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Note

Simultaneous determination of diazepam and its active metabolites in rat serum, brain and cerebrospinal fluid by high-performance liquid chromatography

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Comprehensive investigations of the pharmacokinetics and pharmacodynamics of the benzodiazepines require determination of drug and drug metabolite concentrations in plasma or serum as well as in tissues, particularly the brain and cerebrospinal fluid (CSF) [1–3]. Several analytical procedures have been developed for determining concentrations of benzodiazepines and their metabolites in biological fluids by gas chromatography (GC) [4–6] or high-performance liquid chromatography (HPLC) [7–9]. Some of these methods lack the sensitivity and/or specificity needed for small-animal studies. GC methods combined with electron-capture detection are very sensitive but they require time-consuming extraction and derivatization procedures. The high temperatures required for GC are also inappropriate for heat-labile benzodiazepines such as oxazepam (OXP) and temazepam (TZP) [10,11]. HPLC assays particularly designed for small-animal studies have been described [12–14] but they require radioactive drug or they were intended only for analysis of blood or plasma.

Described here is a sensitive and selective HPLC procedure for the assay of serum, brain and CSF from rats for diazepam (DZP) and its pharmacologically active metabolites desmethyldiazepam (DDZP), OXP and TZP.

EXPERIMENTAL

Chemicals

The reference compounds used in the development of this assay were obtained from the following sources: DZP, DDZP and TZP from Hoffmann-La Roche

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(Nutley, NJ, U.S.A.); OXP from Wyeth (Philadelphia, PA, U.S.A.); and carbamazepine from Ciba-Geigy (Summit, NJ, U.S.A.). For preparing the standard solutions, absolute ethanol (Publicker Chemical, Philadelphia, PA, U.S.A.) was used. All other organic solvents used were of HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Dichlorodimethylsilane (Eastman Kodak, Rochester, NY, U.S.A.) was used to silanize glassware.

Extraction procedure

To 0.5 ml or less of serum in a glass centrifuge tube were added 50 μl ethanol containing 5 μg carbamazepine (internal standard) and 1 ml of 0.2 *M* sodium borate buffer (pH 9). To 0.1 ml CSF were added 10 μl ethanol containing 0.1 μg carbamazepine and 0.5 ml borate buffer. Rat brains were hemisected and weighed. To one half of a brain (approximately 0.5 g) were added 0.1 ml ethanol containing 10 μg carbamazepine and 2 ml borate buffer. Brain samples were homogenized in a motor-driven tissue homogenizer with a PTFE pestle. The samples were mixed and then extracted with 10 ml dichloromethane–pentane (1:1) during 1 min of vortexing. After centrifugation (10 min at 10 000 *g*), the upper organic layer was transferred to a silanized conical evaporation vial and evaporated to dryness at room temperature. The residue was dissolved in 0.05 ml methanol and 25–50 μl were injected onto the HPLC column (maintained at room temperature).

Instrument and chromatographic conditions

A Waters Model 6000A HPLC system (Waters Assoc., Milford, MA, U.S.A.) with a U6K injector and a Waters Model 440 absorbance detector set at 254 nm were used with a Waters 30 cm $\times$ 3.9 mm C_{18} μBondapak (10 μm particle size) column and a 7 cm $\times$ 2.1 mm Whatman C_{18} pre-column (37–53 μm pellicular ODS packing, Whatman, Clifton, NJ, U.S.A.). The mobile phase was methanol–0.025 *M* sodium and potassium phosphate buffer (pH 6) (55:45, v/v), and the flow-rate was 2.0 ml/min. Chromatographic peaks were recorded and integrated on a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Preparation of standard curves

DZP, DDZP, OXP and TZP in known amounts were added to serum, brain or CSF from unmedicated rats. The samples were extracted and analyzed by the described procedures. The ratio of the peak area of drug standard to that of the internal standard was plotted as a function of the known concentrations of each compound and inspected for linearity. Regression equations for these relationships were calculated by the method of least squares. The same assay procedure was followed for determining the extraction yields of these compounds from serum and CSF at various concentrations, except that the internal standard, carbamazepine, was added after extraction and the area ratios were compared to those of unextracted methanolic solutions.

