ELSEVIER

Journal of Chromatography B, 780 (2002) 241–249

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Direct injection micellar liquid chromatographic determination of benzodiazepines in serum

Maria-Elisa Capella-Peiró[a], Devasish Bose[b], Adrià Martinavarro-Domínguez[a], Mayte Gil-Agustí[a], Josep Esteve-Romero[a,*]

[a] *Àrea de Química Analítica, Universitat Jaume I, 12080, Castelló, Spain*
[b] *Department of Criminology and Forensic Sciences, Dr H.S. Gour University, Sagar, India*

Received 5 March 2002; received in revised form 22 July 2002; accepted 22 July 2002

Abstract

A simple micellar liquid chromatographic (MLC) procedure is reported for the determination of several benzodiazepines in serum: bromazepam, diazepam, flunitrazepam, halazepam, medazepam, nitrazepam, oxazepam and tetrazepam. The optimization studies have been made in C_{18} and C_8 columns, using solutions containing sodium dodecyl sulphate (SDS) modified with butanol or pentanol as mobile phases. The method proposed for the determination of the benzodiazepines uses a hybrid micellar mobile phase of 0.06 *M* SDS–5% butanol–0.01 *M* phosphate buffer (pH 7) at 25 °C, and UV detection (230 nm) in a C_{18} column. The serum samples were injected directly, without any pretreatment, and eluted in less than 22 min, in accordance with their relative polarities, as indicated by their octanol–water partition coefficients. The limits of detection (ng ml^{-1}) were within the ranges of 2–6 and 4–18 for aqueous and serum samples, respectively. Repeatability and intermediate precision were tested for three different concentrations of the drugs, and RSD (%) was below 10 for most of the assays. The MLC results were compared with those obtained from a conventional HPLC method using methanol–water 5:5 (v/v) which requires a previous extraction procedure.

Keywords: Benzodiazepines

1. Introduction

Benzodiazepines are used for their properties as anti-convulsants, anesthetics, anti-depressives, hypnotics, tranquillizers and sedatives [1,2]. Bromazepam, diazepam, flunitrazepam, halazepam, medazepam, nitrazepam, oxazepam and tetrazepam are the most frequently prescribed. Most of these benzodiazepines are characterized by the presence of a phenyl ring fused to a partially saturated seven-membered ring with nitrogen at positions 1 and 4 (Fig. 1).

The determination of benzodiazepines has been extensively studied because of the need to detect and quantitate these drugs, especially in physiological fluids and tissues, in clinical or medico-legal studies [3]. Originally, these drugs were determined by UV spectrometry, but nowadays conventional reversed-phase liquid chromatography (RPLC) with aqueous–organic mobile phases are routinely applied in the

*Corresponding author. Tel.: +34-96-472-8093; fax: +34-96-472-8066.
E-mail address: estevej@exp.uji.es (J. Esteve-Romero).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00523-8

