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Short communication

High-performance liquid chromatographic determination of midazolam in rat brain

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Abstract

A high-performance liquid chromatography method for the determination of midazolam in rat brain is described. Midazolam and the internal standard halazepam were extracted with toluene and analyzed isocratically on a reversed-phase column with a mobile phase consisting of methanol, acetonitrile and potassium phosphate buffer. Detection was monitored by ultraviolet absorption at 240 nm. The standard curves were linear over the range of 25–350 ng midazolam per 50 mg brain tissue. The day-to-day coefficient of variation ranged from 1.7 to 6.9%. The limit of quantification was 80 ng/g brain tissue. The method is rapid, simple and reproducible for brain analysis.

Keywords: Midazolam

1. Introduction

Midazolam, an imidazobenzodiazepine derivative, has been reported to be useful for treatment of generalized convulsive status epilepticus [1–3]. To study the efficacy and pharmacokinetics of midazolam in experimental rat models of status epilepticus, determination of midazolam concentrations in brain is necessary. Many high-performance liquid chromatographic methods have been used to measure midazolam concentration in plasma [4–12], but none have been reported for quantitation of midazolam in brain. Brain assays are more difficult with regard to establishing extraction and chromatographic conditions because brain contains high concentration of both water-soluble compounds and lipids. The method described here is a modification of the liquid chromatography method described by Sautou et al. [4]. This assay was used successfully to quantify rat brain midazolam in the studies of efficacy and pharmacokinetics of midazolam in the treatment of experimental status epilepticus.

2. Experimental

2.1. Reagents

Midazolam was obtained from Sigma (St. Louis, MO, USA). Halazepam was kindly supplied by Schering-Plough (Bloomfield, NJ, USA). NaOH, HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Toluene was obtained from Baxter (Muskegon, MI,

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USA). KH_2PO_4 and K_2HPO_4 were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade water was obtained from a Barnstead (Boston, MA, USA) E-pure water purification system.

2.2. Standard solutions

Stock solutions of midazolam and halazepam were made up in methanol (1 mg/ml) and stored at 4°C in amber bottles. The final working concentration of midazolam and halazepam was 1 ng/μl. Potassium phosphate buffer (1 *M*, pH 5.6) was prepared by mixing 5 ml 1 *M* K_2HPO_4 solution with 95 ml 1 *M* KH_2PO_4 solution. Potassium phosphate buffer 0.025 *M* was then made by diluting 25 ml of the 1 *M* buffer to 1 liter with distilled water.

2.3. Apparatus

The study was performed on a Gilson HPLC system (Gilson, Middleton, WI, USA) equipped with two Model 306 pumps, a Model 805 manometric module, a Model 811C dynamic mixer, a Model 231 XL automatic sample injector, a Model 117 ultraviolet detector and a Model 715 system controller software running on an IBM-compatible, 486 25 MHz CPU microcomputer. A Rheodyne Model 7010 valve (Rheodyne, Cotati, CA, USA) with 20-μl loop was used for injection.

2.4. Chromatographic condition

The separation was carried out in a reversed-phase system with a Rainin C_8 Microsorb column (100× 4.6 mm ID, particle size 3 μm) serving as the stationary phase (Rainin, Emeryville, CA, USA). The mobile phase was methanol–acetonitrile–0.025 *M* potassium phosphate buffer (33:37:130, v/v). Detector wavelengths were set at 240 nm with channel A and B sensitivities of 0.005 and 0.01 AUFS. Flow-rate of the mobile phase was maintained at 0.9 ml/min. Run time was 10 min per sample.

2.5. Sample preparation

Cerebral hemispheres of drug-free or experimental rat brain were weighed and homogenized by sonication in 5 ml 0.1 *M* NaOH (pH 13) per gram of brain. The resulting homogenate volume was measured and 250 μl was used for extraction (approximately 50 mg tissue equivalent; the exact weight was calculated for each sample individually).

