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The Screening and Quantitation of Diazepam, Flurazepam, Chlordiazepoxide, and Their Metabolites in Blood and Plasma by Electron-Capture Gas Chromatography and High Pressure Liquid Chromatography

The number and frequency of requests made to toxicologists to analyze blood or plasma samples for benzodiazepines are increasing dramatically, but until recently specific and sensitive procedures were unavailable to meet the demand. Gas-liquid chromatography with electron-capture detection has become the analytical method of choice for these drugs because it meets the necessary sensitivity requirements. Prior to the development of this method the available analytical procedures depended on acid hydrolysis of the benzodiazepines to their corresponding benzophenones [*1*] followed by gas-liquid or thin-layer chromatography. Unfortunately, hydrolysis of several of the benzodiazepines results in the formation of the same benzophenone [*2,3*] so that the individual parent drugs cannot be readily identified.

During the past few years the technique most extensively used has been gas-liquid chromatography with electron-capture detection (GLC-ECD). De Silva et al [*4*] have described a comprehensive extraction scheme using GLC-ECD for the determination of these compounds in blood. However, the procedure has major disadvantages, such as lengthy purification steps and the necessity for relatively large sample volumes (up to 2 ml). Recently, Rutherford [*5*] has described a rapid micro method using GLC-ECD for the determination of diazepam and its metabolites in plasma. The method involves a direct solvent extraction followed by GLC. Although the procedure described in this report is similar, it has been extended to include the quantitative determination of flurazepam and its major metabolite *N*-desalkylflurazepam.

In forensic toxicology the major disadvantage of GLC-ECD procedures is the lack of a suitable confirmatory technique to substantiate the GLC results and provide reliable qualitative identification. Initial investigations by Kopjak et al [*6*] have shown that high pressure liquid chromatography (HPLC) can be used to differentiate the benzodiazepines, and recently Osselton et al [*7*] described an enzymatic digestion method for the analysis of benzodiazepines in tissue which used HPLC as the identifying technique.

This report describes an analytical procedure involving both GLC-ECD and HPLC that can be used to identify, quantitate, and confirm the benzodiazepines and their major metabolites in small-volume plasma and blood samples. Of the benzodiazepines currently available in the United States, clonazepam is the only one that cannot be detected by this

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procedure at very low plasma concentrations, which result from therapeutic dosages of the drug.

The screening method involves direct solvent extraction of a buffered sample followed by GLC-ECD analysis of a small aliquot of the unconcentrated solvent. The blood or plasma is then extracted with additional solvent, which is separated and concentrated. The residue is examined by HPLC to confirm the presence of the benzodiazepines. Quantitation is accomplished in either method by using flunitrazepam as an internal standard. The GLC-ECD method is used for diazepam, desmethyldiazepam, flurazepam, and desalkylflurazepam, and HPLC for chlordiazepoxide and desmethylchlordiazepoxide.

Although the analytical procedure is described for 1 ml of whole blood or plasma, the GLC-ECD screening procedure can be applied to sample volumes as low as 50 μl if necessary.

Equipment and Materials

Materials

Toluene (nanograde), heptane analytical reagent, and sodium borate were purchased from Mallinckrodt Inc., St. Louis, Mo. Isoamyl alcohol and disodium hydrogen phosphate were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Methanol was purchased from Burdick and Jackson Labs, Inc., Muskegon, Mich.

Chlordiazepoxide, desmethylchlordiazepoxide, demoxepam, desalkylflurazepam, diazepam, desmethyldiazepam, flurazepam, and flunitrazepam were generously supplied in pure form by Hoffman-La Roche Inc. (Nutley, N.J.); oxazepam, by Wyeth Laboratories (Philadelphia, Pa.); and prazepam by Warner-Lambert Pharmaceutical Co. (Morris Plains, N.J.).

Equipment

A Hewlett-Packard Model 5730A gas chromatograph fitted with a ^{63}Ni ECD was used. The analytical column was a 1.2-m (4-ft) by 3.2-mm ($\frac{1}{8}$-in.) inside diameter silane-treated gas column packed with 3% OV-17 on Gas Chrom Q, 100-120 (Applied Science Labs, State College, Pa.). Operating conditions were as follows: injector temperature, 300°C; detector temperature, 300°C; oven temperature, 240°C; and nitrogen carrier gas flow rate, 50 ml/min.