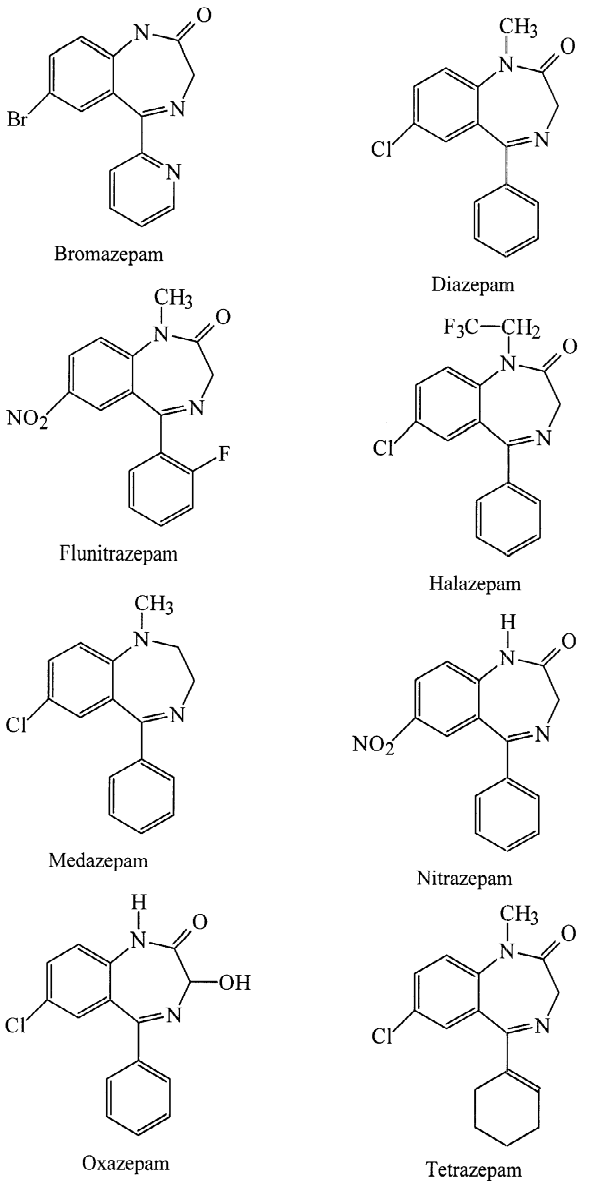

Fig. 1. Structure of the benzodiazepines.

analysis of serum, which requires complex sample pretreatment for the removal of interferences and extraction of the analytes. This work is tedious and frequently leads to low and variable recoveries. Liquid–liquid extraction can be performed using solvents such as chloroform [4], hexane [5] or dichloromethane [6,7]. In some cases solid-phase extraction has also been reported [8,9]. Afterwards, the determination of benzodiazepines by RPLC is performed in aqueous–organic mobile phases containing methanol/water (5:5) [4], (6:4) [5], and (65:35) [9], acetonitrile/methanol/water (25:22:3) [10] and (3:2:5) [11] in a C_{18} column, pH in the 5–7 range and detection around 240 nm, or acetonitrile/water (8:2) in a cyanopropyl column [6]. An HPLC method with acetonitrile/water in gradient (50–70%) allows the selectivity in the determination of some benzodiazepines in serum to be improved [7].

One interesting alternative to the aqueous–organic mobile phases is the use of solutions of surfactants above the critical micellar concentration [12–14]. In this technique, in addition to the formation of micelles, the reversed-phase column packings are covered with a layer of monomers of surfactant that protects and modifies the underlying alkyl-bonded silica phase. Since the solutes partition between three phases, the chromatographic behaviour is more complex than in traditional RPLC. A small amount of an organic modifier is usually added to the mobile phases to increase the elution strength and chromatographic efficiencies. Some attractive advantages of micellar mobile phases are that they allow the direct injection of the serum samples, and are non-toxic, non-flammable, biodegradable and economical, in comparison to aqueous–organic solvents, and compounds of diverse polarity can be analyzed under isocratic conditions. The stable behavior of micellar chromatographic systems permits the accurate prediction of the retention, based on simple models [15,16].

In our laboratory, micellar liquid chromatography (MLC) has been demonstrated to be a useful technique in the control of benzodiazepines in pharmaceutical preparations [17,18] using sodium dodecyl sulphate (SDS) mixed with an organic modifier like butanol. In the literature, a column switching technique was also reported for the extraction and determination of benzodiazepines using micellar mobile phases [19].

The purpose of this work was to develop an MLC procedure with a mobile phase containing SDS and butanol for the resolution of eight benzodiazepines (bromazepam, diazepam, flunitrazepam, halazepam, medazepam, nitrazepam, oxazepam and tetrazepam) with direct injection of untreated serum samples, using UV detection. The performance of MLC is

compared with a conventional procedure using mixtures of methanol–water [4].

2. Experimental

2.1. Chemicals and reagents

The benzodiazepines used in this study were: bromazepam (Roche, Barcelona, Spain), diazepam (Lasa Laboratorios, Barcelona), flunitrazepam, halazepam (Schering Plough, Madrid, Spain), medazepam, nitrazepam, oxazepam (Boehringer Ingelheim, Barcelona) and tetrazepam (Sanofi Winthrop, Barcelona). The drugs were kindly donated by the cited pharmaceutical laboratories. Stock solutions containing 100 μg ml^{-1} were prepared by dissolving the compounds in a few milliliters of methanol, with the aid of an ultrasonic bath (Selecta, Model 617, Barcelona). The water used was nanopure deionized water (Barnstead, Sybron, Boston, MA, USA). The serum samples in blank and with the drug used in this work were provided by the Hospital Verge dels Lliris d'Alcoi (Alacant, Spain). Filtration of the samples was always performed directly in the autosampler vials through 0.45-μm Nylon membranes of 13-mm diameter.

