Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 4/5, pp. 277–284, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Solid-phase extraction of 1,4-benzodiazepines from biological fluids*

M. CASAS, L.A. BERRUETA, B. GALLO† and F. VICENTE

Department of Analytical Chemistry, Faculty of Sciences, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain

Abstract: The solid-phase extraction (SPE) of seven 1,4-benzodiazepines (oxazepam, diazepam, temazepam, nordazepam, brotizolam, adinazolam and midazolam) using prazepam as internal standard was investigated. The 1,4-benzodiazepines were recovered from an aqueous buffer of suitable pH, using C18 Sep-Pak cartridges and mixtures of methanol–water as elution solvent. The recovery of oxazepam using different sorbent materials (C2, C8, C18, cyanopropyl, phenyl and cyclohexyl Bond-Elut) was also examined as a function of pH and the composition of the elution solvent. The SPE of oxazepam was investigated using spiked urine samples and the C2 cartridge gave rise to the cleanest urine extracts. The recoveries of the other 1,4-benzodiazepines from spiked urine and plasma samples using the C2 cartridge was then found to be higher than 90%, without any interference from endogenous compounds of the samples. Finally, the influence of other factors such as drug concentration, sample volume and the number of times the SPE cartridge had been used was also examined.

Keywords: *Solid-phase extraction (SPE); sample preparation; biological samples; 1,4-benzodiazepines; Sep-Pak; Bond-Elut; drug analysis.*

Introduction

Drug analysis in biological fluids (serum, plasma and urine) usually requires the preparation of the samples before they are injected into a liquid chromatographic system. This preparation tends to give the best selectivity in the analysis. Some of these preparation treatments for biological samples are protein removal, enzymatic digestion, ultrafiltration, dialysis, liquid–liquid extraction (LLE) or solid-phase extraction (SPE). In recent years, SPE has been used as an alternative technique to LLE. SPE allows the treatment of smaller sample volumes (50–100 μl) without the problem of possible emulsion formation [1], it is quicker and less expensive than LLE [2], it offers the possibility of automation [3, 4], and provides reproducibility and recoveries at least as good as LLE [5–7].

1,4-Benzodiazepines are the most widely used medicaments in the treatment of anxiety and sleep disturbances [8–10]. Many of the analytical methods for these substances in biological fluids are based on high-performance liquid chromatographic (HPLC) systems, and SPE is frequently used for sample preparation [11–21].

Most of these SPE methods use C18 as the solid-phase material [14–24]. Other sorbent materials used are C2 [4], C8 [13] and silica [17]. However a more exhaustive study of the SPE of 1,4-benzodiazepines has been performed only by Inoue and Suzuki [17] who have investigated the SPE of triazolam and its metabolites using C18 and silica cartridges as solid phases and methanol–water and CH_2Cl_2–methanol mixtures as elution solvents.

In this work, the study of the SPE of several 1,4-benzodiazepines in aqueous and spiked urine and plasma samples is reported. Six 1,4-benzodiazepines (oxazepam, diazepam, nordazepam, temazepam, midazolam and adinazolam) and a thienotriazolo-1,4-diazepine (brotizolam) and six sorbent materials have been investigated using methanol–water mixtures as wash and elution solvents.

Experimental

Reagents

The 1,4-benzodiazepines standards were kindly supplied by Roche (Madrid, Spain) (oxazepam, nordazepam, diazepam and midazolam), Europharma (Madrid, Spain) (brotizolam), Sandoz (Barcelona, Spain)

*This paper is dedicated to the memory of Prof. J.M. Castresana.
†Author to whom correspondence should be addressed.

(temazepam), Upjohn (Madrid, Spain) (adinazolam) and Parke–Davis (Barcelona, Spain) (prazepam). Stock solutions containing 1 mg ml^{-1} of each drug were prepared in methanol (HPLC grade) and stored at 4°C in the dark.

