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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF DIAZEPAM, OXAZEPAM AND N-DESMETHYLDIAZEPAM IN HUMAN BLOOD

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SUMMARY

We describe a rapid method for precisely measuring concentrations of diazepam, oxazepam and N-desmethyldiazepam in blood by high-pressure liquid chromatography. The drugs, together with an internal standard, prazepam, are extracted from 2 ml of blood and analyzed isocratically on a reversed-phase column with a mobile phase consisting of acetonitrile–0.01 *M* sodium acetate buffer (35:65 v/v). The eluted drugs are detected by their absorption at 240 nm. The sensitivity of this method is 30 μg/l for oxazepam and N-desmethyldiazepam, 40 μg/l for diazapam, for 2-ml blood samples. Relative recovery of added drugs to blood varies from 91 to 110%. The day-to-day precision (coefficient of variation) established by 10 replicate analyses was 2.8 to 9.6%.

INTRODUCTION

Benzodiazepines were first introduced in 1959 as psychotherapeutic agents and are still used primarily for this purpose[1]. The current opinion is that more information about concentration–response relationship is necessary for effective monitoring of dosage schedule of diazepam in the treatment of anxiety[2]. N-Desmethyldiazepam, a psychoactive metabolite of diazepam has also been shown to cause concentration-dependent variations in response.

All benzodiazepines appear to undergo biotransformation in the liver prior to excretion. Diazepam is converted to N-desmethyldiazepam and oxazepam. The presence and persistence in the body of both N-desmethyldiazepam and oxazepam may be significant because both possess significant anticonvulsant properties[3,4]. Therefore, it is important to monitor the concentration of all three compounds in the blood for an effective dosage scheduling.

Reliable methods for the determination of diazepam and its metabolites in blood are necessary for studies of absorption, distribution, excretion and biotrans-

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formation of these drugs. Unfortunately, the determination of blood concentrations of diazepam is more difficult than most other anticonvulsants since very low levels result from the low dosage administered.

Several methods involving colorometric[5], spectrometric[6], and gas chromatographic[7,8] techniques have been employed to analyze the benzodiazepines class of compounds in physiological fluids. High-pressure liquid chromatography (HPLC) has particular advantages for the analysis of benzodiazepines in that compounds may be analyzed without an initial derivatization. Analysis of benzodiazepines mixtures by HPLC have been reported by several authors. Mixtures of intact benzodiazepines have been separated on silica[9], anion-exchange[10], cation-exchange resins[11], Durapak OPN[12] and Carbowax 400 [13] coated support. Although many of these procedures have been developed for the separation of diazepam from other benzodiazepines, no technique has been reported for the chromatographic resolution of diazepam and its metabolite by LC. Many of these methods have been used for the separation of microgram quantities of these drugs, which are not suitable for clinical assay. None of these reported methods has been successfully adapted for the analysis of diazepam and its metabolites from blood or other physiological fluids. This communication describes such an analysis performed by HPLC on a reversed-phase column. Our method provides adequate sensitivity (20–30 ng) for therapeutic monitoring of these compounds.

MATERIALS AND METHODS

Chromatography

We used a model 601 (Perkin-Elmer, Norwalk, Conn., U.S.A.) high-pressure liquid chromatograph equipped with a variable-wavelength ultraviolet detector (Perkin-Elmer, LC55), a 1-mV Honeywell Electronic 194 recorder, and a 25 cm × 4.5 mm I.D. reversed-phase column Partisil-10 ODS (Reeve Angel, Clifton, N.J., U.S.A.). The column was eluted with acetonitrile–0.01 *M* sodium acetate buffer (pH 4.6) (35:65, v/v) at the rate of 2.0 ml/min, and the column effluent was monitored at 240 nm. The chromatograph was operated at a pressure of 8.3×10^6 Pa (1200 p.s.i.) and room temperature.

Reagents and standards

All reagents were reagent grade purity and all inorganic reagents were made up in distilled water.

Potassium phosphate buffer (1 *M* was prepared by dissolving) 136.1 g of anhydrous KH_2PO_4 per l of distilled water and adjusting the pH to 7.0 with 1 *M* K_2HPO_4 solution. Diethyl ether was AR grade (Mallinckrodt, St. Louis, Mo., U.S.A.) acetonitrile ACS grade (Eastman-Kodak, Rochester, N.Y., U.S.A.).

Diazepam and N-desmethyldiazepam were kindly supplied by Hoffmann-LaRoche (Nutley, N.J., U.S.A.). Oxazepam was supplied as a gift from Wyeth Labs. (Philadelphia, Pa., U.S.A.). Prazepam was supplied as a gift from Warner-Lambert Research Institute (Morris-Plains, N.J., U.S.A.).

As drug reference standard a standard solution of oxazepam (10 mg), N-desmethyldiazepam (10 mg), diazepam (10 mg), and prazepam (10 mg) was used, which was prepared by dissolving all of them in 100 ml of ethanol. A stock internal standard

of prazepam (10 mg/l) was prepared in ethanol. A working internal standard was prepared by diluting the stock internal standard ten-fold.