In the preparation of the mobile phases the following reagents were used: sodium dodecyl sulphate (99% purity, Merck, Darmstadt, Germany) as the surfactant, and 1-butanol, 1-pentanol (Scharlau, Barcelona, Spain) as modifiers, buffered to pH 7.0 with sodium dihydrogenphosphate (Panreac, Barcelona). After preparation, the mobile phases were filtered through 0.45-μm Nylon membranes (Micron Separations, Westboro, MA, USA). Methanol (Scharlab, Barcelona) was used in the preparation of the aqueous–organic mobile phase and for conditioning the column. Potassium carbonate and chloroform (Fluka, Bucks, Switzerland) were used in the extraction procedure for the serum samples that were injected in the aqueous–organic mobile phase.

2.2. Apparatus

The pH of the mobile phases was measured with a Crison potentiometer (Model micropH 2001; Crison, Barcelona), equipped with a combined Ag/AgCl/glass electrode. Serum samples were centrifuged with a Sorvall RC-5B from DuPont Instruments (Wilmington, DE, USA). UV spectra and absorbance measurements were obtained with a Perkin-Elmer UV–Vis-NIR spectrophotometer (Model Lambda 19; Perkin-Elmer, Norwalk, CT, USA). Maximum wavelengths and molar absorptivities of the drugs are given in Table 1.

An Agilent Technologies model 1100 chromatograph (Palo Alto, CA, USA) was used, equipped with a quaternary pump, an autosampler and a UV–visible detector set at 230 nm. The columns used for the analysis were Eclipse XDBC-8 (Hewlett-Packard, 5 μm particle size, 150 mm×4.6 mm I.D.) and a Kromasil C_{18} (Scharlab, 5 μm particle size, 120 mm×4.6 mm I.D.). The flow-rate used was 1 ml min^{-1}, and the injection volume was set at 20 μl. The chromatographic separations were made in a thermostated module at 25±0.2 °C. The signal was acquired by a PC computer connected to the chromatograph through an HP Chemstation. This was also used for the measurement of peak properties. The dead time was determined as the mean value of the first significant deviation of the base line in the chromatograms of the analytes. Optimization of mobile phase composition was assisted by Michrom software [16].

2.3. Micellar liquid chromatographic method

Blood samples were collected from patients who were treated with the benzodiazepines. The serum was centrifuged for 10 min at 3000 rpm, to be injected directly without any other pretreatment in

Table 1
Values of log K, log $P_{o/w}$, maximum wavelengths and molar absorptivities of the benzodiazepines

Compound	log K	log $P_{o/w}$	λ, nm	ε, l mol^{-1} cm^{-1}
Bromazepam	2.9–11.0	2.05	240	28 900
Diazepam	3.3	2.80	230	31 000
Flunitrazepam	1.8	2.06	230	32 600
Halazepam	NDA	4.47	226	36 500
Medazepam	6.2	4.41	250	27 500
Nitrazepam	3.2–10.8	2.25	220	30 000
Oxazepam	1.7–11.6	2.24	236	32 000
Tetrazepam	NDA	3.2	227	23 500

NDA, no data available.

the C_{18} column at 25 °C, using the optimum mobile phase: 0.06 *M* SDS–5% butanol (v/v) at pH 7.0. The same procedure was used when spiked serum samples were injected.

2.4. Aqueous–organic chromatographic method

For comparison purposes, the benzodiazepines were extracted [4] by mixing 0.5 ml of the serum with 200 μl of 1 *M* dipotassium carbonate and 3 ml of chloroform and shaken for 2 min. The resulting mixture was centrifuged at 1200 rpm for 5 min and afterwards the organic layer was removed by drying at 40 °C under a stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and 20 μl was injected in the chromatographic system using a mobile phase with methanol/water 5:5 (v/v), pH 7.0.

3. Results and discussion

3.1. Selection of pH conditions

In acidic media the benzodiazepines are hydrolized to benzophenone derivatives, and for this reason further experiments were carried out at pH 7.0, which is also more suitable for the conservation of the chromatographic columns.

The protonation constants of most of the selected compounds are shown in Table 1, and two acid–base equilibria with log K_1~11 and log K_2~2–3 have been reported [20]. In the presence of the anionic SDS micelles, we expect both log *K* to increase, owing to stabilization of the positive charge of the protonated drugs. Thus, at pH 7.0, bromazepam, nitrazepam and oxazepam possess one positive charge; diazepam, flunitrazepam and halazepam are in the neutral form; and finally for medazepam and tetrazepam, the two forms could coexist in the micellar media.

3.2. Selection of the column and modifier

The polarities of benzodiazepines change within the range of 2.05–4.47 for bromazepam and halazepam, respectively. The retention of all the benzodiazapines in a C_{18} and C_8 column was excessive when eluted with pure micellar mobile phases of SDS, and after the addition of the modifiers methanol or propanol, even at high concentrations. Elution at appropriate retention times was finally achieved by the addition of an alcohol with a longer chain, such as butanol or pentanol, which permits the elution of diverse hydrophobic compounds [16,17,21–25].

