodensitometric method can be optimized for diazepam and nordiazepam by setting the excitation monochromator at 252 nm. In addition, a better separation may be obtained for diazepam ($R_F \approx 0.58$) and nordiazepam ($R_F \approx 0.47$) from the extracted lipid materials by using benzene—*n*-propanol—conc. ammonium hydroxide (80 : 20 : 1) as the devel-

oping solvent system. However, the resolution of chlordiazepoxide, N-desmethylchlordiazepoxide and demoxepam ($R_F \approx 0.38$, 0.24 and 0.28, respectively) is unsatisfactory. The sensitivity of the assay for diazepam and nordiazepam is not sufficient for the reliable determination of therapeutic levels following single or repeated administration of diazepam [21]* but it sufficient for the determination of blood levels of diazepam and nordiazepam resulting from overdosages of diazepam.

Spectrofluorodensitometry

Quantitation of drugs by fluorodensitometry in situ using either the intrinsic fluorescence of the compound or of a suitable fluorescent derivative is often a very useful means of increasing the sensitivity of the determination [18,19]. The tricyclic antidepressant drugs such as amitriptyline, nortriptyline and imipramine can be determined by spectrofluorodensitometry by spraying the chromatoplate with 10% perchloric acid, and heating the plate at 110° (oven) to generate the fluorescence [17—19]. The formation of fluorescent derivatives of drugs in situ at elevated temperatures by reaction with NH_4HCO_3 at 140—150° was also reported [22]. The qualitative detection of the benzodiazepines on ethanol-saturated TLC plates utilizing their intrinsic luminescence behavior at ambient and cryogenic temperature has been reported [23].

The benzodiazepines I—V were subjected to the above acidic and basic reactions reported for other compounds [18,19,22] and the chromatoplates were examined in the chromatogram analyzer using the fluorescence mode (a 0.500-in. aperture reducer and a No. 7-54 optical filter were inserted into the cylindrical illumination lens assembly. The emission or analyzer monochromator was attached to the analyzer leg, and a No. 3-73 optical filter was used with the 300—800 nm transmission lens in the analyzer leg without any reducers. A RCA 1P-21 photomultiplier was used in conjunction with 10-nm slits at the analyzer).

The fluorescent products formed under both acidic and basic reaction conditions had similar excitation (380 nm) and emission (475 nm) maxima suggesting the formation of the same product, i.e., quinazolines and quinazolinones which possess similar excitation and emission maxima [23].

Although chlordiazepoxide and its metabolites formed fluorescent products in situ under both acidic and basic reaction conditions, the sensitivity limits of detection were no better than that using direct UV spectrophotodensitometry (260 nm), hence fluorodensitometry did not offer any advantage over the former procedure.

Application of the method to biological specimens

Plasma samples from two subjects who had received single 30 mg oral doses of Librium and a third subject who received 10-mg oral doses of Librium at 0, 4, and 8 h (30 mg total) were assayed for intact chlordiazepoxide, by spectrophotodensitometry, by RIA [8] and by DPP [7] respectively. The concentrations of N-desmethylchlordiazepoxide and nordiazepam were also assayed by spectrophotodensitometry and by DPP. Demoxepam was assayed only by

*Editor's note: Therapeutic levels of diazepam in man are reported to be between 100 and 200 ng/ml, following chronic oral administration of 10-mg doses.

spectrofluorometry. Comparison of the results obtained by each method (Tables I—III) gave satisfactory agreement for chlordiazepoxide and N-desmethylchlordiazepoxide. The concentration of N-desmethylchlordiazepoxide for subject No. 2 is below the limits of sensitivity either by spectrophotodensitometry or by DPP.

The 36-h sample of subject No. 3 had the only concentration of demoxepam (0.18 μg/ml) which was measurable by spectrofluorometry. All the other samples assayed gave readings below the sensitivity limits of the assay for this compound. Neither spectrophotodensitometry nor DPP detected the presence of nordiazepam.

The data presented here are in agreement with the pharmacokinetics and biopharmaceutics of chlordiazepoxide in man as reported by Boxenbaum et al. [24].

