Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 7, pp. 625–627, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Short Communication

Determination of plasma diazepam and desmethyldiazepam by solid-phase extraction and reversed-phase high-performance liquid chromatography

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Keywords: *Diazapam; desmethyldiazepam; reversed-phase chromatography.*

Introduction

Diazepam is a commonly used anxiolytic drug and is mainly metabolized by demethylation to desmethyldiazepam. Analysis of diazepam and its desmethyl metabolite in biological fluids has involved the use of gas chromatography with electron capture detection [1–2], radioimmunoassay [3–4] and thin layer chromatography with densitometry [5–6]. High-performance liquid chromatography (HPLC) methods for the determination of diazepam and its desmethyl metabolite have also been published, using liquid–liquid extraction [7–8]. Here, a simple and rapid method for the measurement of diazepam and desmethyldiazepam in plasma has been developed which utilizes solid-phase extraction followed by reversed-phase isocratic high-performance liquid chromatography.

Materials and Methods

Apparatus

The chromatographic system employed was an automated Kontron system consisting of a data system (model 450), a dual piston (model 420 pump), an autosampler (model 460) and an ultraviolet detector (model 432). The column was a C_{18} μBondapack (10 μM particle size, 150 mm × 3.9 mm i.d.; Waters Millipore Corporation, USA).

Chemicals

Diazepam, desmethyldiazepam and lorazepam (internal standard) were purchased from Sigma (St Louis, MO, USA). Methanol was purchased from FSA Laboratory Supplies (Loughborough, UK). Potassium dihydrogen orthophosphate was supplied by BDH (Poole, UK).

High-performance liquid chromatography

All experiments were carried out at ambient room temperature (approximately 20°C). The column was equilibrated with the mobile phase for at least 30 min prior to analysis of samples. The mobile phase was potassium dihydrogen orthophosphate (pH 3.5; 30 mM)–methanol (45:55, v/v) pumped at a flow rate of 1.5 ml min^{-1}, pressure 114 bar (1650 psi). Diazepam, desmethyldiazepam and lorazepam were detected by UV absorbance at 229 nm (AUFS 0.002).

Sample preparation

The standards were prepared by adding appropriate volumes of a mixture of diazepam and desmethyldiazepam solution containing 10% methanol to plasma obtained from healthy subjects. The volumes added were 1–10% of the final volume (1 ml), so that the integrity of the plasma was maintained. The quality control samples were also prepared in the same way, using a separately weighed stock

solution. After aliquoting, 1 ml quality control samples were stored at −20°C until analysis. A 1 μg ml^{-1} solution of lorazepam containing 10% methanol was prepared and 10 μl was added to each sample. The latter was vortex mixed for 10 s and extracted, using Bond Elut C_{18} (3 ml) cartridges (Varian, CA, USA). The cartridges were washed and conditioned prior to the extraction step, by passing through 2 ml methanol, followed by 2 ml water under vacuum (60 kPa), using a Vac Elut manifold (Anachem International, UK). Extracted sample was then desorbed with methanol (250 μl) and a 60 μl aliquot was injected onto the column.

The within-day and between-day precision was established by assaying two different concentrations of diazepam (125 and 250 ng ml^{-1}) and desmethyldiazepam (62 and 125 ng ml^{-1}) in duplicate at six different times.

The assay was evaluated in one subject who received an intravenous dose of diazepam (5 mg) over a 5 min period, via a cannula inserted into a suitable forearm vein. Blood (10 ml) samples were collected at 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 32, 48, 72 and 96 h (post-dose) and were later centrifuged at 2500*g* for 10 min to separate the plasma. Concentrations of diazepam and desmethyldiazepam in plasma were evaluated by determining the ratio of peak area for the compounds to that of the internal standard, then comparing with the standard curves obtained after regression analysis of the calibration samples.

Results and Discussion

The mean recovery for diazapem and desmethyldiazepam was in excess of 98% (relative standard deviation <10%) over the range of the calibration curves for diazepam (20–500 ng ml^{-1}) and desmethyldiazepam (20–250 ng ml^{-1}), when compared to the direct injection of the standard dissolved in water. Figure 1 shows typical elution profiles for blank plasma and a sample containing diazepam and desmethyldiazepam obtained from a subject 1 h following the administration of a single intravenous dose (5 mg) of diazepam. All the compounds were fully resolved and the retention times for diazepam, desmethyldiazepam and lorazepam (internal standard) were 4.87, 6.69 and 8.69 min, respectively. The standard curve for plasma was linear for diazepam and desmethyldiazepam over the range of the two standard curves. The correlation coefficient was greater than 0.99 for each regression line and intercepts were all close to zero (Table 1). The relative standard deviations for diazepam and desmethyldiazepam ranged from 1.2 to 2.5% for within-day and 1.3 to 2.1% for between-day (Table 2). The limit of detection for diazepam and desmethyldiazepam was 5 ng ml^{-1} (signal/noise