A Spectra-Physics Model 3200 liquid chromatograph fitted with a SP8200 ultraviolet detector was used for the HPLC analysis.

The high pressure liquid chromatographic column was a 30-cm by 4-mm Bondapak-C18 reverse phase column (Waters Associates, Melford, Mass.). The solvent flow rate was 2.4 ml/min.

Preparation of Standards

Stock solutions of each drug were prepared by dissolving the appropriate amount of drug in methanol to make a 1 mg/ml solution of the free base. These standards were stable when kept at 0°C. Working solutions of 10 and 100 μg/ml were prepared by serial dilution of the stock solution.

For diazepam, desmethyldiazepam, flurazepam, and desalkylflurazepam, blood or plasma standards of 0.1, 0.3, 0.5, 0.7, and 1.0 μg/ml were prepared from the working solutions. Chlordiazepoxide standards of 0.2, 1.0, 5.0, and 10.0 μg/ml and desmethylchlordiazepoxide standards of 0.2, 0.5, 1.0, and 5.0 μg/ml were also prepared in blood or plasma.

Methods

The extraction scheme used routinely to screen samples for the benzodiazepines by GLC-ECD and subsequent confirmation by HPLC is outlined in Fig. 1. The extracting solvent is a mixture of toluene/heptane/isoamyl alcohol (76:20:4) as described by Zingales [*8*].

Two different eluants were used for the HPLC analyses:

(a) methanol, 0.025*M* disodium hydrogen phosphate (pH 7.5), 58:42; and
(b) methanol, 0.025*M* disodium hydrogen phosphate (pH 7.5), 73:37.

The disodium hydrogen phosphate buffer was prepared by dissolving 3.05 g of the salt in one litre of distilled and deionized water, and adding 1 ml of 0.9*M* phosphoric acid. The pH was then checked with a pH meter. Both solvent mixtures were degassed and filtered before use.

Diazepam, desmethyldiazepam, flurazepam, and desalkylflurazepam are routinely quantitated by using the GLC-ECD procedure with 200 ng of flunitrazepam as the internal standard. For the quantitation of chlordiazepoxide and desmethylchlordiazepoxide by HPLC 3 μg of flunitrazepam is used. For both procedures, quantitation is performed by plotting peak height or area ratios (drug/internal standard) against concentration. The resulting graphs, for duplicate extractions, are shown in Figs. 2 through 5.

A quality control blood sample is routinely analyzed by the GLC-ECD screening procedure. It contains diazepam, desmethyldiazepam, flurazepam, and desalkylflurazepam, each at 0.2 μg/ml concentration.