Aqueous buffers were prepared from analytical grade reagents at two concentration levels, 10^{-2} M for aqueous samples studies and 10^{-1} M for the spiked urine and plasma samples. The buffers were: H_3PO_4–KH_2PO_4 (pH 2.3), HAc–NaAc (pH 4.0), KH_2PO_4–K_2HPO_4 (pH 6.0), KH_2PO_4–K_2HPO_4 (pH 8.0), H_3BO_3–NaH_2BO_3 (pH 9.0), $NaHCO_3$–Na_2CO_3 (pH 10.0) and NaOH (pH 12.0).

Blood and urine samples were obtained from healthy subjects. Blood samples, collected using EDTA as anticoagulant, were centrifuged and the plasma thus obtained was frozen and stored at −20°C. Urine samples were also stored at −20°C.

Instrumentation

The SPE cartridges used were Waters (Barcelona, Spain) Sep-Pak octadecyl (C18) (100 mg) and Analytichem Int. (Harbor City, CA, USA) Bond-Elut octadecyl (C18), octyl (C8), ethyl (C2), cyclohexyl (CH), phenyl (PH) and cyanopropyl (CN) silica bonded phases (100 mg). The SPE was performed with the aid of a Visiprep Vacuum Manifold (Supelco, Bellefonte, CA, USA).

Quantitation was carried out using an HPLC system equipped with a 5140 LKB (Barcelona, Spain) pump, a Rheodyne (Cotati, CA, USA) model 7125 injector fitted with a 20 μl-loop, a 4.6 mm i.d. × 3.5 cm 5-μm-ultrabase C18 column (Scharlau, Barcelona, Spain) and a Waters (Barcelona, Spain) 484 UV–vis detector. A methanol–water (60:40, v/v) mixture was used as the mobile phase and the system was operated at room temperature. Data collection was performed using a microcomputer equipped with a PC-LabCard PCL-812 (Advantech Co., Shing-Tien City, Taiwan) and a BASIC program designed and written in-house. Integration was carried out with the NELSON program (v. 3.5) (Perkin–Elmer, Barcelona, Spain). Prazepam was used as internal standard for quantitation. The different chromatographic conditions used for each compound are shown in Table 1.

SPE studies in aqueous samples

Aqueous samples of 1 μg ml^{-1} (1,4-benzodiazepines are usually found in biological fluids in the range 0.01–10 μg ml^{-1}) were prepared by dilution from stock solutions using the appropriate aqueous buffer. The SPE cartridge was activated with a column volume of methanol and was washed with a column volume of aqueous buffer. After that, 1 ml of aqueous sample was added, the sorbent was washed with three column volumes of water, and finally 1 ml of elution solvent was added to the cartridge and collected for HPLC analysis. For the pH studies, the elution solvent was methanol and for the elution studies (recoveries evaluated as a function of the composition of the elution solvent) methanol–water mixtures were used.

SPE studies in spiked urine and plasma samples

Urine samples were spiked with 1 μg of 1,4-benzodiazepine per ml of urine and buffered with the appropriate 10^{-1} M buffer. The SPE procedure was similar to the case of the

Table 1
Chromatographic conditions used in the quantitation of the different 1,4-benzodiazepines and values of the parameters of the weighted calibration curves attained

Compound	Flow (ml min^{-1})	Wavelength (nm)	Slope ± SD*	Intercept ± SD*	Correlation coefficient	LOD† (ng ml^{-1})
oxazepam	1.00	228	0.26886 ± 0.00095	(−9.9 ± 3.6) 10^{-3}	0.9999	79.2
brotizolam	0.90	240	0.14666 ± 0.00049	(88.9 ± 1.9) 10^{-3}	0.9999	76.1
temazepam	1.10	230	0.24233 ± 0.00095	(−1.04 ± 0.35) 10^{-2}	0.9999	87.8
nordazepam	1.10	227	0.2815 ± 0.0017	(−2.05 ± 6.4) 10^{-3}	0.9998	135
adinazolam	1.00	221	0.16323 ± 0.00058	(0.87 ± 2.18) 10^{-3}	0.9999	79.8
midazolam	1.00	217	0.21748 ± 0.00090	(−8.9 ± 3.4) 10^{-3}	0.9999	92.7
diazepam	1.30	229	0.24787 ± 0.00070	(−2.0 ± 2.6) 10^{-3}	0.9999	63.1

*SD = standard deviation.

