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Solvent-modified solid-phase microextraction for the determination of diazepam in human plasma samples by capillary gas chromatography

Mette Krogh*, Hege Grefslie, Knut E. Rasmussen

Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, Oslo, Norway

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Abstract

This paper describes microextraction and gas chromatographic analysis of diazepam from human plasma. The method was based on immobilisation of 1.5 μl of 1-octanol on a polyacrylate-coated fiber designed for solid-phase microextraction. The solvent-modified fibre was used to extract diazepam from the samples. The plasma sample was pre-treated to release diazepam from the protein binding. The fibre was inserted into the modified plasma sample, adjusted to pH 5.5, an internal standard was added and the mixture was carefully stirred for 4 min. The fibre with the immobilised solvent and the enriched analytes was injected into the capillary gas chromatograph. The solvent and the extracted analytes were evaporated at 300°C in the split–splitless injection port of the gas chromatograph, separated on a methylsilicon capillary column and detected with a nitrogen–phosphorus detector. The method was shown to be reproducible with a detection limit of 0.10 nmol/ml in human plasma.

Keywords: Diazepam

1. Introduction

Sample preparation is the most tedious and time-consuming step in gas chromatographic (GC) analysis of drugs present in biological fluids such as plasma. The principal objectives of sample preparation involve concentration of the analytes and removal of as many interfering compounds as possible. Liquid–liquid extraction and solid-phase extraction are the most commonly used methods for sample preparation in bioanalysis. The drugs are extracted into a volatile solvent, concentrated by evaporation of the solvent and redissolved in a small volume of solvent, prior to injection into the GC system.

Concentration of extracted analyte by evaporation of solvent is avoided in solid-phase microextraction (SPME). In SPME, the solid phase is a non-volatile sorbent coated on a silica fibre [1]. For protection, the fibre is housed in the needle of a micro-syringe. Upon sampling, the micro-syringe plunger is depressed, thus lowering the fibre into the liquid sample for a controlled period of time. The sorption of analytes onto the fibre is enhanced by alteration of the chemical properties of the sample, e.g. pH and by agitation and heating of the sample. After sorption of the analytes, the fibre is withdrawn into the needle. The extracted analytes are then thermally desorbed

*Corresponding author.

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by introducing the fibre into the heated injection port of a gas chromatograph. SPME has successfully been used for the extraction of volatile compounds in environmental samples and foodstuffs [2–15]. The first papers on SPME in bioanalysis of drugs were reported in 1995 [16,17].

SPME was originally designed for the analysis of water samples and difficulties were observed when SPME was applied in the determination of drugs in crude biological samples such as plasma. Drugs are generally compounds with high boiling points, with molecular masses in the region of 200–400 and adsorb slowly onto the SPME fibre. The biological matrices have a high viscosity compared to water and sorption of drugs onto the fibre is significantly reduced in viscous samples. The selectivity of SPME is limited as the SPME fibres are non-selective by nature and the number of commercially available SPME coatings is limited.

In an attempt to improve extraction speed and enrichment, a solvent was immobilised on the SPME fibre and and solvent-modified SPME was investigated. Upon sampling, the fibre with the immobilised solvent is inserted into the sample solution. The chemical properties of the biological matrix are altered (e.g. changing of the pH) to favour partitioning of the analytes onto the solvent-modified fibre. Upon agitation, the partitioning is complete within 4–6 min. The fibre with the enriched analytes is then transferred to the injection port of the GC. The immobilised solvent and the analytes evaporate and separation is performed on the GC column. The total amount of solvent (μl range) used for extraction is injected into the GC and no solvent is wasted.

Benzodiazepines are among the most frequently used sedative and hypnotic drugs and are also among the most commonly abused drugs [18,19]. The chromatographic determination of benzodiazepines in biological matrices is usually performed by high-performance liquid chromatography (HPLC) [20–22], gas chromatography (GC) [23–25] or gas chromatography–mass spectrometry (GC–MS) [26–28].

In this study, the benzodiazepine, diazepam, was chosen as a model compound for solvent-modified SPME. The drug exerts a high degree of binding to plasma proteins (98% in adults) and in total drug analyses, specific measures have to be taken to reduce the protein binding prior to analysis. The purpose of this study was to develop a simple, specific chromatographic method for the determination of diazepam in human plasma for therapeutic drug monitoring and forensic toxicology.