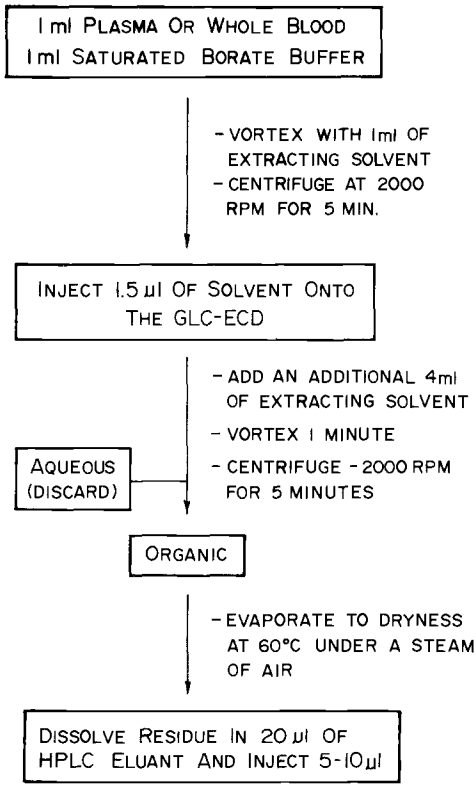

FIG. 1—*Extraction scheme.*

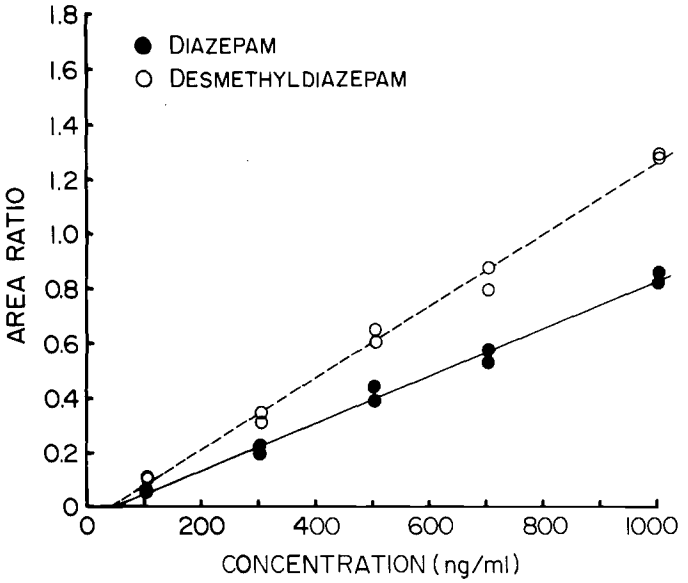

FIG. 2—*Calibration curves for diazepam and desmethyldiazepam.*

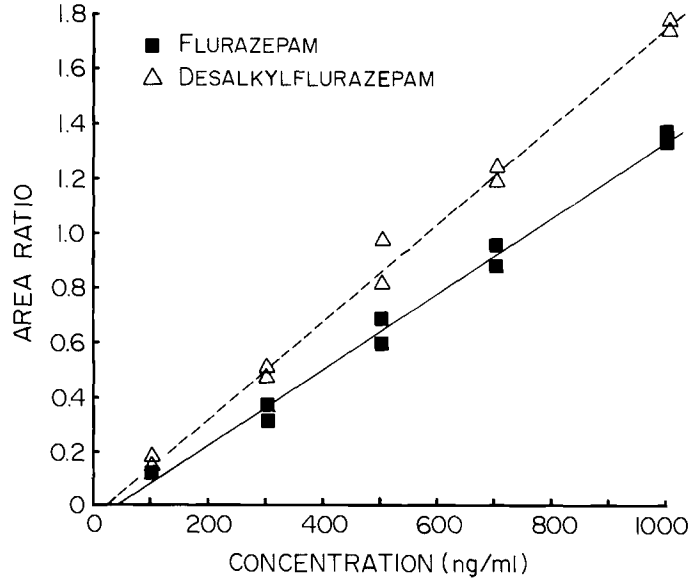

FIG. 3—*Calibration curves for flurazepam and desalkylflurazepam.*

Results and Discussion

De Silva et al [*4*] have demonstrated the usefulness of GLC-ECD when applied to the analysis of blood samples for benzodiazepines and their metabolites. However, the procedures described by these workers are time-consuming and therefore are not applicable to a rapid screening program. This report describes a simple and rapid GLC-ECD screening procedure which because of the sensitivity of the ECD is able to detect therapeutic concentra-