†Limits of detection (LODs) were calculated as the concentration value corresponding to a ratio peak area/internal standard peak area equal to three times the σ of the calibration curve given by the equation: $\sigma = [U/(N_p - 2)]^{1/2}$, where U is the quadratic sum of errors and N_p is the number of points in the calibration plot. If we take into account the preconcentration factor of 5 obtained in the evaporation–reconstitution step, these values must be divided by 5.

aqueous samples, but the cartridge was washed with three column volumes of water and 1 ml of the appropriate washing solvent, prior to elution with 1 ml of elution solvent. Plasma samples were spiked with 1 μg midazolam per ml plasma and buffered at pH 8.0.

All the collected samples from the SPE were evaporated to dryness and reconstituted with 200 μl of mobile phase prior to their injection into the chromatographic system. Recoveries were calculated, in all cases, against the initial amount of drug [1 μg ml^{-1} sample (aqueous or spiked urine or plasma)].

Results and Discussion

The values of the slopes, intercepts, correlation coefficients and limits of detection of the weighted calibration curves used for the chromatographic quantitation of each benzodiazepine in methanol–water (60:40, v/v) in the concentration range 0.1–7 μg ml^{-1} of injected sample are presented in Table 1.

Owing to the aqueous nature of biological fluids (blood, urine), the most suitable sorbent material for the SPE of 1,4-benzodiazepines from this kind of sample is the non-polar type. Therefore the solid phases examined were octadecyl (C18), octyl (C8), ethyl (C2), cyclohexyl (CH), phenyl (PH) and cyanopropyl (CN) silica-bonded phases.

First of all, SPE from aqueous samples was investigated using the most non-polar sorbent material (C18). Recoveries obtained in the pH study are presented in Table 2 and show that all the 1,4-benzodiazepines studied are completely retained over the pH range 2.3–12.0. The recoveries were evaluated as a function of the composition of the elution mixture (methanol–water), fixing the sample pH to 9.0 and the results are shown in Table 3.

The plot of the recovery of each drug versus the methanol percentage of the elution solvent was called the elution curve. The elution curve of each compound gives information about the most suitable washing solvent (the one with the highest methanol percentage without eluting the drug) and the best elution solvent (the lowest methanol percentage that gives complete recovery of the drug) (Fig. 1). The correct selection of the washing and elution solvents will provide the cleanest samples in the SPE process and therefore, the best selectivity of extraction.

Once the complete retention of all the 1,4-benzodiazepines on C18 was checked, the SPE

Table 2
Recoveries from aqueous samples % ± RSD of *n* determinations using the Sep-Pak C18 cartridges for different 1,4-benzodiazepines (*n* = 4), different Bond-Elut cartridges for oxazepam (*n* = 4), and the Bond-Elut C2 cartridges for different 1,4-benzodiazepines (*n* = 3) as a function of the sample pH

