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DETERMINATION OF DIAZEPAM AND ITS PHARMACOLOGICALLY ACTIVE METABOLITES IN BLOOD BY BOND ELUT™ COLUMN EXTRACTION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and quantitative analytical micro method for the determination of diazepam and its major pharmacologically active metabolites utilizing high-performance liquid chromatography (HPLC) is reported. The drug and its metabolites were extracted from 50—100 μl samples of whole blood, serum or plasma using Bond Elut™ C_{18} column and quantitated by high-performance liquid chromatography, using Technicon Fast-LC-C-8 (RP 5 μm) bonded column and a mobile phase consisting of 53% methanol, 1% acetonitrile in KH_2PO_4 buffer and 10 μl/l triethylamine. Methyl nitrazepam and medazepam were used as internal and external standards, respectively. The extraction and recovery of diazepam and its major pharmacologically active metabolites, i.e., 3-hydroxydiazepam, desmethyldiazepam and oxazepam from blood were higher than 88% for all compounds. The minimum detection range of each compound was approximately 2.5 ng per 100-μl sample. This micro method of simultaneous quantitation of diazepam and its major pharmacologically active metabolites provides a valuable technique for the study of diazepam pharmacokinetics in a small animal model without disturbance of normal hemodynamics from excess blood loss, as well as in clinical evaluation of pediatric patients.

INTRODUCTION

Since the introduction of diazepam as an anxiolytic sedative hypnotic in 1959, many benzodiazepines have been used clinically, in a wide spectrum of disorders, for controlling insomnia and anxiety and producing central skeletal muscle relaxation. Likewise, several quantitative analytical procedures for determining benzodiazepines and their metabolites in biofluids have been developed utilizing colorimetry [1], spectrofluorometry [2, 3], gas chromato-

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graphy (GC) [4—6], polarography [7], radioimmunoassay [8, 9], and thin-layer chromatography with densitometry [10, 11].

Among all the methods available in the past, GC has provided adequate sensitivity and resolution for clinical investigation when combined with the electron-capture detector [5]. However, time-consuming extraction followed by derivatization procedures for determination of metabolites are required before application to the GC column. Furthermore, some of the benzodiazepines and their metabolites (for example, oxazepam and chlordiazepoxide are highly thermolabile and cannot be subjected to the high temperature needed for GC analysis [6—21].

Since the development of high-performance liquid chromatography (HPLC), efficient high-performance column packing techniques, and also a choice of a wide variety of bonded phase packing materials were made available, HPLC has become a method of choice for determination of drugs and their metabolites in biofluids, which are thermolabile, and have the distinct hydrophobic physicochemical properties suited for HPLC detection.

Several papers reporting analysis of benzodiazepines and their metabolites by the HPLC [11—16] method have been published. Most of these methods [17—19] are suitable for clinical evaluation of benzodiazepines and their representative metabolites concentrations in human plasma samples of 0.5 to 1.0 ml and therefore, the sample volume is not restricted. However, for the study of the pharmacokinetics of benzodiazepines and their metabolites in small animals, the availability of biofluid in amounts required in the previously published procedures, without disturbing hemodynamics, is a major problem.

Therefore, development of a microanalytical procedure which has a high sensitivity and resolution for drug detection from microquantities (50—100 μl) of biofluid is essential. Accordingly, this study reports the further refinement of presently available methods in the literature, utilizing the HPLC system to fit the analysis of diazepam and its metabolites, for example, from microquantity biofluid samples in conjunction with highly efficient Bond Elut^{TM} C_{18} column extraction.

EXPERIMENTAL

Reagents and drugs

Methanol and acetonitrile of analytical spectrophotometric grades, and water of HPLC grade were purchased from Burdick and Jackson Labs. (Muskegon, IL, U.S.A.) and J.T. Baker (Phillipsburg, NJ, U.S.A.), respectively. Inorganic reagents were reagent grade from Fisher Scientific (Pittsburgh, PA, U.S.A.).

The Bond Elut^{TM} C_{18} extraction column was obtained from Analytichem International (Harbor City, CA, U.S.A.).

Preparation of drug reference standards

Pure crystalline forms of diazepam, 3-hydroxydiazepam (temazepam), desmethyldiazepam (nordiazepam), oxazepam, medazepam and methylnitrazepam for reference standards were generously supplied by Hoffmann-La Roche (Nutley, NJ, U.S.A.).

