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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DIAZEPAM AND N-DESMETHYLDIAZEPAM IN BLOOD

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SUMMARY

High-performance liquid chromatography on porous silica has been employed to determine diazepam and N-desmethyldiazepam in human blood. For forensic purposes, 1.0 ml of blood is sufficient for a quantitative determination of the benzodiazepines in concentrations above 100 ng/ml. In cases where lower levels, 25–100 ng/ml, are of interest, 2.0 ml of blood together with a somewhat more elaborate extraction procedure are necessary.

INTRODUCTION

A large number of papers have been published on the determination of diazepam and its metabolites in body fluids and tissues (for reviews, see refs. 1 and 2). In this laboratory, a similar gas chromatographic method to that of Berlin *et al.*[3] is employed for the determination of diazepam and the metabolite N-desmethyldiazepam in blood. However, under the chromatographic conditions used, an unknown component with the same retention time as diazepam frequently appeared in a number of the blood samples analysed. These diazepam peaks were broader than those obtained from spiked blood samples.

The use of high-performance liquid chromatography (HPLC) to determine benzodiazepines and their metabolites at the microgram level in urine from a dog has been reported by Scott and Bommer[4]. Liquid chromatography of diazepam and some other 1,4-benzodiazepines has also been reported by Weber[5], Rodgers[6] and Macek and Řehák[7] in standard mixtures and in pharmaceutical preparations. The use of HPLC for the quantification of diazepam or its N-desmethyldiazepam metabolite in blood has, to my knowledge, not been employed as a routine method.

In forensic toxicology, it is important to have at least two different and sensitive methods for the determination of unknown drugs in body fluids and tissue. For this purpose, an HPLC method has been developed to supplement the existing gas chromatographic method for the determination of diazepam and N-desmethyldiazepam in blood.

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Waters Associates Model 202/6000 with a U6K Universal injector. The detector was a Perkin-Elmer LC-55 variable wavelength detector connected to a Varian 2500 recorder. The column was a Reeve-Angel steel column (25 cm × 4.6 mm I.D.) packed with Partisil 10 (particle size, 10 μm). The chromatographic conditions were as follows: mobile phase, *n*-heptane–isopropanol–methanol (40:10:1); flow-rate, 1.0 ml/min; pressure, 600 p.s.i.; column temperature, ambient; detector, UV at 232 nm.

Tubes and pipettes were washed with concentrated nitric acid and silanized with 3% v/v dichlorodimethylsilane in toluene.

Chemicals and reagents

Diazepam and N-desmethyldiazepam were donated by F. Hoffmann-La Roche Basle, Switzerland. Pesticide-grade benzene was obtained from Fisher Scientific, New Jersey, U.S.A. The phosphate buffer (pH 7.2) had an ionic strength of 1.5 *M*. All of the other chemicals and solvents were of analytic grade and were used without further purification.

Preparation of blood samples

Solutions of diazepam and N-desmethyldiazepam in methanol (1.0 mg/ml) were diluted ten times with water. These stock solutions were diluted with blood to give standard solutions covering the range 25–1500 ng/ml.

Extraction procedures

In a 16-ml centrifuge tube, 1.0 ml of blood was mixed with 1.0 ml of a saturated solution of potassium chloride, shaken mechanically for 30 min at room temperature with 11.0 ml of benzene and centrifuged. Of the benzene, 10.0 ml were transferred to a 16-ml centrifuge tube and shaken mechanically with 4.0 ml of 6 *N* hydrochloric acid for 30 min at room temperature. After centrifugation, 3.0 ml of the hydrochloric acid were transferred to a 16-ml centrifuge tube and cooled in ice water. The pH was adjusted to 7.2 by the addition of 1.0 ml of phosphate buffer and 2.5–3.0 ml of 6 *N* sodium hydroxide solution. The aqueous phase was extracted at room temperature with 11.0 ml of benzene by shaking it for 30 min. Of the organic layer, 10.0 ml were transferred to a tapered tube, warmed in a water-bath to 40–50° and evaporated to dryness in a stream of nitrogen.

For samples of concentration less than 100 ng/ml, double extractions at each step are necessary. In the range 25–50 ng/ml, one must start with 2.0 ml of blood mixed with 2.0 ml of the saturated solution of potassium chloride.

HPLC analysis

The residue after evaporation was dissolved in 200–400 μl of the mobile phase. Usually, 40–80-μl aliquots were injected into the liquid chromatograph. The concentrations in the unknown blood samples were determined from calibration graphs constructed for extracted blood standards by measuring the peak heights at 5–8 different concentrations. After each fifth injection, the column was run at a flow-rate of 3–4 ml/min for *ca.* 10 min.