	pH					
	2.4	4.0	6.0	8.0	10.0	12.0
C18						
oxazepam	95.9 ± 1.7	99.0 ± 2.5	99.1 ± 2.2	91.6 ± 3.2	96.5 ± 4.0	92.08 ± 0.42
brotizolam	95.5 ± 1.6	101.2 ± 1.5	102.3 ± 1.0	100.3 ± 2.0	99.7 ± 1.4	99.5 ± 1.2
temazepam	93.7 ± 1.4	97.6 ± 1.6	96.5 ± 1.8	95.3 ± 2.4	96.2 ± 2.1	101.98 ± 0.70
nordazepam	98.6 ± 2.3	103.1 ± 1.6	101.6 ± 4.4	98.7 ± 1.5	104.0 ± 1.4	105.3 ± 2.9
adinazolam	94.3 ± 2.2	95.1 ± 2.1	101.2 ± 1.9	98.0 ± 2.2	102.7 ± 1.0	98.2 ± 2.9
midazolam	97.3 ± 4.1	104.5 ± 1.5	96.5 ± 1.4	97.2 ± 2.0	106.43 ± 0.65	98.2 ± 1.9
diazepam	104.0 ± 1.6	107.0 ± 1.2	106.44 ± 0.47	96.5 ± 1.9	101.7 ± 2.7	104.5 ± 1.5
Oxazepam						
C18		96.96 ± 0.65	98.4 ± 1.5	97.9 ± 1.1	96.9 ± 1.4	
C8		99.35 ± 0.67	100.2 ± 1.3	98.6 ± 1.5	96.7 ± 3.1	
C2		97.7 ± 2.5	100.69 ± 0.72	93.2 ± 1.6	92.2 ± 2.1	
CH		98.4 ± 2.2	98.16 ± 0.60	84.7 ± 4.2	91.5 ± 2.7	
PH		98.2 ± 2.5	97.2 ± 1.9	87.4 ± 5.6	99.6 ± 2.4	
CN		13.8 ± 1.5	12.75 ± 0.61	9.16 ± 0.71	7.4 ± 1.7	
C2						
oxazepam		97.7 ± 2.5	100.69 ± 0.62	93.2 ± 1.6	92.2 ± 2.1	
brotizolam		93.3 ± 1.2	100.6 ± 3.2	87.6 ± 1.3	89.9 ± 4.3	
temazepam		93.7 ± 2.7	96.5 ± 3.9	89.6 ± 5.1	90.2 ± 6.1	
nordazepam		97.1 ± 1.6	100.5 ± 3.2	99.13 ± 0.84	100.2 ± 4.7	
adinazolam		93.0 ± 4.9	100.81 ± 0.97	92.9 ± 3.4	75.3 ± 1.6	
midazolam		<1.9	95.4 ± 4.1	99.60 ± 0.58	98.7 ± 2.6	
diazepam		94.6 ± 5.5	99.8 ± 2.7	93.4 ± 4.7	98.2 ± 5.5	

Table 3
Recoveries from aqueous samples (% ± RSD of *n* determinations) using the Sep-Pak C18 cartridges for different 1,4-benzodiazepines at pH 9.0 ($n = 4$), different Bond-Elut cartridges for oxazepam at pH 6.0 ($n = 4$), and the Bond-Elut C2 cartridges at pH 6.0 for all compounds except for midazolam (pH 8.0) ($n = 3$) as a function of the composition of the elution mixture (methanol–water)