Ten milligrams each of diazepam, 3-hydroxydiazepam, nordiazepam and

oxazepam were weighed and dissolved in 10 ml methanol in a siliconized volumetric flask (1 mg/ml concentration). The solution was further diluted under sonication to reach the final concentration ranging from 35 ng/ml to 25 μg/ml, either in mobile phase solution (for direct injection into HPLC), or in HPLC-grade water, or in blood, plasma or serum (for extraction and recovery studies). Ten milligrams each of medazepam (external standard) and methylnitrazepam (internal standard) were also weighed and dissolved individually in 10 ml methanol. Medazepam was further diluted in the mobile phase solution to achieve a final concentration ranging from 100 ng/ml to 10 μg/ml. Methylnitrazepam was further diluted into HPLC-grade water under sonication to achieve a final concentration of solutions ranging from 25 ng/ml to 25 μg/ml.

Preconditioning of column and extraction procedure

Bond Elut C_{18} columns (1 ml capacity) were positioned in luerlock fittings on the cover of a Vac-Elut™ system. Vacuum pressure was adjusted to 15 in. of mercury. Each column was prewashed repeatedly with a column volume of methanol and then with water through open-and-close processes of the vacuum pressure line.

After the last water wash the column was buffered to pH 9.5 by 100 μl 0.1 *M* sodium borate buffer (pH 9.5). Finally, the column was heparinized by 50 μl 1000 units/ml heparin. The preconditioned columns can be capped and refrigerated for future use.

For extraction, 50 of 100 μg of whole blood, plasma or serum containing the drug and its metabolites were applied to the column. Internal standard methylnitrazepam 100 ng in a 10-μl volume was applied to the column at this step. The sample was drawn through the column by vacuum and the drug and the metabolites were adsorbed on the column matrix. The matrix was then washed twice with water, followed by 50 μl of methanol. This small amount of methanol displaces water from the matrix, but does not elute the drug from the column. Vacuum was then turned off.

A microcentrifuge tube was placed under each extraction column in the Vac-Elut rack for drug elution procedures. With vacuum off, 200 μl of methanol were added to each column in which the drugs were adsorbed. After 2 min of equilibration, vacuum was turned on to elute the materials into the collection tube. Vacuum was turned off and the column was allowed to come to atmospheric pressure. An additional 100 μl of methanol were placed onto the column and the same procedure of elution was repeated in order to complete the elution process. The vacuum was left on for 30 sec in order to collect all the eluent. The eluent totalling 300 μl was dried under a stream of nitrogen. The residue was reconstituted in 25 μl of mobile phase liquid, or with external standard. A 10-μl volume of this reconstituted sample extract solution was injected into the chromatograph.

HPLC analytical procedure

A Hewlett-Packard high-performance liquid chromatograph Model No. 1081, fitted with a Rheodyne syringe loading sample injector with a 10-μl loop was used. The Schoeffel variable-wavelength ultraviolet detector was attached and the wavelength was set at 240 nm (0.002 AUFS).

The column was the Technicon Fast-LC-C-8 (RP 5 μm) bonded prepacked column (150 mm × 4.6 mm; Technicon, Tarrytown, NY, U.S.A.).

The HPLC mobile phase consisted of methanol—2 m*M* KH_2PO_4—acetonitrile (53:46:1). The flow-rate was maintained at 1.3 ml/min at 51 bar. The column temperature was set at 35°C.

Under reversed-phase chromatography conditions, the capacity factor (k') of diazepam was calculated and was 3.90 as defined by $k' = (V_2 - V_0)/V_0$, where V_2 = retention volume. Separation factor (α) values given in Table I were calculated for each compound by dividing the k' value of that compound by the k' value of diazepam.

The chromatogram was recorded on an *X*—*Y* recorder (1 mV gain 0.25 in./min speed) and the data processor was used to calculate the area and/or peak height of each compound analyzed.

RESULTS

Recovery of diazepam and its major metabolites from biofluid by Bond Elut C_{18} column

Fig. 1B shows a chromatogram of diazepam and its metabolite standards from blood extract through a Bond Elut C_{18} column. Blood containing 100 ng each of methylnitrazepam (internal standard), oxazepam, 3-hydroxydiazepam, nordiazepam and diazepam was extracted through the Bond Elut C_{18} column and chromatographed. Fig. 1A shows the chromatogram of the control blood sample which did not contain any of these compounds, but was extracted through the Bond Elut column.