Procedure

Deliver 2.0 ml of 1 *M* phosphate buffer (pH 7.0) into 40-ml centrifuge tube. Deliver 2.0 ml of the working internal standard (1 mg/l) into each tube, followed by 2.0 ml of blood. Vortex-mix each tube 5–10 sec to thoroughly mix the buffered aqueous phases. Deliver 20 ml of anhydrous diethyl ether into each tube, shake for 5 min, and then centrifuge at 210 *g* (2000 rpm) for 5 min. Separate the ether phase and discard the aqueous phase. Extract the ether phase with 2.5 ml of 6 *M* HCl and centrifuge at 210 *g* (2000 rpm) for 5 min. Aspirate the ether layer and discard. Add 6 *M* NaOH to the aqueous phase to achieve a basic pH, add 2.0 ml of 1 *M* phosphate buffer (pH 7.0), and deliver 20 ml of anhydrous diethyl ether. Shake for 5 min, then centrifuge at 210 *g* (2000 rpm) for 5 min. Aspirate the ether phase, and evaporate slowly to dryness at 37° with a light stream of nitrogen. Dissolve the residue in 30 μl of ethanol. Inject 10–15 μl into the liquid chromatograph. A simple extraction of the buffered blood solution into anhydrous diethyl ether could be employed for diazepam and N-desmethyldiazepam; however, the analysis of oxazepam is interfered with by extraneous peaks.

RESULTS

We evaluated various chromatographic conditions by injecting a mixture of oxazepam, N-desmethyldiazepam, diazepam and prazepam, 1.0 μg of each in 10 μl of ethanol, varying the composition of the mobile phase, pH, and the molarity of the buffer.

Mobile-phase variation included acetonitrile–water and acetonitrile–sodium acetate buffer. Elution order of the compounds was unaffected, but retention times and resolution of oxazepam and N-desmethyldiazepam were affected. Although, a mobile phase containing the same ratio of acetonitrile–water will elute these drugs, the drug peaks showed substantial tailing. Replacing water with 0.01 *M* sodium acetate eliminated the tailing of these peaks.

The effect of buffer molarity and pH were minimal. We found pH 4.6 and 0.01 *M* sodium acetate to be optimal for the separation of these drugs.

The detector wavelength, 240 nm, is the absorbance maximum for most of benzodiazepines. We compared absorbance at this wavelength and at 254 nm, the wavelength commonly used in fixed wavelength detectors. Although 254 nm could be used in most circumstances, the absorbance is only about 50% of that at 240 nm.

Analytical variables

Standards. 1 μg of the internal standard (prazepam) and of each of the drugs in the standard mixture described above were injected into the column. Fig. 1A shows the resulting chromatogram. Analysis time from the point of injection was approximately 13 min.

A mixture of drug free blood plus known quantities of diazepam (1 mg/l), oxazepam (1 mg/l) and N-desmethyldiazepam (1 mg/l) was taken through the procedure as described. Fig. 1B shows the resulting chromatogram.

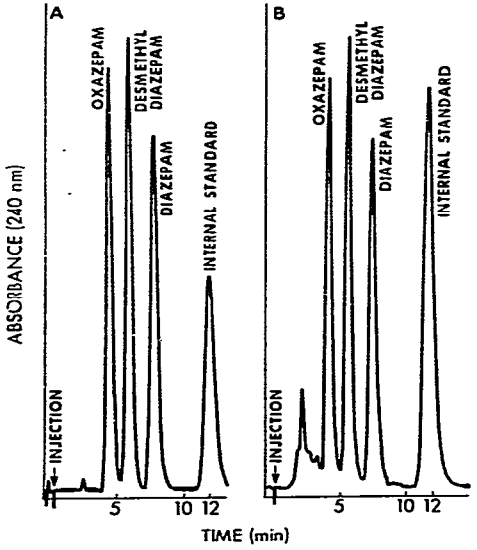

Fig. 1. (A) Chromatogram of a standard mixture of drugs. Quantity chromatographed, 1 μg each of all drugs. (B) Chromatogram of an extract of a mixture of blood and drugs, oxazepam 1 mg/l; N-desmethyldiazepam 1 mg/l; diazepam 1 mg/l; prazepam 2 mg/l.

Quantitation and identification of drugs. We evaluated unknowns by measuring their peak heights relative to that of the internal standard. For the compounds studied, a linear relationship between peak height ratio and concentration was obtained for the range 0.05–10 mg/l. The drugs and internal standard were identified by their relative retention time.

Detection and sensitivity. The drugs and internal standard were detected at 240 nm, the lower limit of detection being 20 ng for oxazepam and N-desmethyldiazepam, and 30 ng for diazepam. The sensitivity of the method allowed for easy quantitation of 50 μg/l of these drugs in a 2-ml blood sample.