The retention times and efficiencies of benzodiazepines increase when butanol and the C_{18} column are used in comparison with pentanol and C_8, respectively. Asymmetry factors (B/A) are in the range of 1–2 and 1–4 for all the mobile phases used in the C_{18} and C_8 columns, respectively. For example when bromazepam and halazepam (the most hydrophilic and hydrophobic substances, respectively) are chromatographed in 0.1 *M* SDS–4% butanol, pH 7.0, the retention times (t_R), efficiencies (N) and B/A, were 5.0, 1700, 1.47 and 19.2, 2400, 1.04 in the C_{18} column, or 3.32, 900, 1.87 and 15.2, 350, 2.73 in the C_8 column. For the same two compounds, in 0.1 *M* SDS–4% pentanol, pH 7.0, t_R, N and B/A, were 2.9, 900 and 2.41 and 9.9, 700, 2.05 in the C_{18} column, or 1.8, 400, 3.72 and 6.8, 1850, 1.82 in the C_8 column.

The peaks of diazepam and oxazepam, on the one hand, and those of halazepam and tetrazepam, on the other, could not be resolved with pentanol. Butanol was thus preferred to optimize the separation of the eight drugs.

The hydrophilic layer formed by the sulphate head groups of SDS above the surface of the silica influences the retention of the compounds [26]. The hydroxyl groups on the silica surface play a less important role in the separation as a result of SDS adsorption. Since the hydrophilic layer exists above the silica surface, the association kinetics, which is controlled primarily by the electrostatic interaction, is easier than ion-exchange processes involving the silanol groups on the silica surface. Furthermore, the interaction of the protonated benzodiazepines with the hydrophilic layer formed by SDS reduces the penetration depth of the compounds into the bonded phase. The net effect is an improvement in efficiency when a micellar mobile phase is employed since the role of the silanol groups on the silica surface have been diminished with respect to their participation in the retention mechanism. This can explain the higher values of the efficiencies obtained in pentanol for

halazepam and medazepam, in comparison with the use of butanol.

3.3. Optimization of the mobile phase

The usual behavior in MLC is that when the concentration of surfactant increases, the retention time and efficiencies decrease and when the concentration of alcohol increases, retentions decrease and efficiencies are enhanced. The elution strength was similar for butanol and SDS, in the 1–7% butanol and 0.05–0.15 *M* SDS concentration ranges.

The accurate prediction of the retention behavior, based on a checked model, can speed up the process of finding the optimal composition of the mobile phase, for a given compound. The following equation has proved to be adequate to describe the retention of many compounds in MLC with hybrid mobile phases, with errors in the 2–4% range [27]:

$$k = \frac{K_{AS}\dfrac{1 + K_{SD}\varphi}{1 + K_{AD1}\varphi + K_{AD2}\varphi^2}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD1}\varphi + K_{AD2}\varphi^2}[M]} \quad (1)$$

where k is the retention factor, $[M]$ and φ are the concentrations of surfactant and modifier, respectively; K_{AS} and K_{AM} describe the association equilibria between the solute in bulk water and stationary phase or micelle, respectively; K_{SD}, K_{AD1}, K_{AD2} and K_{MD} are constants that measure the relative variation in the concentration of solute in bulk water and micelles, due to the presence of modifier, and refer to a pure micellar solution (without modifier).

On the basis of the selected pH, column and modifier, an optimization study was carried out using Michrom [16], which allowed the rapid and reliable simulation of chromatograms based on equations that describe the retention (such as Eq. (1)), and peak shape [27]:

$$h(t) = H\exp\left(-\frac{1}{2}\frac{(t - t_R)^2}{[s_0 + s_1(t - t_R)]^2}\right) \quad (2)$$

where *h(t)* is the height at diverse times, H the peak height, t_R the retention time, s_0 is a measurement of peak width at the maximum, and s_1 a distortion factor. These coefficients were obtained from the values of retention time, efficiency and asymmetry factor. The two latter parameters were interpolated by weighting the inverse of the distance between the predicted and available experimental mobile phases. With Michrom, the changes in the predicted retention times with mobile phase composition can easily be observed owing to the high simulation speed. It has been checked, for several groups of compounds, that the agreement between predicted and experimental chromatograms is good.