Fig. 2 plots the concentration response relationships of diazepam and its three major metabolites, 3-hydroxydiazepam, nordiazepam, and oxazepam, which were spiked into 100 μl blood, recovered from the Bond Elut C_{18}

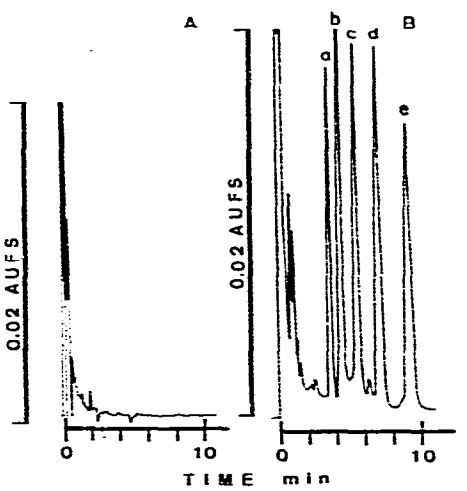


Fig. 1. Chromatograms of (A) blank control blood sample (B) diazepam and its metabolite standards. Blood containing 100 ng each of (a) methylnitrazepam (internal standard), (b) oxazepam, (c) 3-hydroxydiazepam, (d) N-desmethyldiazepam and (e) diazepam was extracted through the Bond Elut C_{18} column.

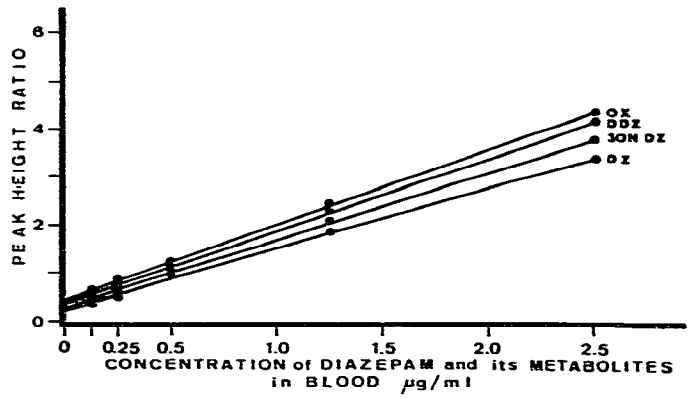


Fig. 2. Recovery concentration response curves of diazepam and its metabolites. Abscissa: initial concentrations of compound in 100 μl whole blood. Ordinate: response expressed as peak height ratio of each compound vs. medazepam as external standard. Curves: OX = oxazepam, DDZ = N-desmethyldiazepam, 3-OHDZ = 3-hydroxydiazepam and DZ = diazepam. Regression slope value ± S.E. (slope) was 1.75 ± 0.07 for oxazepam, 1.59 ± 0.06, for nordiazepam, 1.49 ± 0.06 for 3-hydroxydiazepam and 1.34 ± 0.05 for diazepam. Correlation coefficient for each slope was > 0.994.

TABLE I

HPLC EVALUATION OF SEPARATED DRUG DIAZEPAM AND METABOLITES ON TECHNICON FAST-LC-C-8 BONDED REVERSED-PHASE COLUMN

Compound	Retention time (min) ± S.E.	Capacity factor (k')	Resolution factor (α)
Methylnitrazepam	4.04 ± 0.009	1.05	0.27
Oxazepam	4.84 ± 0.009	1.45	0.37
3-Hydroxydiazepam (Temazepam)	5.99 ± 0.01	2.04	0.52
Desmethyldiazepam (Nordiazepam)	7.57 ± 0.01	2.84	0.73
Diazepam	9.65 ± 0.02	3.90	1.00

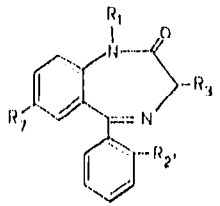
column, and were analyzed by HPLC with medazepam as external standard. As shown in Fig. 2, the relationship was linear for each compound and the quantities tested.

Table I shows HPLC evaluation of separated drugs, methylnitrazepam (internal standard), diazepam, oxazepam, 3-hydroxydiazepam, nordiazepam, and diazepam; retention time, k' and α for each of the above compounds are shown.

Table II shows the efficiency of the extraction and recovery of diazepam and its three major metabolites, i.e., 3-hydroxydiazepam, nordiazepam and oxazepam spiked into 100 μl blood, extracted and recovered through the Bond Elut C_{18} column. The quantities of these compounds tested ranged from 2.5—1000 ng (concentration range 62.5 ng/ml—2.5 μg/ml). As shown in Table II, the percentages of each compound which were recovered were consistently above 88%, on average 93%. Additionally, the recovery efficiency of methylnitrazepam, internal standard was tested and was 96.97 ± 0.54% S.E. at 100-ng quantities.