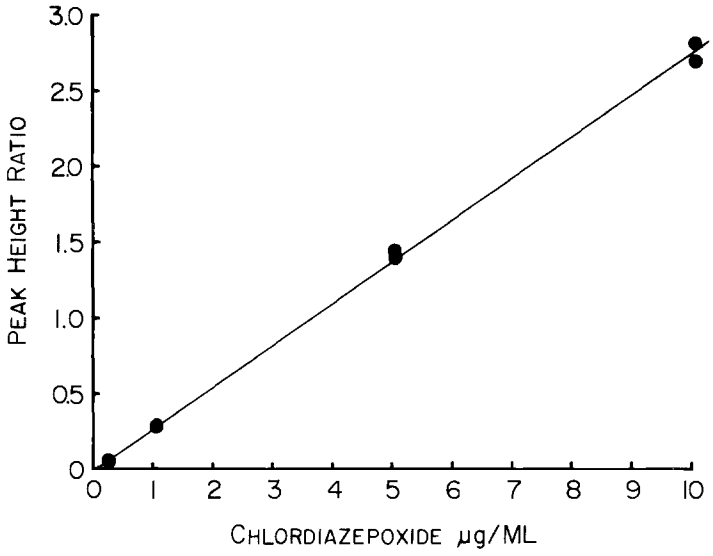

FIG. 4—*Calibration curve for chlordiazepoxide.*

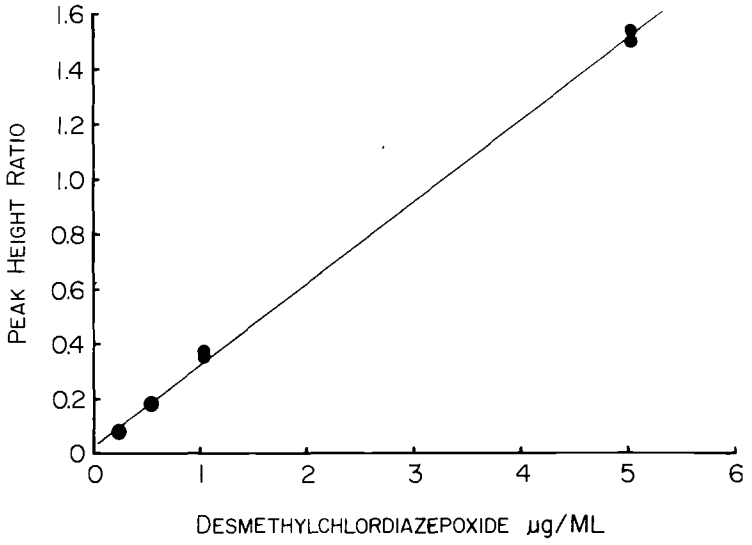

FIG. 5—*Calibration curve for desmethylchlordiazepoxide.*

tions of the benzodiazepines and their primary metabolites in sample volumes as low as 50 µl. The ability to use small specimens is extremely important when clinical samples from pediatric patients are to be examined. Recently, Rutherford [5] described a similar procedure which he applied to diazepam and its metabolites, but it did not include quantitation of flurazepam and desalkylflurazepam.

During the initial development of the gas chromatographic criteria it was found that the peak shape of desmethyldiazepam improved after several injections of blood or plasma extracts. This probably results from the occupation of some of the active sites on the packing

material by fatty acids or lipids extracted from the blood. Similar observations have been made following injections of lecithin[2] and tristearin [9].

Because of this improvement in gas chromatographic characteristics the column used was routinely "primed" before use with several injections from an extract of blank plasma or blood. The retention times of the benzodiazepines on a 3% OV-17 column with an oven temperature of 240°C and a carrier gas flow of 50 ml/min are shown in Table 1.

Blank blood and plasma samples when carried through the GLC-ECD screening procedure gave no peaks that interfered with any of the commonly encountered benzodiazepines (Fig. 6*a*). Figures 6*b* and *c* show the chromatograms obtained when two positive plasma samples were carried through the GLC-ECD quantitation procedure.

As can be seen from Figs. 2 and 3, the peak height ratio/concentration plots are linear over the range of 0.1 to 1.0 μg/ml. Because of the possibility of saturating the ECD at concentrations greater than 1 μg/ml, samples which fall outside this range are diluted with blank plasma or blood and re-extracted.

Chlordiazepoxide and desmethylchlordiazepoxide have been reported to be thermally labile [10] and because of this, and the wide range of blood and plasma concentrations encountered, HPLC was used to quantitate these particular benzodiazepines. In addition, it was decided to use this technique to confirm the presence of the other benzodiazepines detected and presumptively identified by GLC-ECD.