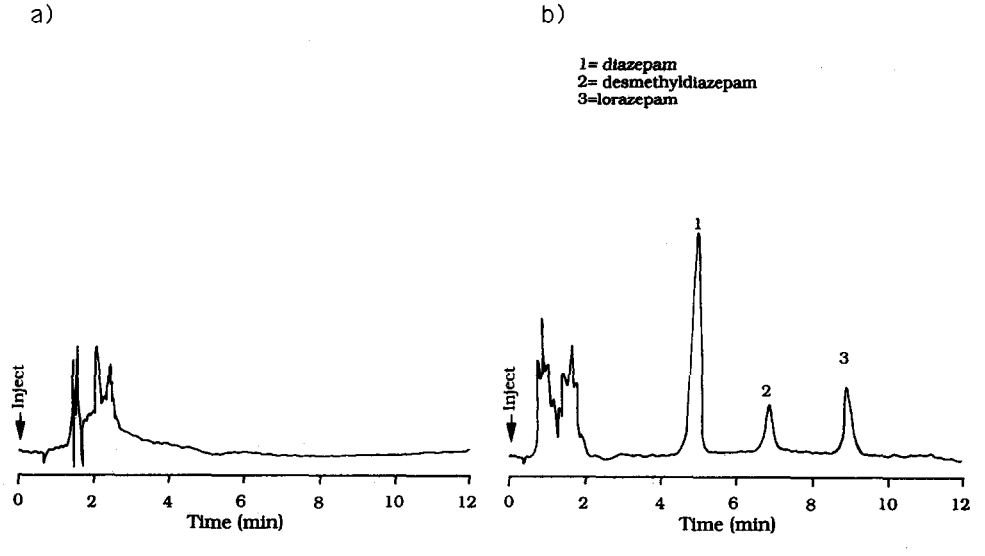

Figure 1
Elution pattern for (a) blank plasma sample and (b) diazepam (80 ng ml^{-1}), desmethyldiazapem (20 ng ml^{-1}) and lorazepam (10 ng ml^{-1}) extracted from plasma on a μBondapack column (10 μM particle size, 150 mm × 3.9 mm i.d.). Flow rate 1.5 ml min^{-1}; λ = 229 nm. Eluent — potassium dihydrogen orthophosphate (30 mM; pH 3.5)–methanol (45:55, v/v).

Table 1
Mean (±SD) linear calibration curve for diazepam and desmethyldiazepam. Experiments were carried out on six different days

	Equation	Correlation coefficient
Diazepam	$y = 0.0038\ (0.0003)\ x + 0.0274\ (0.0234)$	0.9989 (0.0018)
Desmethyldiazepam	$y = 0.0049\ (0.0002)\ x + 0.0013\ (0.0145)$	0.9981 (0.0011)

y = compound/I.S. peak area ratio.
x = concentration of each compound.

Table 2
Precision data for diazepam and desmethyldiazepam in plasma

	Within-day (n = 6) conc. (mean ± SD) (ng ml^{-1})	RSD (%)	Between-day (n = 6) conc. (mean ± SD) (ng ml^{-1})	RSD (%)
Diazepam	122.0 ± 1.0	1.2	126.0 ± 1.2	1.5
	248.0 ± 0.6	1.4	254.0 ± 0.5	1.3
Desmethyldiazepam	61.0 ± 2.9	1.8	65.0 ± 3.2	2.1
	123.0 ± 2.0	2.5	122.0 ± 1.4	1.7

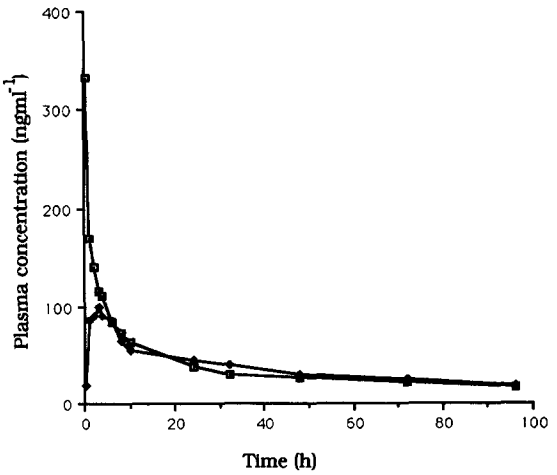

Figure 2
Concentration vs time profile for diazepam (⊡) and desmethyldiazepam (◆) in a subject receiving 5 mg diazepam intravenously.

ratio = 5). Figure 2 represents the concentrations of diazepam and desmethyldiazepam in plasma over a 96 h period from a subject who had received an intravenous dose (5 mg) of diazepam.

Conclusions

An HPLC assay for the measurement of diazepam and its major metabolite, desmethyldiazepam in plasma has been developed involving solid-phase extraction of the compounds followed by isocratic HPLC analysis. The diazepam and desmethyldiazepam peaks were well resolved and were eluted within 10 min, following sample injection, without interference from other endogenous compounds. This simple and rapid assay for diazepam and desmethyldiazepam can readily be adopted for routine laboratory analysis.

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[Received for review 14 October 1992; revised manuscript received 15 December 1992]