2. Experimental

2.1. Chemicals

Diazepam was obtained from Apothekernes Laboratorium (Oslo, Norway). Prazepam was a gift from the National Institute of Forensic Toxicology (Oslo, Norway). Sodium acetate and acetic acid were from Merck (Darmstadt, Germany). Methanol was supplied by Rathburn (Walkerburn, UK). 1-Octanol, 1,2-butandiol, 2-octanone, pentylacetate and dibutylether were purchased from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) was supplied by Sigma (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

2.2. Gas chromatography

The chromatographic analyses were performed on a Shimadzu GC-14A capillary gas chromatograph (Kyoto, Japan), equipped with a 30 m×0.2 mm I.D. DB-1 (methylsilicon) column (0.25 μm film thickness, Supelco, Bellefonte, PA, USA) and either a flame ionisation detector (FID) or a nitrogen–phosphorus detector (NPD). A deactivated retention gap (2 m×0.25 mm I.D.) was coupled between the injection port of the GC and the analytical column. The temperature of the injector and the detector was set at 300°C. Helium was used as the carrier gas at a flow-rate of 1 ml/min (150°C). The detector gases were hydrogen (4 ml/min) and air (63 ml/min). Helium was used as the make-up gas at a flow-rate of 25 ml/min.

A SPME device equipped with a polyacrylate (PA)-coated fiber (film thickness, 85 μm) or a polydimethylsiloxane (PDMS)-coated fiber (film thickness, 7 and 100 μm) was supplied by Supelco. New fibres were conditioned prior to use according to the conditioning procedure recommended by the manufacturer. At the beginning of each day, the fibres were cleaned by heating them at 250°C for 5

min in the injection port of a second GC to prevent the injection of impurities onto the capillary column.

2.3. Preparation of standards

Stock standard solutions (2 μmol/ml) of diazepam and prazepam (internal standard, I.S.) were prepared in methanol. Working standard solutions of diazepam (5 nmol/ml) were prepared in 0.1, 1.0 and 5.0 *M* acetate buffer, pH 5.5, from the stock solution. Plasma samples spiked with diazepam (0.25–7.5 nmol/ml) and prazepam (I.S.) (5 nmol/ml) were prepared from the stock standard solutions. The spiked plasma samples were prepared freshly or kept at −20°C until analysis.

2.4. Sample pretreatment

To an aliquot of plasma (450 μl) was added 50 μl of methanol containing the I.S. The mixture was agitated for 1 min. The addition of methanol to plasma reduces the protein binding of the benzodiazepines. The plasma proteins were thereafter precipitated by addition of 100 μl of 1 *M* TCA. After centrifugation at 13 000 rpm for 10 min, 100 μl of 5 *M* acetate buffer, pH 7, were added to the supernatant (400 μl), which ensured a final pH in the plasma supernatant of 5.5. The modified plasma sample (150 μl) was transferred to a conical glass insert (200 μl) (Chromacol, Trumball, CT, USA).

2.5. SPME

The SPME assembly was clamped in place above the glass vial (200 μl) and the fiber was inserted into the plasma sample for microextraction of diazepam and prazepam (I.S.). The sample was stirred during enrichment to enhance partitioning.

2.6. Solvent-modified SPME

The SPME assembly was clamped in place as described in Section 2.5 and the fiber was inserted into the 1-octanol (2 ml) to allow solvent sorption and immobilisation. After 2 min, the solvent-modified fiber was withdrawn and inserted into the plasma sample for microextraction of diazepam and prazepam (I.S.). The sample was stirred during enrichment to enhance partitioning, as described above. After 4 min of exposure, the fiber was withdrawn into the SPME holder.

2.7. Capillary GC analysis

The SPME fiber was inserted through the septum and into the heated (300°C) splitless injector for 1 min, to thermally desorb the immobilised solvent and the extracted analytes into the capillary GC system for chromatographic separation and detection. After desorption was complete (1 min), the fiber was withdrawn into the SPME holder, removed from the injection port and the split vent was opened. The chromatographic separation was achieved by temperature programming. The temperature was held at 150°C for 1 min and then increased at 40°C/min to 300°C, the final temperature being held for 3 min. The chromatograms were recorded on a Shimadzu CR3-A integrator.

2.8. Determination of enrichment factor/amount extracted

The enrichment factor was determined by comparison of peak-heights and areas obtained by syringe injection of standard solutions of diazepam versus SPME/solvent-modified SPME. The amount of diazepam enriched on the fibre was correlated to the volume of sample with the equivalent amount of diazepam.