The coefficients of the retention model given by Eq. (1) (Table 2) were calculated for each compound, using the retention factors obtained for a set of seven mobile phases with SDS (*M*) and 1-butanol (%, v/v): 0.05–1, 0.05–7, 0.1–4, 0.15–1, 0.15–7, 0.075–3.2 and 0.1–2.5, all containing the phosphate buffer at pH 7.0. Peak positions and shapes were then predicted, in the whole factor space. The composition of the mobile phase giving any desired retention time can easily be predicted using Eq. (1) with the coefficients given in Table 2 for each substance.

Fig. 2a shows the resolution diagram for the eight benzodiazepines in serum samples. These com-

Table 2
Coefficients of Eq. (1) used to predict the chromatographic behavior of the benzodiazepines

Compound	K_{AS}	K_{AM}	$K_{MD}\times10^7$	K_1	K_{SD}	K_2
Bromazepam	445.2	10.06	1.00	−277.3	133.4	21 168
Diazepam	186.9	0.20	1.12	−162.1	120.6	17 312
Flunitrazepam	65.0	0.021	6.60	−152.1	179.0	15 212
Halazepam	153.5	0.0001	5.90	−134.3	66.1	5711
Medazepam	241.1	0.002	3.17	−119.3	27.7	2784
Nitrazepam	139.5	0.00056	6.66	−199.5	14.6	408
Oxazepam	59.4	0.0066	4.92	−123.6	51.8	3728
Tetrazepam	175.0	0.022	5.38	−150.2	82.6	8538