Precision and accuracy. Within-run precision was established on two drug-free blood pools. Oxazepam, diazepam and N-desmethyldiazepam were added at two different concentrations. Within-run precision data are included in Table I.

TABLE I

PRECISION OF ASSAYS FOR OXAZEPAM, N-DESMETHYLDIAZEPAM AND DIAZEPAM IN WHOLE BLOOD

S.D. = standard deviation; C.V. = coefficient of variation.

Drug	*Within-run (n = 10)*		*Day-to-day (n = 10)*	
	mg/l (±S.D.)	*C.V. (%)*	*mg/l (±S.D.)*	*C.V. (%)*
Diazepam	0.979 ± 0.030	3.7	0.996 ± 0.060	6.0
	0.214 ± 0.013	5.3	0.201 ± 0.019	9.5
Oxazepam	0.914 ± 0.051	5.6	0.890 ± 0.025	2.8
	0.184 ± 0.012	6.3	0.185 ± 0.012	6.5
N-Desmethyldiazepam	0.940 ± 0.060	6.0	0.930 ± 0.063	6.7
	0.196 ± 0.010	5.1	0.196 ± 0.019	9.6

Similarly, day-to-day precision was established at two different concentrations for these drugs. Table I shows day-to-day precision data.

The absolute recovery from blood of these drugs and the internal standard was evaluated in the following manner. The three compounds were added to drug-free blood to achieve concentrations of 0.1, 0.5 and 1.0 mg/l. These samples were then analyzed by our procedure without any internal standard. Carefully measured aliquots of the extract were then chromatographed and the peak heights determined. Absolute recovery was calculated by comparing these peak heights with peak heights obtained by the direct injection of pure compounds. Absolute analytical recoveries of the drugs ranged from 70 to 75% for oxazepam and 75 to 80% for N-desmethyldiazepam, prazepam and diazepam. In Table II we have shown that the relative recoveries of three drugs at various concentrations vary between 91 and 116%.

TABLE II

RECOVERY OF OXAZEPAM, N-DESMETHYLDIAZEPAM AND DIAZEPAM FROM WHOLE BLOOD ($n = 5$)

Drug	*Drug added (μg)*	*Drug recovered (μg)*	*Recovery (%)*
Diazepam	0.05	0.058	116
	0.1	0.110	110
	0.2	0.197	99
	0.5	0.502	101
	1.0	1 030	103
	2.0	1.950	98
Oxazepam	0.05	0.055	110
	0.1	0.106	106
	0.2	0.190	95
	0.5	0.470	94
	1.0	0.910	91
	2.0	1.930	97
N-Desmethyldiazepam	0.05	0.057	114
	0.1	0.103	103
	0.2	0.194	97
	0.5	0.475	95
	1.0	0.940	94
	2.0	1.890	95

Patients specimens. Blood specimens drawn 1.5 h following a single oral dose of 10 mg of diazepam were assayed for diazepam and N-desmethyldiazepam. The concentration of diazepam varied from 80 to 150 μg/l, while N-desmethyldiazepam concentration ranged 50 to 80 μg/l. We could not detect any measurable amount of oxazepam in these specimens.

Background. Ten drug-free blood samples were processed to determine if any normal component(s) of blood in the extract interfered with the determination of these drugs. The background was negligible in all areas except in 2 samples which revealed peaks in the oxazepam region that were approximately 5–10 μg/l, a level totally insignificant relative to therapeutic levels.

Interference. We checked for possible interference from other commonly used drugs (see Table III). Propoxyphene, methadone, imipramine, phencylidine and desipramine were not detected under the conditions used. Phenobarbital, phenytoin and primidone were eluted from the column well before oxazepam. Carbamazepine is eluted from the column at the same time as oxazepam, and may interfere with

oxazepam analysis. Amitriptyline elutes from the column at 7.5 min, and may interfere with diazepam analysis. Flurazepam is not eluted from the column under the conditions used. Medazepam is eluted at 22.0 min, well after the internal standard.

TABLE III

RETENTION TIMES OF SOME OTHER DRUGS

Drug	*Retention time (min)*
Ethosuximide	4.5
Carbamazepine	4.0
Methaqualone	4.5
Glutethimide	3.5
Phenytoin	3.0
Phenobarbital	2.5
Primidone	2.5
Amitriptyline	7.5
Propoxyphene	N.D.
Methadone	N.D.
Imipramine	N.D.
Desipramine	N.D.
Oxazepam	4.0
N-Desmethyldiazepam	5.5
Diazepam	7.5
Prazepam (internal standard)	12.0
Flurazepam	Not eluted
Medazepam	22.0

N.D. = not detected.

DISCUSSION

The method described is adequately sensitive to monitor diazepam and its metabolites at the low therapeutic concentrations normally found in blood. These drugs were quantifiable in the blood of individuals 1.5 h following a single oral dose of 10 mg of diazepam. Although the simultaneous extraction of all three drugs from blood requires several steps, diazepam and N-desmethyldiazepam can be analyzed following a single solvent extraction.

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