2.9. Validation of the method

The calibration graphs for the determination of diazepam in plasma were based on peak-height measurements versus the peak-height of the I.S. The limit of detection was determined at a signal-to-noise ratio of 3 ($S/N=3$).

3. Results and discussion

3.1. Extraction of diazepam from aqueous standards

The enrichment of diazepam from 1 *M* acetate buffer, pH 5.5, was studied. The enrichment was dependent on the ionic strength, and both lower and higher ionic strengths resulted in lower enrichment.

At a pH value of 5.5 or above, partitioning of diazepam ($pK_a = 3.5$) into the hydrophobic phase and the immobilised solvent is enhanced compared to lower pH values. The enrichment of diazepam from the acetate buffer (1 *M*) at pH 5.5 by SPME is shown in Fig. 1. An enrichment factor of 40 was found with the PA-coated SPME fibre (time allowed for extraction was 6 min). An enrichment factor of 40 equals the complete extraction of diazepam in 40 μl of sample onto the fibre.

Several solvents were investigated for the modification of the SPME fibres, but the fibres were found to be incompatible with most organic solvents. Only 1-octanol and 2-octanone were satisfactorily immobilised on SPME fibres that were coated with either PDMS (7 and 100 μm film thickness) or PA (85 μm film thickness). The PA coating was able to immobilise 1.5 μl and 3.0 μl of 1-octanol and 2-octanone, respectively, within 2 min (Fig. 2). The amount of immobilised solvent was determined by comparison of peak-heights obtained by syringe injection of solvent (FID). A further increase in solvent exposure time resulted in either no change or a decrease in the amount of immobilised solvent. The PDMS coatings were able to immobilise between 0.3 and 0.8 μl of 1-octanol and 2-octanone, respectively. Immobilisation of solvent occurred rapidly and no increase in the amount of solvent immobilised was observed with increased exposure time.

Enrichment factors of approximately 90 were achieved with solvent-modified SPME (PA coating) and 1-octanol and 2-octanone, as shown in Fig. 2. Twice the amount of diazepam was enriched on the solvent-modified fibre compared to the untreated SPME fiber. No significant difference in enrichment was observed between 1-octanol and 2-octanone, despite the difference in immobilised volume, i.e.,