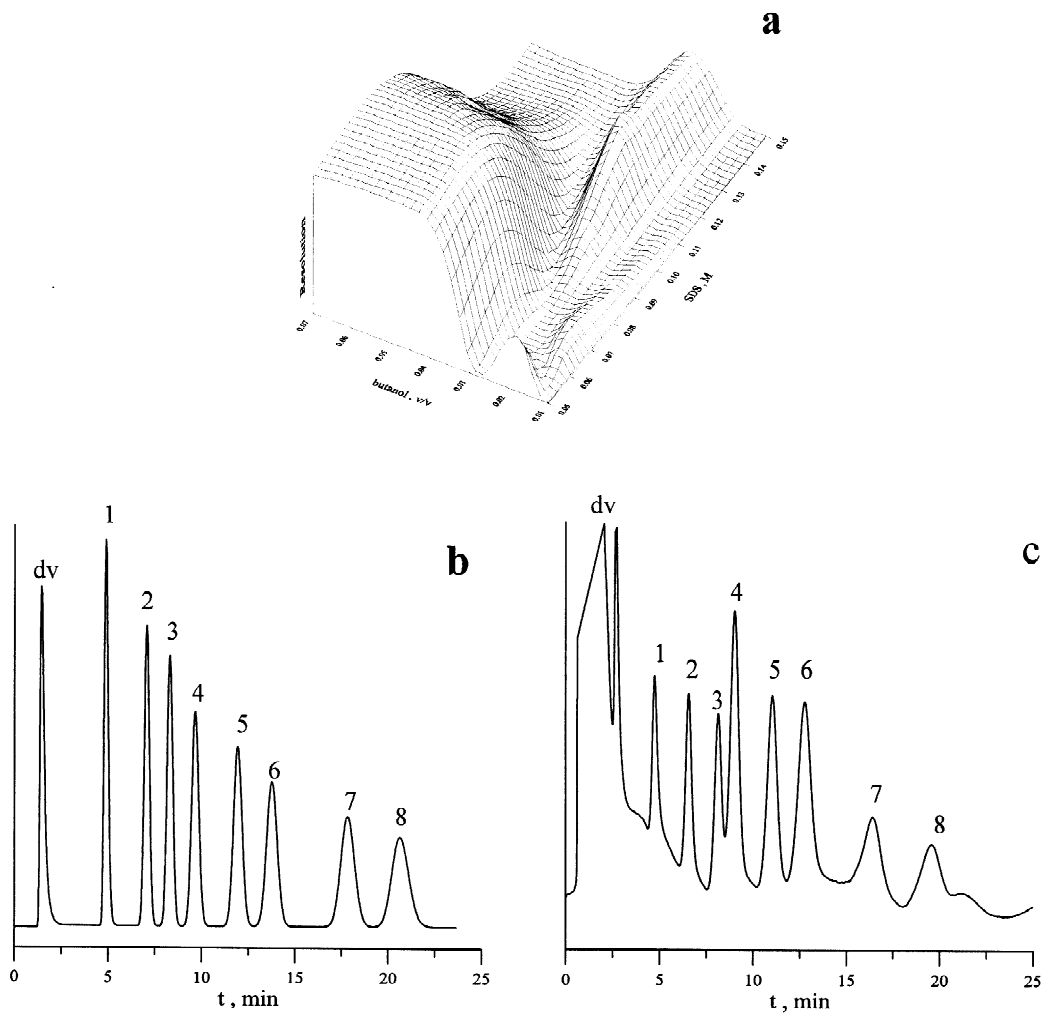

Fig. 2. Resolution diagram for the separation of the eight benzodiazepines (a), predicted (b) and real chromatograms (c) for the optimum mobile phase (0.06 *M* SDS–5% butanol, pH 7). Compounds are: dead volume (dv), bromazepam (1), flunitrazepam (2), nitrazepam (3), diazepam (4), oxazepam (5), tetrazepam (6), halazepam (7), and medazepam (8). Concentration of the benzodiazepines is 750 ng ml^{-1}.

pounds can be resolved in micellar mobile phases containing 0.05–0.083 *M* of SDS and 4.5–7% of butanol. The dead volume, which contains proteins and other unretained compounds of the serum, does not overlap in any mobile phase with the first peak corresponding to bromazepam. The low resolution observed in Fig. 2a is due to the overlapping of two pairs of substances, nitrazepam–diazepam and oxazepam–tetrazepam. No other overlappings are observed in the simulated chromatograms.

It was found that a single mobile phase of 0.06 *M* SDS–5% butanol at pH 7.0 (0.01 *M* phosphate buffer) allowed the elution of the eight benzodiazepine drugs in appropriate times. No shorter retention times can be obtained by increasing the volume fraction of butanol or the concentration of SDS. The retention times (min) and efficiencies for the selected mobile phase were the following: bromazepam (4.8, 1750), flunitrazepam (6.6, 2700), nitrazepam (8.1, 3050), diazepam (9.2, 2600), oxazepam (10.8, 2800), tetrazepam (12.8, 2500), halazepam (16.5, 2400), medazepam (19.5, 2050). Determination of the eight benzodiazepines can be performed in 21 min. As observed, the drugs eluted in accordance

with their relative polarities represented by their log $P_{o/w}$ (Table 1). Fig. 2b and c shows the predicted and real chromatograms, respectively, of the eight benzodiazepines in a serum matrix.

3.4. Calibration

To determine the linearity of response of the system for the eight analytes, ten standard solutions were prepared in water and serum, and each was injected in triplicate. These were designed to cover the anticipated ranges of concentration expected in real serum samples obtained in the Hospital Verge dels Lliris d'Alcoi: 150–1500 ng ml^{-1} for diazepam, medazepam and oxazepam, 50–250 for bromazepam, halazepam and nitrazepam, and 50–500 for flunitrazepam and tetrazepam. Standard calibrations were determined on 5 successive days. Table 3 shows the regression calibration parameters obtained in water and serum for the eight benzodiazepines when the peak areas were measured. The regression coefficients were always $r > 0.999$.

3.5. Limits of detection

The limits of detection (LODs) for the analytes were determined by subjecting low concentrations of the analytes to the proposed micellar liquid chromatography method. Limit of detection was estimated as the concentration resulting in a signal-to-noise ratio of three (3-s criterion). Table 3 shows the LODs, which are in the 2–6 and 4–18 ng ml^{-1} ranges for water and serum samples, respectively.

Table 4
Repeatabilities and intermediate precision (RSD%, $n=10$) obtained in the determination of the benzodiazepines eluted with 0.06 *M* SDS–5% butanol, pH 7

Compound	Repeatability			Intermediate precision		
	c_1	c_2	c_3	c_1	c_2	c_3
Bromazepam[a]	4.8	8.6	4.6	12.7	2.33	1.54
Diazepam[b]	3.0	4.3	8.1	4.8	1.11	1.64
Flunitrazepam[c]	10.9	1.8	5.4	6.2	1.9	1.41
Halazepam[a]	2.6	1.6	1.1	2.3	4.5	0.40
Medazepam[b]	1.1	2.9	1.3	2.7	4.5	0.71
Nitrazepam[a]	1.0	1.7	4.9	3.7	4.6	0.77
Oxazepam[b]	2.1	3.3	4.2	6.7	9.1	0.87
Tetrazepam[c]	1.6	2.7	2.1	5.4	8.0	0.73

[a] c_1, c_2, c_3 were 150, 200 and 250 for flunitrazepam and tetrazepam.
[b] c_1, c_2, c_3 were 250, 500 and 1500 for flunitrazepam and tetrazepam.
[c] c_1, c_2, c_3 were 100, 250 and 500 for flunitrazepam and tetrazepam.