	%MeOH							
	20	30	40	50	60	70	80	90
C18								
oxazepam		<1.6	2.77 ± 0.56	24.3 ± 2.5	88.0 ± 2.1	98.3 ± 2.1	97.3 ± 1.1	—
brotizolam		<1.5	<1.5	13.7 ± 1.7	78.8 ± 0.8	96.1 ± 1.4	102.4 ± 1.2	—
temazepam		<1.8	1.98 ± 0.26	12.6 ± 1.6	76.9 ± 1.6	92.51 ± 2.5	96.61 ± 0.95	—
nordazepam		—	<2.7	6.5 ± 1.3	61.9 ± 1.1	90.4 ± 3.6	96.9 ± 2.5	100.0 ± 1.5
adinazolam		—	<1.6	4.9 ± 2.3	48.3 ± 1.3	92.5 ± 1.3	97.5 ± 1.4	97.4 ± 1.4
midazolam		—	<1.9	<1.9	36.6 ± 4.8	89.9 ± 3.0	98.5 ± 1.4	99.6 ± 1.7
diazepam		—	<1.3	4.46 ± 0.83	70.9 ± 3.2	97.1 ± 1.7	104.5 ± 2.7	100.5 ± 0.6
Oxazepam								
C18	—	<1.6	<1.6	16.4 ± 2.7	91.3 ± 1.7	94.8 ± 1.0	96.58 ± 0.73	
C8	—	<1.6	2.1 ± 1.5	14.6 ± 2.0	97.1 ± 1.3	97.0 ± 1.8	—	
C2	<1.6	1.73 ± 0.56	28.2 ± 1.4	87.2 ± 5.8	100.6 ± 1.4	97.24 ± 0.71	—	
CH	—	<1.6	<1.6	13.8 ± 2.7	96.9 ± 1.2	95.8 ± 2.5	—	
PH	—	<1.6	1.70 ± 0.20	48.8 ± 5.5	97.4 ± 3.6	94.2 ± 1.0	—	
C2								
oxazepam	<1.6	1.73 ± 0.56	28.2 ± 1.4	87.2 ± 5.8	100.6 ± 1.4	97.0 ± 1.8	—	—
brotizolam	—	<1.5	5.44 ± 0.46	84.1 ± 2.4	94.1 ± 1.6	92.6 ± 1.1	89.58 ± 0.90	95.43 ± 0.66
temazepam	—	<1.8	16.7 ± 6.5	82.1 ± 5.0	99.0 ± 4.3	95.6 ± 1.2	92.4 ± 2.8	94.8 ± 1.7
nordazepam	—	<2.7	3.5 ± 2.6	64.4 ± 2.7	95.2 ± 3.0	92.5 ± 1.1	93.05 ± 0.84	96.28 ± 0.26
adinazolam	—	<1.6	<1.6	34.9 ± 6.8	94.3 ± 1.6	91.06 ± 0.10	87.2 ± 4.9	98.7 ± 1.5
midazolam	<1.9	<1.9	<1.9	56.5 ± 3.1	87.9 ± 4.2	94.14 ± 0.87	95.0 ± 1.1	94.7 ± 1.6
diazepam	—	<1.3	7.43 ± 10.9	61.2 ± 3.7	97.9 ± 1.9	96.4 ± 1.9	96.64 ± 0.63	99.6 ± 1.6

from aqueous samples of oxazepam, the least retained on C18, was studied using different sorbent materials. The pH study results (Table 2) show that the solid phases investigated retain oxazepam quantitatively in the pH range 4.0–10.0, excluding CN which gave poor recoveries, due to the relatively polar character of this sorbent. The elution studies were performed having adjusted the sample pH to 6.0 and the results are presented in Table 3.

The recoveries obtained with all solid phases were complete, except for CN. In order to decide which of them was the most suitable for use with biological samples, the cleanliness of the extract obtained with each sorbent was tested using urine samples spiked with 1 μg oxazepam per ml urine. The SPE conditions used were obtained from Tables 2 and 3 and are summarized in Table 4.

The results shown in Table 4 appear to indicate a general decrease in the recovery of oxazepam from spiked urine samples in relation with aqueous samples. This fact can be attributed to the partial overlap between oxazepam and endogenous peaks in the chromatogram, producing an erroneous integration of the oxazepam peak. The sorbent providing the least interfering peaks was the C2 material (Fig. 2).

Hence, C2 was chosen as the best sorbent, and sample pH and composition of the elution solvent were investigated for all the studied compounds. The pH study for SPE from aqueous samples using the C2 cartridge (Table 2) shows quantitative recoveries for the seven 1,4-benzodiazepines within the pH range 4.0–10.0, except for midazolam which needs a minimum pH of 6.0. This fact can be explained

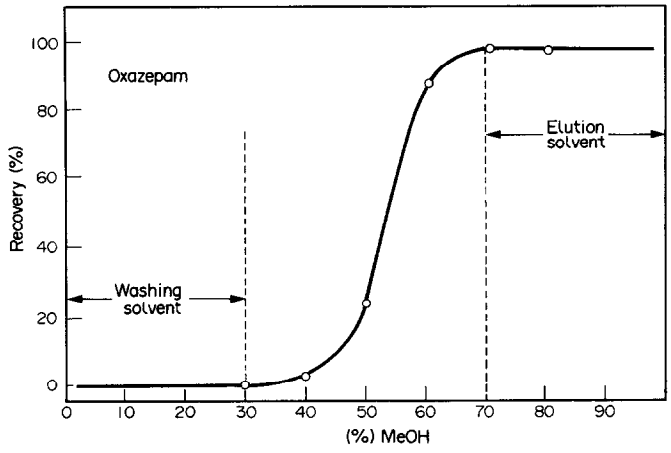

Figure 1
Elution curve corresponding to the SPE of oxazepam at pH 9.0 using the Sep-Pak C18 cartridge.