Assay specificity

Specificity of the assay was investigated by two separate methods. One method involved connecting a 229-nm detector to the 254-nm detector of the HPLC in

series. Peak-area ratios (relative to the internal standard) were determined for each wavelength, using two integrators. The ratio of the peak-area ratios, 229 to 254 nm, was calculated. This procedure was followed with solutions of authentic standards and with extracts of serum samples obtained from rats treated with DZP (samples were collected approximately 19 min after the start of an infusion of DZP at a rate of 0.206 mg/min). The values of the ratio of peak-area ratios thus obtained were compared. The other method used to test for assay specificity consisted of determining the partition ratio profile of authentic standards and of the benzodiazepines in the serum extracts. Partitioning of the drug between equal volumes of the aqueous phase (pH 9 borate buffer) and an organic phase of dichloromethane–pentane (in six different proportions) was examined. Drug concentrations in the aqueous and organic phases after equilibration were determined as previously described.

Animal studies

Preliminary studies on rats were performed to determine if the assay could detect concentrations of DZP and its active metabolites in serum, brain and CSF at onset of loss of righting reflex. Nine inbred female Lewis rats (Charles River Breeding Labs., Wilmington, MA, U.S.A.), weighing approximately 200 g and maintained on Charles River Rat–Mouse–Hamster formula, were used. The animals were infused with DZP solution (1 mg/ml in 33% polyethylene glycol 3350–distilled water) through the right jugular vein at a rate of 0.206 mg/min, using PTFE tubing. The infusion was stopped when the animals lost their righting reflex. CSF, blood and brain were obtained immediately for determination of DZP and metabolite concentrations.

RESULTS AND DISCUSSION

Assay procedure

Chromatograms of blank serum, brain and CSF are shown in Fig. 1. The blanks are free of interfering peaks at the retention times of interest. The chromatograms in Fig. 1 of blank samples spiked with a mixture of DZP, DDZP, OXP and TZP show a peak at 5.2 min for the internal standard carbamazepine and then for OXP at 6.8 min, TZP at 8.4 min, DDZP at 11 min and DZP at 13.5 min. Thus, all five compounds were cleanly separated in less than 15 min. Serum, brain and CSF samples from rats infused with diazepam, also shown in Fig. 1, exhibit peaks that are sharp, symmetrical and cleanly separated, with no interferences apparent.

The standard curves for all four compounds were linear in the concentration range of 0.025 μg/ml to at least 1.0 μg/ml for CSF and 0.1 μg/ml to at least 10 μg/ml for serum and brain, with r^2 values of 0.980 or higher. The coefficient of variation of the slope value (five standard curves per compound) was about 4% for serum, 5% for brain and about 10% for CSF. Average extraction yields were higher than 90% for all four compounds in serum and CSF in the concentration range 0.025–1 μg/ml for CSF and 0.1–10 μg/ml for serum. The intra-day variability (coefficient of variation based on ten replicates of each sample) ranged from 2 to 8% for all compounds, with the highest variability at the lowest con-