For liquid–liquid extractions, 60 ng of halazepam as internal standard was added to 10 ml FEP teflon tubes. Standard curves were prepared by spiking with 25, 50, 100, 200 or 350 ng of midazolam. Tubes were dried in a water bath (50°C) under a stream of nitrogen followed by addition of drug-free brain homogenate to the standard curve tubes and experimental rat brain homogenate to tubes containing only the internal standard. NaOH (0.1 *M*, pH 13, 250 μl) was added and the tubes vortexed thoroughly. Toluene (2.0 ml) was then added to each tube. Tubes were mixed by 50 inversions and centrifuged at 15 000 rpm at 4°C for 20 min. The organic phase was transferred to a glass conical tube and dried under nitrogen. A second 2.0-ml volume of toluene was added to the aqueous phase and the mixing and centrifugation steps repeated. The second organic phase was added to the first in the conical tube and dried. The dried residue was redissolved in 100 μl of mobile phase and 40 μl was injected onto the chromatography system.

2.6. Calculation

The concentration of midazolam was determined by calculating the linear regression of the amount of the added midazolam in nanogram versus peak-height ratio (midazolam/halazepam) for the standard curve. The regression coefficients were then used to quantify the drug concentrations of experimental samples, using the peak-height ratio and weight of tissue in the sample. Each sample was assayed in duplicate and final concentration was calculated from their mean value.

3. Results and discussion

Typical chromatograms of brain samples are shown in Fig. 1, with (A) drug-free rat brain, (B) brain spiked with 50 ng of midazolam and 60 ng of halazepam and (C) brain from a rat which was treated with midazolam and spiked with 60 ng of halazepam as internal standard before extraction. The

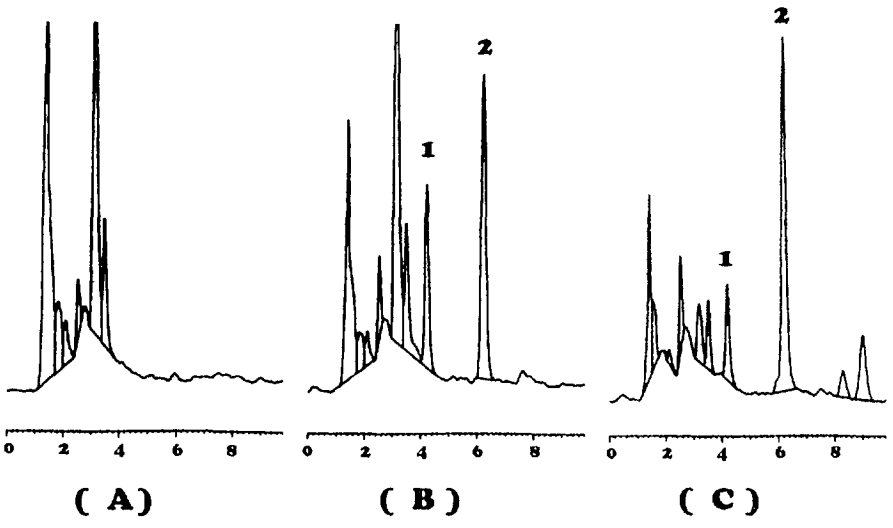

Fig. 1. Chromatograms in brain samples: (A) drug-free rat brain; (B) rat brain spiked with 50 ng midazolam and 60 ng halazepam; (C) brain from a rat treated with midazolam and 60 ng halazepam was added as internal standard prior to extraction. Peaks: 1=midazolam; 2=halazepam.

chromatograms show that midazolam and halazepam are separated completely and none of the brain endogenous components interfere with the assay. Under the conditions described for the assay, the drug and internal standard eluted at 4.2 and 6.2 min, respectively. Standard curves were linear over the range from 25 to 350 ng midazolam. The correlation coefficient of standard curves constructed over a period of five days varied from 0.997 to 1.000. The day-to-day slope averaged 106.296 with a coefficient of variation of 6.7%. Reproducibility of the method was determined by assaying two pools of brain homogenate (each of which had been spiked with a different concentration of midazolam) over a period of five days. Results are shown in Table 1. The coefficient of variation ranged from 1.7 to 2.2% for within-day and from 5.6 to 6.9% for between-day determinations. The recovery of midazolam from brain was 98%, which was estimated by studying peak height ratios of 50 ng midazolam/60 ng halazepam in 12 extracted samples (50 mg brain) and 12 spiked references without extraction. The limit of quantification was 500 ng/g brain when 50 mg brain was used, but can be decreased to 80 ng/g by using up to 1 g brain.

