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Determination of benzodiazepines in human urine and plasma with solvent modified solid phase micro extraction and gas chromatography; rationalisation of method development using experimental design strategies

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Abstract

Solid phase micro extraction (SPME) and gas chromatographic analysis was used for the analysis of several benzodiazepines (oxazepam, diazepam, nordiazepam, flunitrazepam and alprazolam) in human urine and plasma. Several factors likely to affect the analyte recovery were screened in a fractional factorial design in order to examine their effect on the extraction recovery. Parameters found significant in the screening were further investigated with the use of response surface methodology. The final conditions for extraction of benzodiazepines were as follows: Octanol was immobilised on a polyacrylate fibre for 4 min. The fibre was placed in the sample and extraction took place at pH 6.0 for 15 min. Urine samples were added to 0.3 g ml^{-1} sodium chloride. In plasma, the extraction recovery was less than in urine and releasing the benzodiazepines from plasma proteins followed by protein precipitation was found necessary prior to sampling. The method was validated and found linear over the range of samples. The limits of detection in urine were determined to be in the range 0.01–0.45 μmol l^{-1}. The corresponding limits of detection in plasma were in the range 0.01–0.48 μmol l^{-1}. Finally, the method developed was applied to determine some benzodiazepines after administration of a single dose. This method offers sufficient enrichment for bioanalysis after a single dose of high dose benzodiazepines as diazepam, but for low dose benzodiazepines as flunitrazepam, further sensitivity is needed.

Keywords: Benzodiazepines; Chromatography; Octanol; SPME

1. Introduction

Belardi and Pawliszyn first described the solvent free extraction technique, solid phase micro extraction (SPME), in the late 1980s [1]. SPME is

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based on the partitioning of analytes between an extracting phase; usually a polymeric material immobilised on a fused silica fibre, and a sample. Absorption is carried out for a well-defined time or until equilibrium is reached. In gas chromatography (GC), exposing the fibre into the injection port thermally desorbs the analytes. If coupled to high performance liquid chromatography (HPLC), the analytes are resolved in an organic solvent [2].

Since it was first discovered, SPME has been applied in many different areas in analytical chemistry, mainly for extraction of volatiles and semi-volatiles in environmental samples and foodstuffs. Overviews of the methods and applications have been provided [2–4]. Also, the determination of a number of compounds, e.g. cocaine, barbiturates, amphetamine, valproic acid, methadone, nicotine, antidepressants, benzodiazepines and local anaesthetics in biological matrices have been published [5–16].

The sensitivity of an SPME method is mostly governed by the partition coefficient of an analyte between the coating and the sample matrix. Selectivity can be achieved by choosing the appropriate type of polymeric material. Other factors known to improve the analyte recovery onto the fibres are absorption time, alteration of pH and the addition of salt [2]. Immobilisation of Octanol onto the SPME fibre prior to sampling has been reported to improve the enrichment of diazepam in human plasma [8]. Although a relatively large numbers of factors are known to influence the analyte recovery, little emphasis has been applied on their systematic optimisation by the advantages of experimental design strategies; as most of the existing literature is based on univariate investigations of factors. In this paper several benzodiazepines were selected to study SPME in urine and plasma using experimental design strategies. Benzodiazepines are one of the most frequently prescribed drugs used as tranquillisers, sleep inducers, hypnotics, anticonvulsants and anxiolytics. The chemical structures and physicochemical properties of these benzodiazepines are listed in Table 1. The benzodiazepines are amphoteric and relatively polar compounds. These properties restrict to some extent the possibilities of analysing underivatised benzodiazepines by GC. Many different approaches for the analysis of this group of compounds by GC, HPLC, thin-layer chromatography and other methods have already been reported [17–22]. The sample preparation step usually is laborious and time consuming. SPME would therefore be a simple and reliable alternative.

In this study, several factors likely to affect the analyte recovery, such as absorption and desorption time, fibre type, immobilisation of Octanol, pH, addition of salt and the volume of the urine sample were screened in a fractional factorial design in order to examine their effect on the extraction recovery onto the fibre. Parameters found significant in the screening were further investigated with response surface methodology. The screening study and the response surface investigation were both performed in urine, and attempts to extrapolate the results to plasma will be discussed. Finally, the analysis conditions were validated and used to determine benzodiazepines in real samples.