RESULTS AND DISCUSSION

Chromatographic conditions

Liquid chromatography of benzodiazepines has been performed on columns packed with coated supports or pellicular or porous silica adsorbents and with a variety of solvents. In the present paper, high-performance 10-μm porous silica was used with *n*-heptane–isopropanol–methanol (40:10:1) as the mobile phase. The variable UV detector was set at 232 nm, *i.e.*, the wavelength of maximum absorbance for both diazepam and N-desmethyldiazepam. With a 25 cm $\times$ 4.6 mm I.D. column and a solvent velocity of 1.0 ml/min, the retention volumes were 6.2 ml for N-desmethyldiazepam and 7.0 ml for diazepam and with a near to baseline separation. With mixtures of the pure compounds, 2 ng of each component gave peaks which were about four times as intense as the noise level. A plot of peak height *versus* injected amount showed a linear relation up to at least 1500 ng. Using linear regression analyses, correlation coefficients of 0.9994 were obtained for both diazepam and N-desmethyldiazepam.

Extraction

The extraction of diazepam and N-desmethyldiazepam from blood is similar to the method used by Berlin *et al.*[3]. According to this procedure, complete extraction of diazepam and N-desmethyldiazepam is obtained when 100 μl of plasma and 100 μl of a saturated solution of potassium chloride are shaken with twenty times the volume of benzene. In HPLC, using a UV detector, it is necessary to use 1.0 ml or more of blood in order to be able to determine the low blood levels of diazepam and N-desmethyldiazepam. A direct scale-up of the procedure of Berlin *et al.*[3] to 1–2 ml of blood is not practical because of the large volumes of benzene which would be necessary. However, the partition of diazepam and N-desmethyldiazepam between equal volumes of the blood–potassium chloride solution and benzene has been shown to give complete distribution of diazepam and 94% distribution of N-desmethyldiazepam into the organic phase. Consequently, with a four-fold excess of benzene, a better than 98% extraction should be obtained.

Because of the selectivity of the electron-capture detector in gas chromatography, a single extraction is sufficient to determine the benzodiazepines in question. In HPLC, a further purification of the benzene extract was found to be necessary. The extraction of diazepam and N-desmethyldiazepam into hydrochloric acid was studied by De Silva and Puglisi[8] and by Zingales[9]. They stated that complete extraction of the drugs into 6 *N* hydrochloric acid can be accomplished without decomposition. The benzodiazepines were consequently extracted from benzene into hydrochloric acid, the aqueous phase was neutralized and the drugs were re-extracted into benzene. The extract was evaporated to dryness and the residue was dissolved in the liquid phase and chromatographed.

Recovery

The recoveries at the 100 ng/ml and the 500 ng/ml levels in blood were determined from 10 parallel extractions and found to be 92 $\pm$ 7% (standard deviation) for both diazepam and N-desmethyldiazepam.

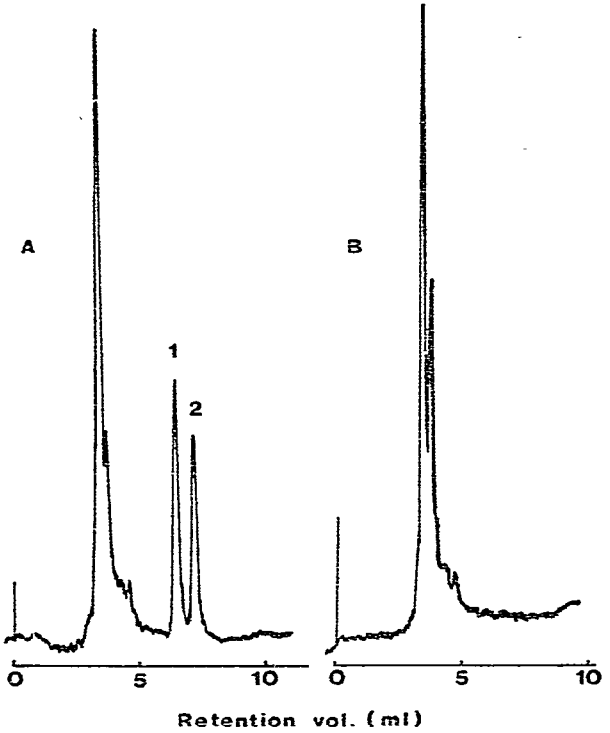

Fig. 1. HPLC chromatograms showing extracts from 1.0 ml of human blood. A, 300 ng per ml of blood of each of diazepam (2) and N-desmethyldiazepam (1); B, blood blank.