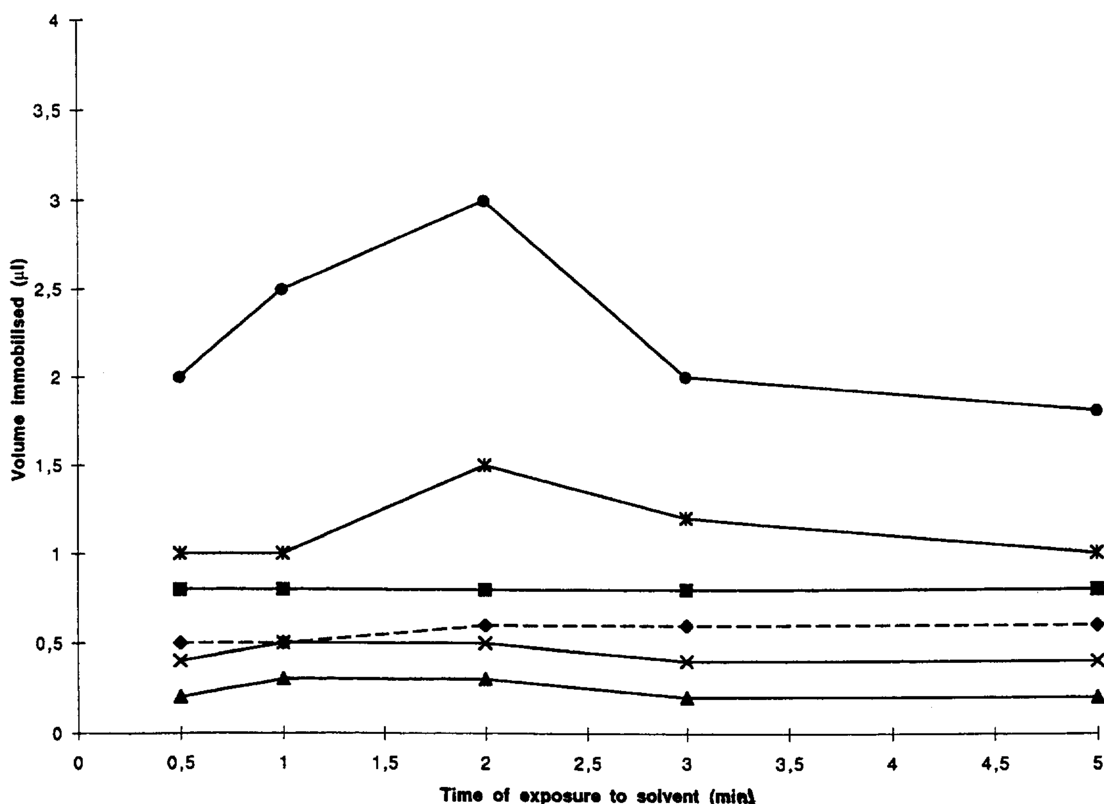

Fig. 1. The volume of immobilised solvent versus exposure time: PA fibre (85 μm) with (●) 2-octanone and (*) 1-octanol; PDMS fibre (100 μm) with (■) 2-octanone and (♦) 1-octanol; PDMS fibre (7 μm) with (×) 2-octanone and (▲) 1-octanol.

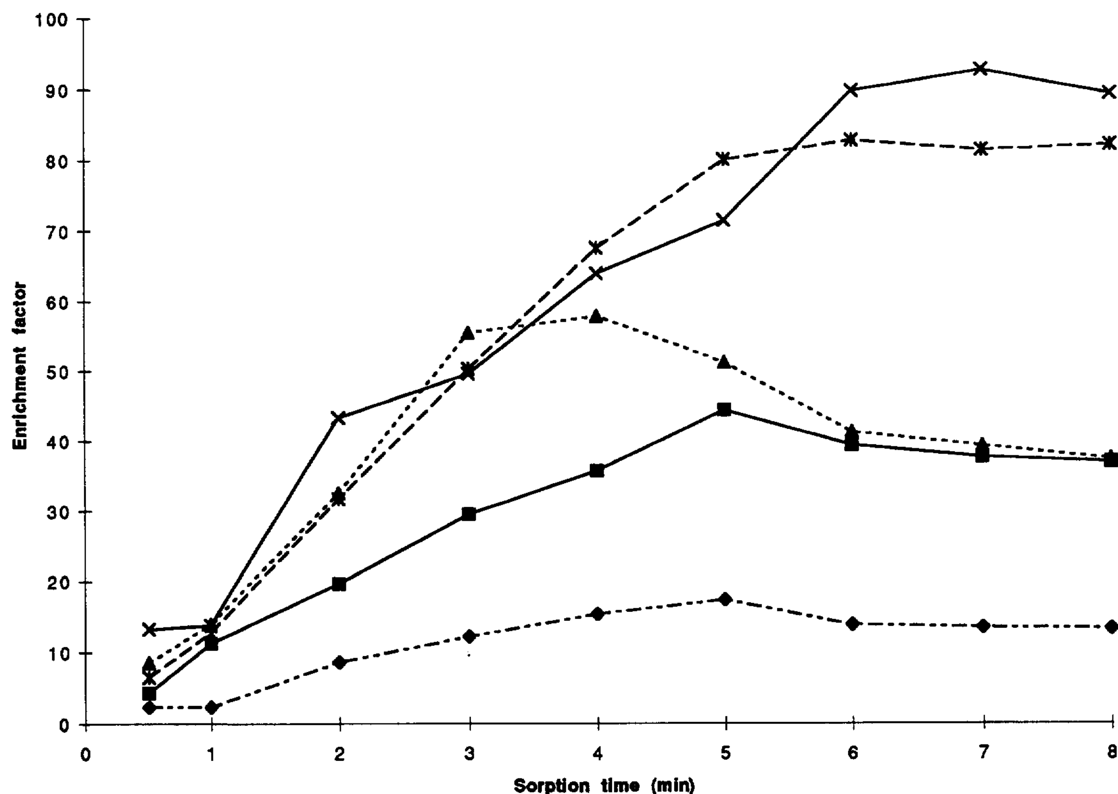

Fig. 2. Enrichment factors obtained by solvent-modified SPME and SPME of diazepam in 1 M acetate buffer, pH 5.5, versus sorption time. PDMS fibres (100 μm): (♦) SPME and (■) SPME modified with 1-octanol; PA fibres (85 μm): (▲) SPME, (*) SPME modified with 1-octanol and (×) 2-octanone.

1.5 and 3 μl of 1-octanol and 2-octanone, respectively. This demonstrated the different selectivities of the two solvents towards diazepam.