Although several workers [11,12] have described HPLC procedures for the determination of individual benzodiazepine drugs and their metabolites, they did not extend the methods to the identification of other benzodiazepines. Initial work with normal phase chromatography on a silica column, as used by Vree et al [11] and Grassi et al [12], indicated that the separation of desmethyldiazepam and desalkylflurazepam would be difficult. Preliminary investigations by Kopjak et al [6] have shown that a reverse phase column could be used for the identification of some of the benzodiazepines. Further work with a Bondapak-C18 column and methanol/disodium hydrogen phosphate eluants has proved to be more useful, in that all of the commonly encountered benzodiazepines can now be separated.

Two different eluants had to be used, but these differ only in the percentage of methanol. When the more polar solvent system (Eluant a) was used both prazepam and flurazepam

TABLE 1—*Chromatographic properties of the benzodiazepins.*

Drug	GLC-ECD Retention Time, min[a]	HPLC, Retention Volume, ml	
		Eluant a[b]	Eluant b[c]
Chlordiazepoxide	9.95 (major)	12	<5
Desmethylchlordiazepoxide	NR	9.6	<5
Demoxepam	NR	6.7	<5
Diazepam	2.2	16.1	<5
Desmethyldiazepam	3.3	12.7	<5
Flurazepam	4.6	>25	11.0
Desalkylflurazepam	2.5	10.8	<5
Oxazepam	NR	9.8	<5
Prazepam	3.8	>25	8.5
Flunitrazepam	3.8	8.2	<5

[a]NR = not run.
[b]Eluant a = methanol/phosphate buffer (58:42).
[c]Eluant b = methanol/phosphate buffer (73:37).

[2]Hoffman-La Roche, Nutley, N.J., personal communication.

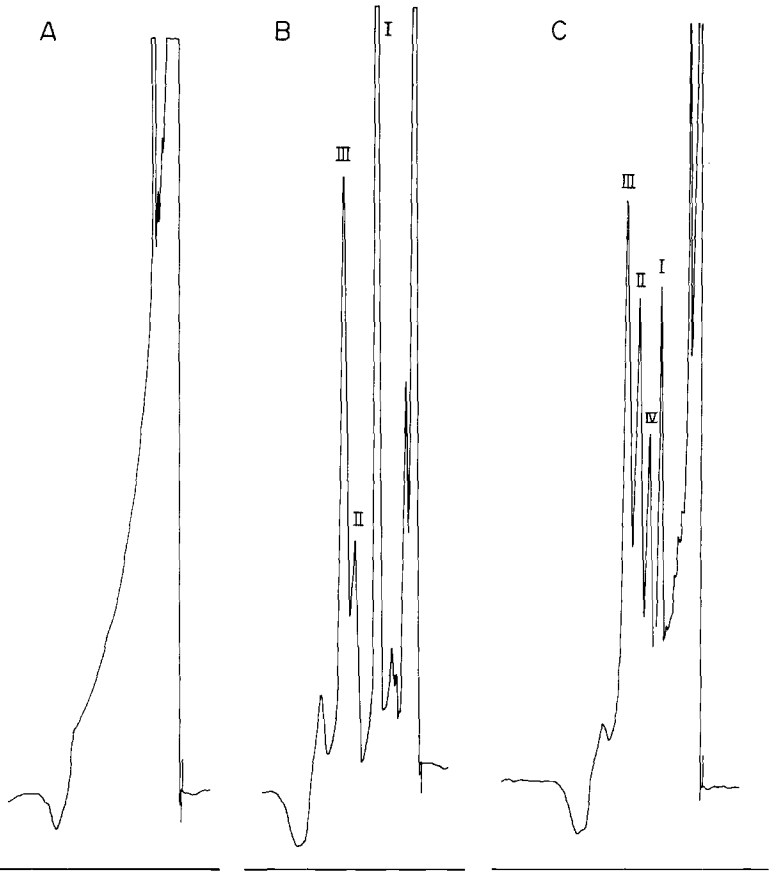

FIG. 6—(A) *Extracted blank plasma.* (B) *Plasma screened positive for diazepam* (I), *desmethyldiazepam* (II), *and flunitrazepam* (*internal standard*) (III). (C) *Plasma screened positive for diazepam* (I), *desmethyldiazepam* (II), *desalkylflurazepam* (IV), *and flunitrazepam* (*internal standard*) (III).

had retention volumes greater than 25 ml. Increasing the methanol concentration to 73% resulted in the elution of these two benzodiazepines. The retention volumes of the benzodiazepines are shown in Table 1.