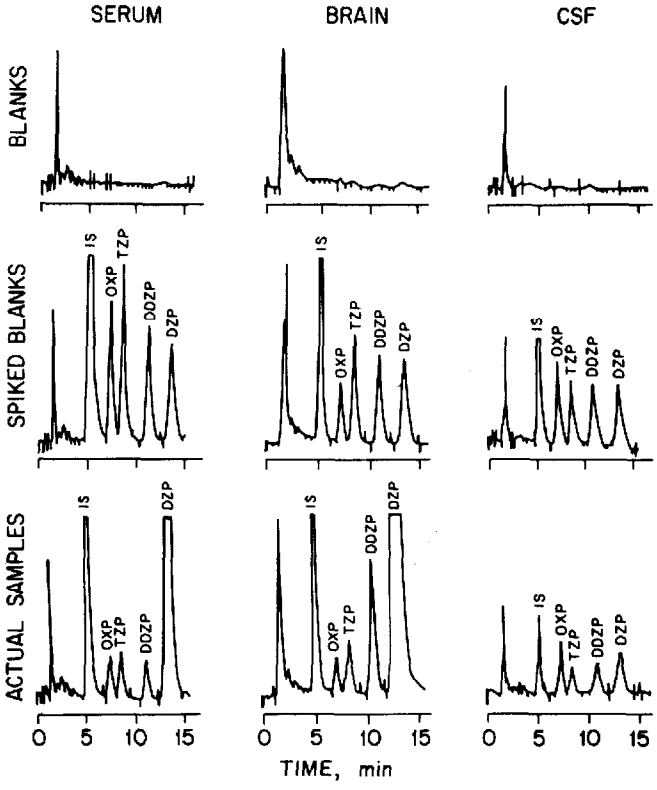

Fig. 1. Chromatograms of serum, brain and CSF from unmedicated rats (upper row), serum, brain and CSF from unmedicated rats with added benzodiazepines and internal standard (middle row), and serum, brain and CSF from rats after a short infusion of DZP. Peaks: DZP = diazepam; DDZP = desmethyldiazepam; OXP = oxazepam; TZP = temazepam; IS = internal standard (carbamazepine).

centration (0.025 μg/ml). Inter-day variability was somewhat higher, ranging from 4 to 12%, the latter at the lowest concentration (0.025 μg/ml). The limit of detection of the assay was 2.5 ng for all four compounds.

TABLE I

CONCENTRATIONS OF DIAZEPAM AND ITS ACTIVE METABOLITES IN RATS AT ONSET OF LOSS OF RIGHTING REFLEX

Compound	Concentrations (mean ± S.D., $n = 9$) (μg/ml or g)		
	Serum	Brain	CSF
Diazepam	4.75 ± 0.75	17.2 ± 2.2	0.36 ± 0.08
Desmethyldiazepam	0.33 ± 0.14	1.19 ± 0.26	0.13 ± 0.13
Oxazepam	0.49 ± 0.16	0.40 ± 0.09	0.09 ± 0.09
Temazepam	0.13 ± 0.07	0.26 ± 0.07	0.04 ± 0.03

Assay specificity

Using two different wavelengths, the ratio of peak-area ratios obtained for each of the four benzodiazepines in serum was not significantly different from the corresponding ratio obtained with pure standards. Thus, no co-chromatographing peaks with a different absorption spectrum were detected. To determine the possible presence of interfering metabolites with essentially the same absorption spectrum but different polarity, partition ratio profiles were determined at pentane–dichloromethane volume ratios of 0:1, 0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2 and 1:0. There were no apparent differences between the profiles of authentic standards and those of the corresponding compounds in serum from rats treated with DZP.

Animal studies

The concentrations of DZP and its active metabolites in the serum, brain and CSF of nine rats at onset of loss of righting reflex are shown in Table I. Despite the fact that the biologic samples were obtained immediately at the end of a short DZP infusion period, all three active metabolites were found in serum, brain and CSF, and the concentrations of DZP and DDZP in brain exceeded those in the serum. The coefficient of variation of the average concentrations reflects both inter-animal and assay variability. The effect of inter-animal variability is probably least on DZP concentrations soon after administration of this drug. It is of interest, in that context, that the coefficient of variation of the plasma and brain DZP concentrations was less than 16 and 13%, respectively, in our study.

The assay method described here should be suitable for pharmacokinetic and pharmacodynamic studies not only of DZP, but of DDZP, OXP and TZP which are marketed benzodiazepine drugs in their own right. Presumably, the assay can be used not only for analysis of fluids and tissues from rats but also from other species. However, this has not been tested. We have used the assay procedure for three years in investigations of the effect of disease states on the pharmacodynamics of benzodiazepines [1,15,16].

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