Sensitivity

For the purposes of forensic toxicology, I have made routine analyses by HPLC of blood samples containing diazepam and N-desmethyldiazepam for *ca.* 1 year. Because of the high extraction yields and the high molar extinction coefficients of the benzodiazepines at 232 nm, 1.0-ml blood samples were sufficient for the determination of drug levels down to 100 ng/ml. A chromatogram obtained after analysis of a spiked blood sample containing 300 ng/ml of each compound is given in Fig. 1 together with a chromatogram of a blood-blank extraction. In the cases where low therapeutic levels (down to 25 ng/ml) were of interest, 2.0 ml of blood and a somewhat more elaborate extraction procedure were necessary.

Determination in blood

Unknown blood samples containing the benzodiazepines were determined from a calibration graph by measuring the peak heights. The calibration graphs were constructed for a set of extracted standards covering the range 100–1500 ng/ml. Each sample and standard (usually five) was injected twice and the calibration graphs were computed using linear regression analysis. Calibration graphs based on six standards are depicted in Fig. 2.

Some analyses of blood containing less than 100 ng/ml of diazepam and N-desmethyldiazepam were made. In this range also, linear calibration graphs covering

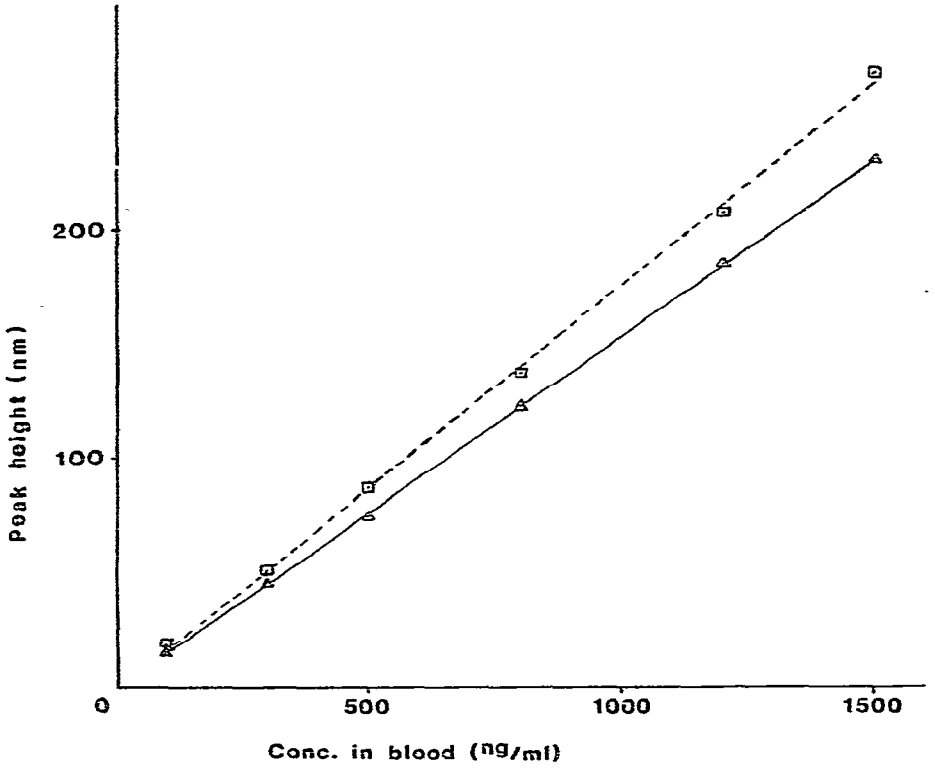

Fig. 2. Calibration graphs for diazepam (△—△), correlation coefficient 0.9998, and for N-desmethyldiazepam (□- - - -□), correlation coefficient 0.9994, added to human blood.

the concentration range 25–100 ng/ml were obtained. Occasionally, the blood extracts contained components with rather high retention volumes. In order to avoid interference between these components and the benzodiazepines, it was necessary to "empty" the column. As a routine procedure, the column was run at an increased flow-rate of 3–4 ml/min for *ca.* 10 min after each fifth injection.

Comparison with gas chromatography

In a recent paper, Whelpton and Curry[10] showed how chlorpromazine interferes with the gas chromatographic determination of diazepam on OV-17 columns and how improved separation can be achieved with a column of OV-225. Our OV-17 column has now been replaced by an OV-225 column. With this column, complete separation between diazepam and the unknown component has been obtained. The determination of diazepam and N-desmethyldiazepam by gas chromatography and by liquid chromatography now shows very good agreement.

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