The joint determinations of chlordiazepoxide and its N-desmethyl metabolite were subjected to straight-line analysis [25]. The results are presented in Table IV. In comparing the TLC spectrophotodensitometric results to those of the other methods the fitted intercepts and slopes were not significantly different ($p \leqslant 0.05$) from 0 and 1 respectively, with high correlation coefficients in each case. Therefore, the plasma concentrations of chlordiazepoxide measured by either spectrophotodensitometry, RIA, or DPP are equivalent while the plasma concentrations of N-desmethylchlordiazepoxide measured by either spectrophotodensitometry or by DPP are also equivalent.

TABLE I

PLASMA LEVELS OF CHLORDIAZEPOXIDE AND ITS N-DESMETHYL METABOLITE FOLLOWING ORAL ADMINISTRATION OF A SINGLE 30-mg DOSE OF LIBRIUM TO SUBJECT No. 1

Nil = Below sensitivity limits, (<0.02 μg/ml for RIA; <0.03—0.10 μg/ml for TLC—SPD; <0.05—0.10 μg/ml for DPP); N.A. = not analyzed.

Time after dose (h)	Chlordiazepoxide (μg/ml)			N-Desmethylchlordiazepoxide (μg/ml)	
	Radio-immuno-assay	TLC—spectro-densito-metry	Differ-ential pulse polaro-graphy	Spectro-densito-metry	Differential pulse polarography
0.0	Nil	Nil	Nil	Nil	Nil
0.25	0.15	0.20	0.14	Nil	Nil
0.5	0.82	0.90	0.85	0.08	Nil
0.75	1.14	1.15	1.10	0.12	N.A.
1.0	1.22	1.22	1.30	0.18	N.A.
1.5	1.38	1.04	1.32	0.20	0.20
2	1.03	1.07	1.15	0.18	0.18
3	0.88	1.07	1.02	0.30	0.30
4	0.88	1.00	1.00	0.26	0.30
8	0.67	1.00	0.96	0.40	0.30
12	0.60	0.80	0.54	0.40	0.38
24	0.24	0.26	0.19	0.50	0.30
30	0.17	0.15	0.10	0.45	0.30
36	0.11	0.07	0.06	0.24	0.26
48	0.05	0.05	N.A.	0.23	0.19
72	Nil	Nil	Nil	0.13	0.12

TABLE II

PLASMA LEVELS OF CHLORDIAZEPOXIDE FOLLOWING ORAL ADMINISTRATION OF A SINGLE 30-mg DOSE OF LIBRIUM TO SUBJECT No. 2

Nil = Below sensitivity limits (<0.02 μg/ml for RIA; <0.03—0.10 μg/ml for TLC—SPD; <0.05—0.10 μg/ml for DPP); N.A. = not analyzed

Time after dose (h)	Chlordiazepoxide (μg/ml)		
	Radioimmunoassay	TLC—spectrodensitometry	Differential pulse polarography
0.0	Nil	Nil	Nil
0.5	1.72	1.30	1.50
1.0	1.38	1.20	1.18
1.5	1.14	1.00	1.27
2.0	1.30	1.00	1.00
3.0	1.14	0.85	0.91
4.0	1.10	0.87	0.91
5.5	1.00	0.81	0.85
7.0	0.90	0.82	1.09
8	1.04	0.72	1.09
9.5	0.80	0.83	0.74
11	1.00	0.71	1.00
13	0.96	0.72	0.59
15	0.85	0.61	0.68
24	0.53	0.54	0.50
30	0.74	0.61	0.59
36	0.72	0.62	NA
48	0.62	0.43	0.41
72	0.26	0.30	NA

Plasma and serum samples from subjects suspected of ingesting an overdose of Valium were assayed for diazepam and nordiazepam by the spectrophotodensitometric assay (optimized for these compounds). Comparison of these results to those obtained either by DPP or GLC—ECD (Table V) gave satisfactory agreement.