Table 4
Optimum conditions for the SPE of the different 1,4-benzodiazepines using Bond-Elut cartridges extracted from Tables 2 and 3 and recoveries (% ± RSD of four determinations) obtained from spiked urine samples

	Sample pH	Washing solvent 1 ml MeOH–H_2O	Elution solvent 1 ml MeOH–H_2O	Recovery from spiked urine
Oxazepam				
C18	6.0	30:70	80:20	86.2 ± 4.0
C8	6.0	30:70	60:40	83.0 ± 2.2
C2	6.0	20.80	60:40	89.5 ± 1.3
CH	6.0	30:70	60:40	83.5 ± 2.8
PH	6.0	30:70	60:40	83.5 ± 2.0
C2				
oxazepam	6.0	20:80	60:40	85.5 ± 1.3
brotizolam	6.0	30:70	60:40	101.2 ± 2.8
temazepam	6.0	30:70	60:40	103.6 ± 1.3
nordazepam	6.0	30:70	60:40	95.6 ± 2.2
adinazolam	6.0	30:70	60:40	97.5 ± 1.0
midazolam	8.0	30:70	70:30	93.2 ± 1.0
diazepam	6.0	30:70	60:40	99.09 ± 0.32

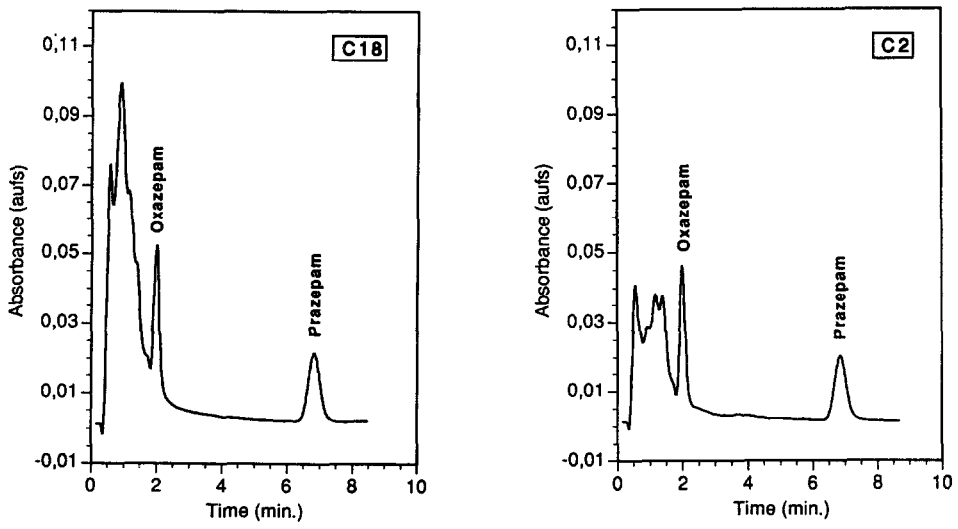

Figure 2
Chromatograms corresponding to the SPE of urine samples spiked with 1 μg oxazepam per ml urine using the Bond-Elut C18 and C2 cartridges.

in terms of the acid–base equilibrium of midazolam, which has a deprotonation constant of 5.5, while the others have dissociation constants between 1.5 and 3.5 [25]. On one hand, the C18 material retains the protonated form of midazolam due to its high hydrophobic character, providing hydrophobic interactions with the analyte greater than the electrostatic forces between midazolam and the sample solvent (water). On the other hand, the C2 sorbent is not able to retain the positively charged form of midazolam because of its less hydrophobic character, thus offering more selectivity than C18.