The HPLC characteristics of the benzodiazepines on a reverse phase column are similar to those described by Osselton et al [7] when they used a Spherisorb-ODS column to identify these drugs following enzymatic digestion of liver tissue.

It was found that blank blood and plasma gave no interfering peaks on either HPLC solvent system when carried through the screening and confirmation procedures (Fig. 7*a*). Figures 7*b* and *c* show liquid chromatograms obtained from plasma samples that were positive for diazepam, desmethyldiazepam, and desalkylflurazepam by the GLC-ECD screening procedure. The HPLC method has proved particularly useful for the quantitation of chlordiazepoxide and its major metabolite. Symmetrical peaks are obtained (Fig. 7) and the linear range is adequate to cover anticipated blood and plasma concentrations (Figs. 4 and 5) that are likely to be encountered in toxicology cases.

In cases of overdose as little as 250 μl of sample is sufficient to quantitate chlordiazepox-

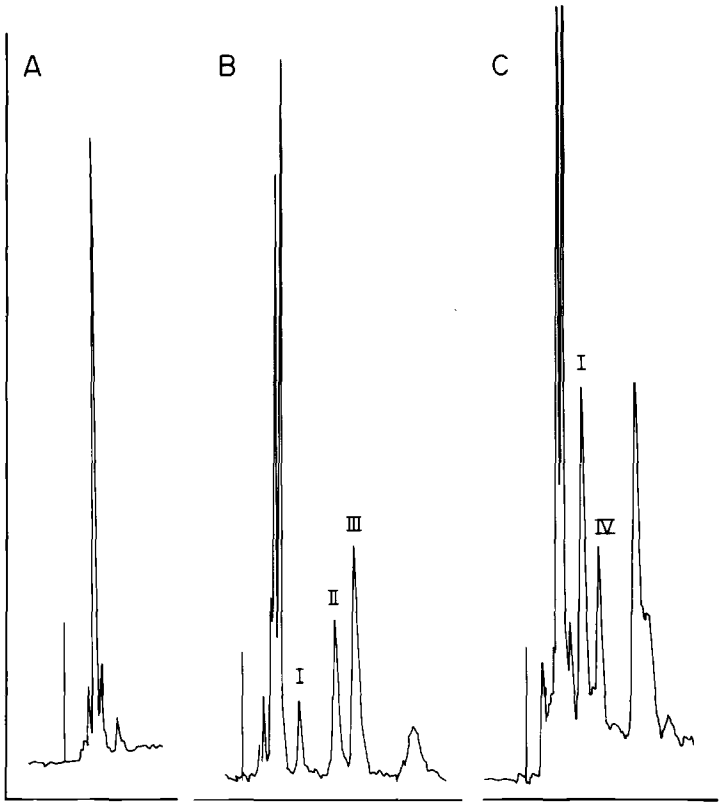

FIG. 7—(A) *Extract by HPLC of blank plasma.* (B) *Extract by HPLC of plasma containing 1.1 μg/ml diazepam* (II), *0.6 μg/ml desmethyldiazepam* (III), *and 0.2 μg of flunitrazepam* (I). (C) *Extract by HPLC of plasma containing desalkylflurazepam* (IV) *and 0.2 μg of flunitrazepam* (I).

ide and desmethylchlordiazepoxide. Unfortunately, HPLC confirmation of the other benzodiazepines requires 1 ml of specimen, although the use of a variable-wavelength ultraviolet detector may very well permit reduction of this sample volume.

Summary

A combined GLC-ECD and HPLC procedure has been developed for the analysis of the most commonly encountered benzodiazepine drugs and has been applied to both plasma and postmortem blood samples. There is no doubt that since their introduction the use of these sensitive analytical methods have resulted in an increase in the incidence of detection of these drugs in both clinical and forensic toxicology cases.

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