Table 1
Reproducibility of midazolam as say in rat brain

Added (ng)	Within-day[a]		Between-day[a]	
	Found (mean) (ng)	C.V. (%)	Found (mean) (ng)	C.V. (%)
55	55.2	1.7	52.1	6.9
310	316.5	2.2	338.5	5.6

[a] $n=5$.

Fig. 2 compares the brain concentrations of midazolam over 15 min after intravenous drug injection to those found with similar administration of diazepam or lorazepam [13]. The value of measuring these drugs in brain is clear: the rapid onset of action of midazolam, like diazepam, is a result of its very rapid brain entry; its limited duration of anticonvulsant action is the result of rapid redistribution, causing brain levels to drop precipitously. In contrast, lorazepam enters brain more gradually and the brain concentration is maintained over time, leading to a prolonged duration of anticonvulsant action.

Midazolam is a highly lipophilic benzodiazepine, which is rapidly and widely distributed after intravenous injection and easily crosses blood–brain barrier. The significance of these physiochemical properties of midazolam is shown in Fig. 2, where it

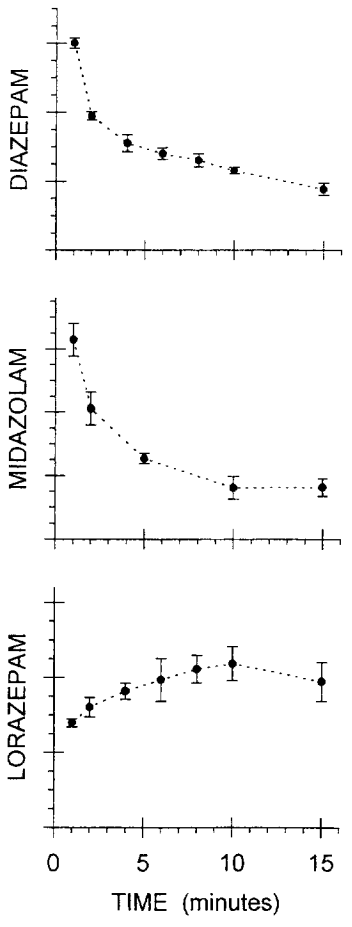

Fig. 2. Entry and exit of three benzodiazepines from brain over 15 min following i.v. injection. Benzodiazepine concentrations have been normalized to ng/g per mg/kg injected, because different drug doses were used for the different benzodiazepines. Each data point is the mean±standard error for 4 rats. The top panel shows diazepam, the middle panel shows midazolam and the bottom panel shows lorazepam.

can be seen to behave much like diazepam in its rate of brain entry and rapid redistribution. These characteristics produce a rapidity and brevity of anticonvulsant action that represent both major advantages and disadvantages as treatments for status epilepticus. Lorazepam, with physiochemical properties that both slow its entry into brain and prevent extensive redistribution, displays a prolonged duration of action against status epilepticus, even though its serum elimination half-life is considerably shorter than that of diazepam [14]. After intravenous injection, midazolam is extensively metabolized in liver to form 1-hydroxymethylmidazolam, the principle metabolite, and 4-hydroxymidazolam, both of which rapidly conjugate with glucuronic acid. The unconjugated metabolites occur only in trace amounts in plasma and are not detectable by either high-performance liquid chromatography or gas chromatography, while the conjugated compounds can be detected in both plasma and urine [4,7,12]. However, the conjugated metabolites do not cross the blood–brain barrier to any significant extent and their potential of suppressing status epilepticus is still not established. We did not analyze these metabolites in brain.

Although many liquid chromatographic methods for midazolam determination have been reported, they were established for assay of plasma samples [5–12]. Sautou et al. [4] recommended solid-phase extraction of midazolam from plasma because of its rapidity, convenience and the small amount of plasma required. Other investigators have used liquid–liquid extractions. In our assay, toluene was used to extract midazolam instead of dichloromethane [5] or a combination of diethyl ether and dichloromethane [8] because we found that toluene extracted many fewer unwanted, endogenous compounds from the brain matrix. Solid-phase extraction sample clean-up was not required with this assay, as can be seen from the chromatograms in Fig. 1. Endogenous components co-extracted with midazolam and halazepam did not interfere with the assay. The method we developed for brain midazolam measurement is rapid (10 min per run), simple and reproducible. It is also sensitive enough for use when the drug is given at doses appropriate for treatment of experimental status epilepticus.

Acknowledgments

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