No significant dissolution of immobilised solvent in the sample was apparent, as no loss of immobilised solvent was determined by GC–FID.

3.2. Extraction of diazepam from plasma

Diazepam is strongly bound to proteins in plasma and must be released prior to analysis. In the plasma pretreatment procedure, diazepam was released from the plasma proteins prior to protein precipitation. No significant difference in enrichment was found when the plasma sample was spiked with diazepam prior to, or after, protein precipitation, i.e., no loss of diazepam occurred during the plasma pretreatment procedure.

The sorption curve of diazepam from plasma by SPME is shown in Fig. 3. The enrichment was less efficient than the enrichment of diazepam obtained from acetate buffer. The biological sample contained several endogenous compounds and exerted a higher degree of viscosity than the aqueous buffer solution. An enrichment factor of six was found by SPME (PA coating), i.e., the complete extraction of diazepam in 6 μl of sample. Macromolecules were found to adsorb onto the SPME fibre and the fibres had to be replaced after every ten injections.

The solvent-modified PA-coated SPME fibres were found to be more efficient in the extraction of diazepam than the PDMS-coated SPME fibres. With solvent-modified SPME (PA coating), an enrichment factor of about twenty was found, as shown in Fig. 3.

A sorption time of 4 min was chosen as it was sufficient to detect sub-therapeutic concentrations of diazepam while keeping within the time frame of the capillary GC separation. The time involved in the

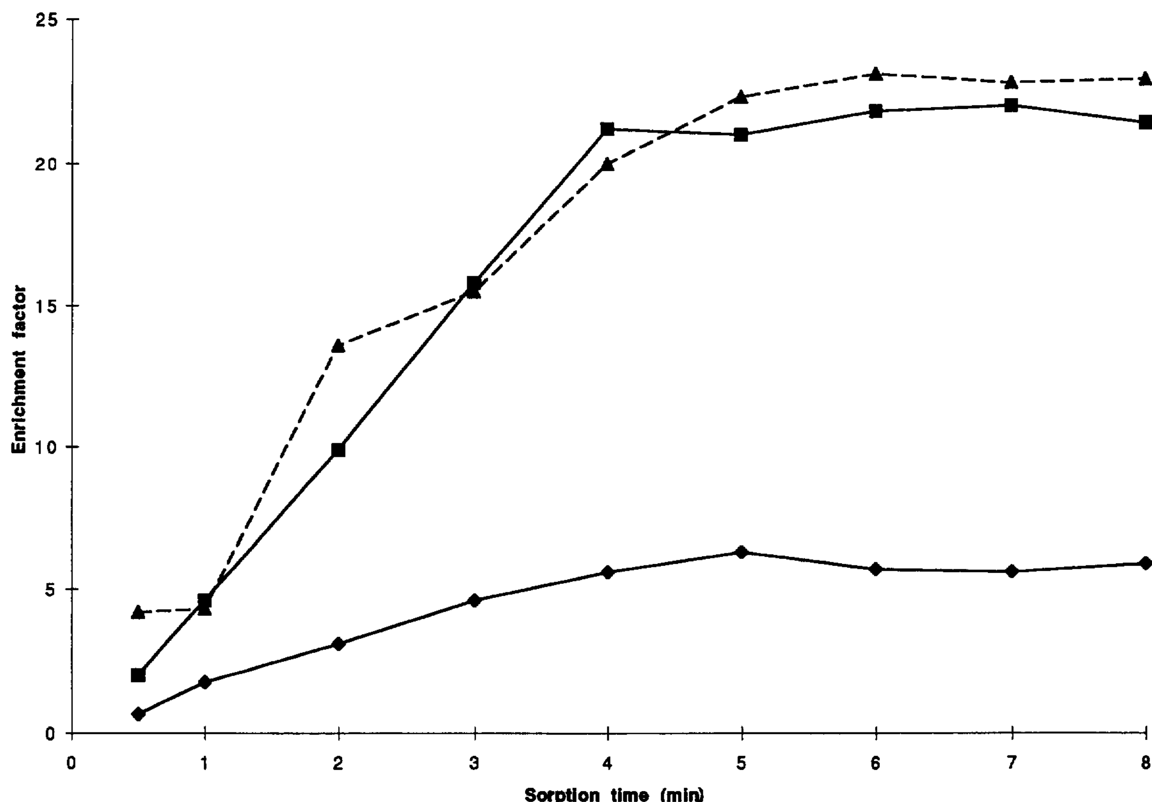

Fig. 3. Enrichment factors obtained by solvent-modified SPME and SPME of diazepam in plasma versus sorption time. PA fibres (85 μm): (♦) SPME, (▲) SPME modified with 1-octanol and (■) with 2-octanone.

sample preparation procedure should not exceed the GC run time, to ensure concurrent analysis. The solvent-modified SPME fibres had to be replaced every 40 samples, due to a significant decrease in reproducibility and enrichment.