3.6. Repeatability and intermediate precision

Three test solutions in the plasma matrix were prepared, according to the ICH Harmonised Tripartite Guideline, to assess the repeatability or intra-assay precision and intermediate precision of the assay for the eight analytes. The repeatability was determined by assaying these three test solutions ten times in the same day. The intermediate precision was the average of ten measurements of intra-assay values taken on 10 days over a 3-month period and made by different analysts and equipment, at three different drug concentrations, within the therapeutic ranges. The results in Table 4 show how the relative

Table 3
Slope, intercept and correlation coefficient (*r*) for the calibration curves of the benzodiazepines, spiked in water and serum, eluted with 0.06 *M* SDS–5% butanol, pH 7

Compound	Water				Serum			
	Slope	Intercept	*r*	LOD	Slope	Intercept	*r*	LOD
Bromazepam	83.3±2.7	−27.8±34.1	0.9998	2	85.8±2.0	−12.0±9.5	0.9997	10
Diazepam	159.9±1.5	−18.5±10.3	0.9996	3	164.9±2.8	6.7±10.2	0.9997	10
Flunitrazepam	70.6±2.6	−1.5±29.8	0.9993	3	71.3±1.5	9.4±7.2	0.9999	9
Halazepam	78.1±0.83	−10.1±2.5	0.9999	5	84.8±9.6	37.9±19.6	0.9994	18
Medazepam	112.4±4.2	21.5±21.7	0.9999	6	111.2±2.1	7.6±11.2	0.9999	11
Nitrazepam	92.1±0.79	−7.2±2.78	0.9997	2	89.8±3.1	−4.1±21.2	0.9995	4
Oxazepam	148.3±0.82	−3.2±1.55	0.9999	2	158.2±6.7	−31.4±10.2	0.9998	7
Tetrazepam	153.7±2.5	−14.8±27.7	0.9992	3	148.9±1.4	−11.5±8.9	0.9995	10

Limits of detection (LOD) are given in ng ml^{-1}.

Table 5
Determination of benzodiazepines in serum spiked samples ($n=10$) at three different concentrations (ng ml^{-1}) within the therapeutic range of each substance

Compound	Added			Found		
	c_1	c_2	c_3	c_1	c_2	c_3
Bromazepam	150	200	250	148.1±2.6	201.4±1.9	250±2.0
Diazepam	250	500	1500	247.5±4.2	502.0±3.5	1449±5
Flunitrazepam	100	250	500	104.8±6.2	252.1±2.4	497.5±4.2
Halazepam	150	200	250	151.9±4.7	203.1±5.2	249.4±3.7
Medazepam	250	500	1500	246.8±2.9	505.3±6.2	1503±8
Nitrazepam	150	200	250	148.2±3.8	203.3±4.9	248.0±2.5
Oxazepam	250	500	1500	251.3±3.3	503.2±4.2	1505±7
Tetrazepam	100	250	500	103.9±3.6	254.2±5.1	502.9±3.8

standard deviations (RSD) were in the 0.4–12.7% range.

3.7. Analysis of serum samples

To demonstrate the usefulness of this procedure, blank plasma samples were spiked with known amounts of each drug at three different concentrations within their therapeutic range. The data obtained (Table 5) showed satisfactory recoveries for the eight benzodiazepines. The accuracy of the MLC method was also confirmed by comparison with the reference method [4] when used for real serum samples provided by the Hospital Verge dels Lliris d'Alcoi. As shown in Table 6, serum concentrations of the eight benzodiazepines obtained by the two methods showed good correlation, indicating that there was no substantial difference between the two in the practical sense of clinical analysis.

To determine the specificity of the assay, blank plasma samples, collected from healthy adult volunteers, were analyzed using the reported procedure. No interfering peaks appear in the chromatograms when inspected at the same retention times as the benzodiazepines.

Finally it can be concluded that our MLC method is simple, does not require any pretreatment of the sample and is able to accurately determine serum benzodiazepines at any concentration observed in clinical analysis.