TABLE II

INTRA-ASSAY RECOVERY OF DIAZEPAM AND ITS METABOLITES FROM BLOOD



Compounds	Chemical structure				Retention time ± S.E. (min)	Spiked blood concentration (ng/100 μl)					
	R_1	R_3	R_7	$R_{2'}$		250	125	50	25	12.5	6.25
						Recovery ± S.E. (n = 12)					
Oxazepam	H	OH	Cl	H	4.84 ± 0.008	93 ± 2.0	93 ± 2.4	96 ± 3.1	88 ± 3.6	92 ± 3.2	98 ± 4.8
3-Hydroxydiazepam	CH_3	OH	Cl	H	5.99 ± 0.01	92 ± 1.63	94 ± 2.2	89 ± 3.3	90 ± 2.7	90 ± 3.29	94 ± 6.0
Desmethyldiazepam	H	H	Cl	H	7.57 ± 0.01	93 ± 1.02	99 ± 2.1	98 ± 2.9	91 ± 2.9	92 ± 3.3	93 ± 5.1
Diazepam	CH_3	H	Cl	H	9.65 ± 0.02	95 ± 2.0	95 ± 2.5	93 ± 2.9	91 ± 2.7	90 ± 3.7	89 ± 4.6

DISCUSSION

The analytical method for simultaneous quantitation of diazepam and its major metabolites from microquantities of blood reported herein, was based on a combination of two processes. One used the Bond Elut C_{18} column to achieve high extraction efficiency and recovery of the parent drug and its metabolites from microquantities of biofluids (50—100 μl); the second used the HPLC system with a Technicon Fast-LC-C-8 (RP 5 μm) column and the mobile phase to identify these compounds with high resolution and accuracy. As shown in the Results section, the quantity response relationship between the amount of diazepam and its metabolites in biofluid and the peak heights (or areas) registered on the HPLC tracings was linear in the quantity range between 2.5 ng and 1 μg. This quantity range will approximate the actual biofluid concentration ranges (50 ng/ml—25 μg/ml) of diazepam and its metabolites which are achieved in clinical situations, and also experimental laboratory conditions, utilizing small animals, from 50—100 μl volume samples, especially since, recently, the smaller volume loop for sample injection has become available for the HPLC system.

The Bond Elut column system was preferable to many extraction systems hitherto reported because of its reproducibility for extraction, and for the simplicity of the procedure. It requires only a one-step extraction and elution and, hence, significantly minimizes loss of drugs. Good and Andrews [20] have recently characterized the Bond Elut column and have extended their studies to determine the type of drugs which can be retained by the Bond Elut column and, hence, be used in combination with the HPLC system for quantitation of the drugs and their major metabolites in biofluid samples. In agreement with these investigators, the efficiency of elution was maximal with a 200-μl methanol volume followed by an additional 100-μl methanol volume in completing elution of benzodiazepines. The column can be preconditioned with an appropriate pH buffer; in our studies pH 9.5 was found to be most appropriate for adsorption of diazepam and its metabolites. When the column was preconditioned with heparin, the collected blood could go directly into the extraction column. Furthermore, on-column hemolysis of red blood cells is accomplished by simple application of HPLC grade water onto the column. Other benzodiazepines and their metabolites such as chlordiazepoxide and flurazepam can be extracted by a Bond Elut column and quantitated by HPLC from a small quantity of biofluid.

Although medazepam has been found suitable as an external standard for the consistency and linearity of detection responses in wide concentration ranges, it was found inadequate as an internal standard because of its poor recovery from the Bond-elut column extraction system. In this respect methylnitrazepam was found most suitable as an internal standard in reflecting extractions and recoveries of diazepam and its major metabolites.

In summary, an HPLC method in conjunction with a Bond Elut C_{18} column for efficient extraction of diazepam and its major metabolites from microquantities of biofluid has been reported. The method is relatively simple and also reproducible, therefore, suitable for pharmacokinetic studies in small laboratory animals and/or in pediatric patients where the biofluid sample

volume is the major limiting factor, particularly if normal hemodynamics is to be maintained. Simultaneous detection and measurement of the parent compound and its major metabolites is also essential for other benzodiazepine type drugs because they are biotransformed into several pharmacologically active metabolites and, hence the overall pharmacologic action is considerably complex.

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