CONCLUSIONS

The sensitivity and specificity of the spectrodensitometric assay renders it suitable either for monitoring plasma levels obtained following single or chronic oral administration of chlordiazepoxide hydrochloride, or as a rapid toxicological procedure in determining cases of overdosage of either chlordiazepoxide hydrochloride or diazepam. In addition, it is more rapid than any of the existing assay procedures [1,3,6—8,13]. The assay is non-destructive and the compounds may be eluted for further characterization. Alternatively, spray tests for the unambiguous identification of the compounds may be carried out on the plate [18,19,23].

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TABLE III

PLASMA LEVELS OF CHLORDIAZEPOXIDE AND ITS N-DESMETHYL METABOLITE FOLLOWING ORAL ADMINISTRATION OF A 10-mg DOSE OF LIBRIUM (3-times a day) AT 0,4, AND 8 h TO SUBJECT No. 3

Nil = Below sensitivity limits (<0.02 μg/ml for RIA; <0.03—0.10 μg/ml for TLC—SPD; <0.05—0.10 μg/ml for DPP).

Time (h)	Chlordiazepoxide (μg/ml)			N-Desmethylchlordiazepoxide (μg/ml)	
	Radio-immuno-assay	TLC—spectro-densito-metry	Differ-ential pulse polaro-graphy	TLC—spectro-densito-metry	Differential pulse polarography
0.0*	Nil	Nil	Nil	Nil	Nil
0.5	Nil	Nil	Nil	Nil	Nil
1.0	0.32	0.26	0.24	Nil	Nil
1.5	0.22	0.27	0.14	Nil	0.05
2	Nil	0.21	0.20	Nil	Nil
3	Nil	0.20	0.20	Nil	Nil
4*	Nil	0.21	0.19	0.04	0.05
5.5	0.40	0.42	0.33	0.08	0.05
7	0.64	0.39	0.40	0.10	0.05
8*	0.63	0.40	0.42	0.10	0.15
9.5	Nil	0.34	0.35	0.11	0.15
11	Nil	0.37	0.26	0.15	0.14
13	0.51	0.40	0.53	0.19	0.17
15	0.67	0.50	0.51	0.23	0.22
24	0.40	0.25	0.33	0.23	0.20
30	0.37	0.26	0.23	0.34	0.33
36	0.46	0.20	0.13	0.28	0.27
48	0.25	0.08	Nil	0.17	0.18
72	Nil	Nil	Nil	0.07	0.08

* Aministration of 10 mg of Librium

TABLE IV

RESULTS OF STRAIGHT-LINE ANALYSIS OF THE JOINT DETERMINATIONS

SPD = Spectrophotodensitometry; RIA = radioimmunoassay; DPP = differential pulse polarography.

Compound	Methods compared	Range of levels compared (μg/ml)	Intercept (μg/ml)	Slope	Correlation coefficient
Chlordiazepoxide	SPD versus RIA	0.05—1.72	0.081	1.00	0.914
Chlordiazepoxide	SPD versus DPP	0.05—1.72	0.061	1.11	0.957
N-Desmethylchlor-diazepoxide	SPD versus DPP	0.04—0.50	0.022	0.786	0.897

TABLE V

PLASMA AND SERUM CONCENTRATIONS OF DIAZEPAM AND ITS N-DESMETHYL METABOLITE FOLLOWING SUSPECTED INGESTION OF OVERDOSES OF VALIUM

Case No.	Biological fluid	Diazepam (μg/ml)		N-Desmethyldiazepam (μg/ml)	
		Spectrophoto-densitometry	Level found previously	Spectrophoto-densitometry	Level found previously
528	Plasma	0.90	1.0*	0.2	0.29*
536	Serum	0.35	0.51**	0.85	0.67**
538 A	Plasma	1.80	2.01**	3.55	3.90**
538 B	Plasma	2.00	1.96**	3.75	3.30**
538 C	Plasma	1.45	1.41**	3.90	2,89**

* Differential pulse polarography.

** Electron-capture gas—liquid chromatography.

the drawings of the figures presented. Karl Bratin was a 1975 A.C.S. Summer Intern, from Clarkson College, Potsdam, N.Y.

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