Table 6
Regression parameters ($Y = aX + b$) for the analysis of serum samples by the MLC method versus the HPLC reference method [4]

Compound	a	b	r
Bromazepam	0.975	2.1	0.9572
Diazepam	0.895	3.6	0.9167
Flunitrazepam	0.933	1.5	0.9428
Halazepam	0.919	2.6	0.9092
Medazepam	0.957	3.4	0.9368
Nitrazepam	1.012	2.8	0.8965
Oxazepam	0.946	4.7	0.9741
Tetrazepam	0.923	1.8	0.9619

Acknowledgements

This work was funded by Project BQU 2001-3770 (MCYT) and P1A2000-13 (BANCAIXA) from Spain. Devasish Bose, Mayte Gil-Agustí and Maria-Elisa Capella-Peiró would like to thank the Bancaixa for their research grants. Thanks also to Hospital Verge dels Lliris d'Alcoi (Spain) for providing the serum samples.

References

[1] R.J. Shader, D.J. Greenblatt, Am. J. Psychol. 134 (1977) 652.
[2] American Hospital Formulary Service, American Society of the Board of Health-System Pharmacists, Bethesda, MD, 1998.
[3] O.H. Drummer, J. Chromatogr. B 713 (1998) 201.
[4] E. Tanaka, M. Terada, S. Misawa, C. Wakasugi, J. Chromatogr. B 682 (1996) 173.
[5] I. Barbero-Rodriguez, J. Rodriguez-Procopio, L. Hernandez-Hernandez, Fresenius' Z. Anal. Chem. 328 (1987) 117.

[6] P. Odou, H. Robert, M. Luyckx, C. Brunet, T. Dine, B. Gressier, M. Cazin, J.C. Cazin, Biomed. Chromatogr. 11 (1997) 19.
[7] C. Le-Guellec, M.L. Gaudet, M. Breteau, J. Chromatogr. B 719 (1998) 227.
[8] G. Theodoridis, E.H.M. Koster, G.J. de Jong, J. Chromatogr. B 745 (2000) 49.
[9] K.M. van Rij, D. Compas, E.L. Swart, P.N.F.C. de Goede, D.J. Touw, Ther. Drug. Monit. 21 (1999) 416.
[10] E.I. Minder, R. Schaubhut, C.E. Minder, D.J. Vonderschmitt, J. Chromatogr. B 63 (1987) 135.
[11] http://www.chrompack.com
[12] M.J. Medina-Hernández, M.C. García-Alvarez-Coque, Analyst 117 (1992) 831.
[13] M.G. Khaledi, J. Chromatogr. A 780 (1997) 3.
[14] H. Nishi, J. Chromatogr. A 780 (1997) 243.
[15] M.C. García-Alvarez-Coque, J.R. Torres-Lapasió, J.J. Baeza-Baeza, J. Chromatogr. A 780 (1997) 129.
[16] A. Berthod, M.C. García-Alvarez-Coque, Micellar Liquid Chromatography, Marcel Dekker, New York, 2000.
[17] M. Gil-Agustí, S. Carda-Broch, M.C. García Alvarez-Coque, J. Esteve-Romero, J. Chromatogr. Sci. 38 (2000) 521.
[18] M. Gil-Agustí, S. Carda-Broch, M.C. García Alvarez-Coque, J. Esteve-Romero, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 1387.
[19] M.J. Koenigbauer, M.A. Curtis, J. Chromatogr. B 71 (1988) 277.
[20] C. Hansch, in: R.G. Sammes, J.B. Taylor (Eds.), Comprehensive Medicinal Chemistry, Vol. 6, Pergamon Oxford, 1990, pp. 325–535; 567–738; 842.
[21] S. Torres-Cartas, M.C. García-Alvarez-Coque, R.M. Villanueva-Camañas, Anal. Chim. Acta 302 (1995) 163.
[22] M.C. García-Alvarez-Coque, E.F. Simó-Alfonso, G. Ramis-Ramos, J.S. Esteve-Romero, J. Pharm. Biomed. Anal. 13 (1995) 237.
[23] M. Gil-Agustí, J.R. Torres Lapasió, M.C. García Alvarez-Coque, J. Esteve-Romero, J. Chromatogr. A 866 (2000) 35.
[24] M. Gil-Agustí, M.E. Capella-Peiró, Ll. Monferrer-Pons, M.C. García-Alvarez-Coque, J. Esteve-Romero, Analyst 126 (2001) 457.
[25] M. Gil-Agustí, Ll. Monferrer-Pons, M.C. García-Alvarez-Coque, J. Esteve-Romero, Talanta 54 (2001) 621.
[26] B.K. Lavine, S. Hendayana, I. Tretreault, Anal. Chem. 66 (1984) 3458.
[27] M.C. García-Alvarez-Coque, J.R. Torres-Lapasió, J.J. Baeza-Baeza, J. Chromatogr. A 780 (1997) 129.