The elution studies from aqueous samples were performed at pH 6.0 for all the compounds except for midazolam, for which the sample pH was set to 8.0. The results are shown in Table 3. The best sample pH and washing and elution solvents for the SPE of each 1,4-benzodiazepine using the C2 cartridge are presented in Table 4.

Using these optimum conditions, the SPE of 1,4-benzodiazepines from spiked urine and plasma samples was studied. The recoveries obtained from spiked urine samples are similar to those from aqueous samples, except for oxazepam as has been explained above (Table 4). Recovery from the plasma sample spiked with 1 μg midazolam per ml plasma was 96.5%, similar to those obtained from aqueous (94.14 ± 0.87) and spiked urine (93.2 ± 1.0) samples. Figure 3 shows the chromatogram corresponding to the spiked plasma sample after the SPE process. No interfering peaks of endogenous compounds from urine or plasma samples were found in the chromatograms in the regions of the drug and internal standard peaks, with the exception of the slight interference in the case of oxazepam, mentioned previously.

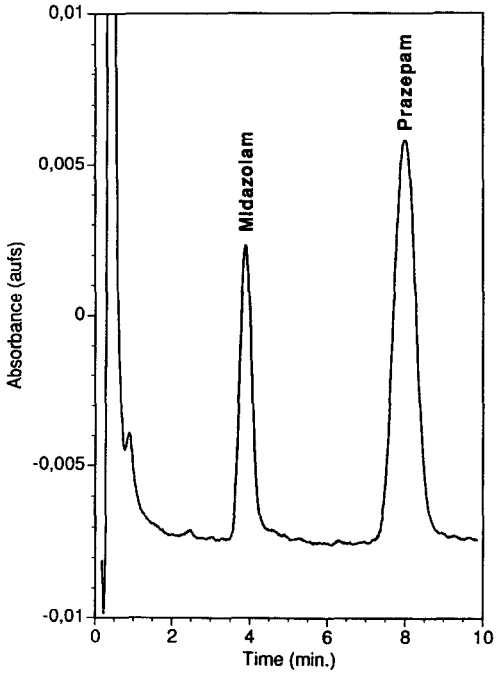

Figure 3
Chromatogram corresponding to the SPE of a plasma sample spiked with 1 μg midazolam per ml plasma using the Bond-Elut C2 cartridge.

Table 5
Recoveries of midazolam from spiked urine samples (% ± RSD of n determinations) using the Bond-Elut C2 cartridges as a function of the concentration of midazolam in the sample (n = 3), the sample volume (n = 3) and the number of uses of the same cartridge (n = 1)

Midazolam conc. (μg ml^{-1})	0.05	0.1	0.5	1	5	10	15	20				
% Recovery ± RSD	97.2 ± 12.1	94.0 ± 4.6	99.6 ± 4.0	93.2 ± 1.0	93.5 ± 1.7	94.1 ± 1.5	94.53 ± 0.99	94.90 ± 0.53				
Sample volume (ml)	1	2	3	4	5	6	8	10				
% Recovery ± RSD	93.2 ± 1.0	92.1 ± 2.8	95.0 ± 3.5	94.3 ± 3.0	96.36 ± 0.74	98.85 ± 0.25	95.5 ± 1.7	93.03 ± 0.71				
Number of uses	1	2	3	4	7	9	10	11	12	13	14	15
% Recovery	93.2	93.9	97.5	95.7	95.5	90.5	96.5	95.6	90.7	99.1	99.0	94.6

Finally, the effect of other factors on SPE was examined using urine samples spiked with 1 μg midazolam per ml urine, the C2 cartridge and the optimum conditions from Table 4. The results of this study are presented in Table 5 and they show that the recovery of midazolam remained constant under the following conditions: in the concentration range studied (0.05–20 μg of midazolam per ml of urine) the mean % recovery was 95.1 ± 2.3 (RSD); for all the sample volumes investigated (1–10 ml of urine) the mean % recovery was 94.8 ± 2.3; that, provided the C2 cartridge is washed with two columns volumes of methanol between each sample, cartridges may be used up to 15 times, with mean % recovery of 95.1 ± 3.1.