3.3. Capillary GC analysis

A satisfactory separation of the compounds was achieved on a cross-linked methylsilicon capillary column within 8 min and no interfering peaks were detected in the analysis of drug-free plasma samples. The deactivated retention gap of 2 m was sufficient to provide reconcentration of the analytes by solvent effect and enabled a high initial column temperature (150°C) to be used and thereby resulted in shorter analysis times. A total of six to seven analyses could be performed per hour. Chromatograms of a drug-free plasma sample and a plasma sample spiked with 5 nmol/ml of diazepam and prazepam (I.S.) are shown in Fig. 4.

3.4. Validation of the method

The calibration graphs were linear in the concentration range 0.25–7.5 nmol/ml of diazepam, with correlation coefficients of r=0.9994 or better. The relative standard deviations were between 3.2 and 6.5% (n=6). The intra- and inter-assay validation of the procedure is shown in Table 1. The method was found to be reproducible. The limit of detection is determined by the sorption time, the volume of immobilised solvent and the nature of the solvent. The limit of detection at a signal-to-noise ratio of 3 was 0.10 nmol/ml for diazepam in human plasma.

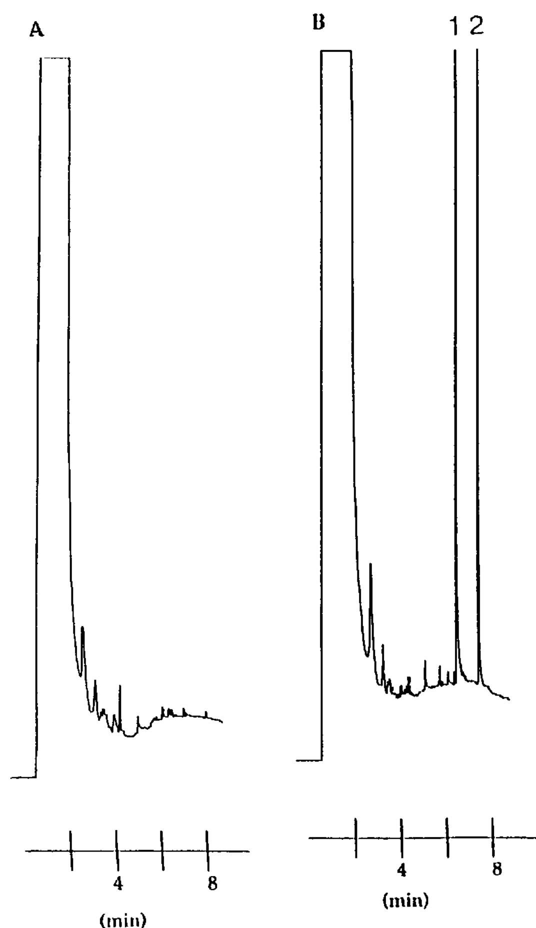

Fig. 4. Chromatogram of (A) a blank plasma sample and (B) a plasma sample spiked with 5 nmol/ml of diazepam and prazepam (I.S.). Peaks: 1=diazepam and 2=prazepam.

Table 1
Intra- and inter-assay variations after solvent-modified SPME

Concentration added (nmol/ml)	Measured concentration (mean±S.D.) (nmol/ml)	R.S.D. (%)
Intra-assay (n=6)		
0.50	0.48±0.02	4.2
3.00	2.92±0.17	5.8
5.00	4.96±0.16	3.2
Inter-assay (n=6)		
0.50	0.48±0.03	6.3
3.00	2.94±0.19	6.5
5.00	4.98±0.28	5.6

Results are expressed as the mean of parallel samples±standard deviation (S.D.) and relative standard deviation (R.S.D.).

4. Conclusions

A solvent-modified SPME method was developed for the analysis of diazepam in human plasma and was shown to be reproducible. Solvent-modified SPME offers sufficient enrichment for bioanalysis, high selectivity and short sample preparation time. The potential of solvent-modified SPME is limited by the incompatibility of the SPME coatings with most organic solvents.

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