Using a sample volume of 10 ml it is possible to get a preconcentration factor of 50.

Conclusions

The SPE of seven 1,4-benzodiazepines has been investigated, showing that this technique is suitable for the preparation of biological fluids such as urine and plasma. The selection of the convenient extraction conditions (sample pH, composition of the washing and elution solvents and sorbent material used) provides the cleanest samples for later chromatographic analysis and can be an important factor in the selectivity of the entire analytical method.

Acknowledgements — The authors wish to thank Prof. N. Etxebarria for his kind collaboration in the preparation of the manuscript. These studies were supported by the Dirección General de Investigación Científica y Técnica (DGICYT, project PB87-0733) and the University of the Basque Country (UPV, project 171.310-E070/90).

References

[1] R.D. McDowall, J.C. Pearce and G.S. Murkitt, *J. Pharm. Biomed. Anal.* **4**, 3–21 (1986).
[2] R.D. McDowall, G.S. Murkitt and J.A. Walford, *J. Chromatogr.* **317**, 475–481 (1984).
[3] L. Yago, *Int. Lab.* **18**, 40–51 (1985).
[4] P. Mura, A. Piriou, P. Fraillon, Y. Papet and D. Reiss, *J. Chromatogr.* **416**, 303–310 (1987).
[5] J.T. Stewart, T.S. Reeves and I.L. Honigberg, *Anal. Lett.* **17**, 1811–1826 (1984).
[6] A.C. Mehta, *Talanta* **33**, 67–73 (1986).
[7] M.R. Harkey, *Chem. Anal. (N.Y.)* **100**, 59–85 (1989).
[8] H. Schutz, *Benzodiazepines. A Handbook. Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature.* Springer, Berlin (1982).
[9] H. Schutz, *Benzodiazepines II. A Handbook. Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature.* Springer, Berlin (1989).
[10] S. Caccia, T. Mennini, A. Spagnoli and S. Garattini, *Medicina* **5**, 379–404 (1985).
[11] P. Heizmann, R. Geschke and K. Zinapold, *J. Chromatogr.* **310**, 129–137 (1984).
[12] T. Kozu, *J. Chromatogr.* **310**, 213–218 (1984).
[13] S.K. Gupta and E.H. Ellinwood, *Pharm. Res.* **5**, 365–368 (1988).
[14] S.N. Rao, A.K. Dhar, H. Kutt and M. Okamoto, *J. Chromatogr.* **231**, 341–348 (1982).
[15] R.H.B. Sample, A. Chulk, C. Kolanowski and J.C. Baenziger, *Clin. Chem.* **32**, 1055–1055 (1986).
[16] S.E. Patterson, *J. Pharm. Biomed. Anal.* **4**, 271–274 (1986).
[17] T. Inoue and S.-I. Suzuki, *J. Chromatogr.* **422**, 197–204 (1987).
[18] G. Carlucci, *J. Liq. Chromatogr.* **11**, 1559–1568 (1988).
[19] N. De Giovanni and M. Chiarotti, *J. Chromatogr.* **428**, 321–329 (1988).
[20] M. Mazhar and S.R. Binder, *J. Chromatogr.* **497**, 201–212 (1989).
[21] T.J. Good and J.S. Andrews, *J. Chromatogr.* **19**, 562–566 (1981).
[22] J. Rodríguez Procopio, P. Hernández Hernández and L. Hernández Hernández, *Analyst* **112**, 79–82 (1987).
[23] F. Urrea Sánchez, J. Rodríguez Procopio and L. Hernández Hernández, *Anal. Chim. Acta* **199**, 227–232 (1987).
[24] O. Suzuki, H. Seno and T. Kumazawa, *J. Forens. Sci.* **33**, 1249–1253 (1988).
[25] M.M. Orive, B. Gallo, R.M. Alonso, F. Vincente, J.C. Viré and G.J. Patriarche, *Mikrochim. Acta* **I**, 181–190 (1989).

[Received for review 20 January 1992; revised manuscript received 13 October 1992]