2. Experimental

2.1. Chemicals

Diazepam, nordiazepam, oxazepam, flunitrazepam, alprazolam cut, prazepam and trichloroacetic acid (TCA) were obtained from Sigma, St. Louis (MO). Sodium chloride (NaCl), sodium acetate and hydrochloric acid (HCl) were purchased from Baker B.V (Deventer, The Netherlands). Methanol and Octanol were obtained from Lab Scan (Dublin, Ireland). Acetic acid and glycerol were purchased from Merck (Darmstadt, Germany), β-Glucuronidase/arylsulfatase from Helix pomatia was purchased from Boehringer Mannheim (GmbH, Germany). Deionized water was obtained from a Milli-Q plus 185 water-purification system from Millipore (Bedford, MA, USA).

2.2. Equipment

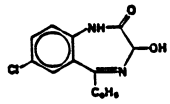
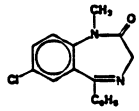
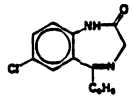
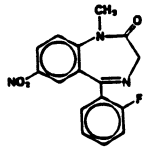
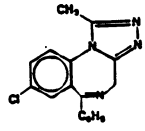
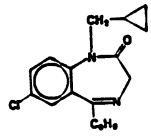
SPME fibre assemblies, 100 μm polymethylsiloxanes (PDMS), 85 μm polyacrylate (PA) and

65 μm Carbowax/template, polyethylene glycol/template poly (divinylbenzene resin) (CAX) fibres and the fibre holders were purchased from Supelco (Bellefonte PA, USA). Samples were extracted from clear glass vials 2.0 ml from Hewlett Packard (HP), (Palo Alto, CA) and 5.0 ml, 5 SEPEX, Bio Merieux, (Mercy l'Etoile, France). Samples were stirred using a stirrer. Measurements of pH were made with a Metrohm pH-meter 744. Samples were stirred with a Teflon coated 5 × 3 mm stir bar.

2.3. Preparation of standards

Working standard solutions (10–300 μmol l^{-1}) of diazepam, nordiazepam, flunitrazepam, oxazepam, alprazolam and prazepam were prepared in methanol. During the study, urine and plasma samples were prepared by adding the same amount of working standard solutions in order to keep the amount of methanol in the sample constant.

Table 1
Description of the structures: Physicochemical properties for the benzodiazepines

Name	Chemical	Molecular weight	Melting point	pK_a (20°C)	log P (20°C)	(Oct/water) pH = 7.4
Oxazepam		286.7	≅198	1.7, 11.6	2.2	
Diazepam		284.7	131-135	3.3	2.7	
Nordiazepam		270.7	≅216	3.5, 12.0	-	
Flunitrazepam		313.3	≅170	1.8	2.4	
Alprazolam		308.8	225 - 231	2.4	3.2	
Prazepam		324.8	143 - 148	2.7	3.7	

2.4. SPME sampling in urine

Firstly, 0.3 g ml^{-1} of solid NaCl was weighed into empty vials and a magnetic stir bar was added. The vials were then added, i.e. 1350 μl urine, 150 μl 1 M acetate buffer and 15 μl of the standard solution. The PA coated fibre was placed into Octanol (1.5 ml) to allow solvent absorption and immobilisation for 4 min. The fibre was then placed into the sample solution where extraction took place for 15 min. In the hydrolysis experiment, the urine samples were added. 30 μl 3-glucuronidase/arylsulfatase and heated at 55°C for 2 h prior to sampling as described above.

2.5. SPME sampling in plasma

3000 μl plasma was added 30 μl of the drug standard solution. 250 μl 1 M HCl in 25% glycerol was added and the mixture was shaken for 1 min and 665 μl 1 M TCA was added. Centrifugation at 1200 *g* for 10 min was performed and 1.5 ml of the supernatant was transferred into a glass vial with a magnetic stir bar. The solution was buffered to pH 6.0 with 5 M-acetate buffer. The solution was then sampled in the same manner as outlined for urine analysis.

2.6. Maintenance of fibres

Fibres were conditioned in a GC injector port prior to use, according to the manufacturer's recommendation. Fibres were cleaned by immersing them in a water solution and stirred for 2 min and then reconditioned in the GC injector for 3 min after each sample. At the end of the day, fibres were reconditioned for 30 min, and a fibre blank was performed daily to check the baseline.

2.7. Gas chromatographic analysis

All GC separations were performed on a HP 5890 equipped with a nitrogen phosphorous detector (NPD) with a narrow insert (73.0 × 5.5 mm × 0.1 mm i.d.). Helium was used as the carrier-gas at a flow-rate of 1 ml min^{-1} at 150°C. Method development and validation was carried out using a CPSIL 8 CB column (25 × 0.25 mm i.d., 0.25 μm) from Chrompack, (Middelburg, The Netherlands). The injector temperature and the NPD detector temperature were maintained at 300°C. The fibres were desorbed in the GC injector for 1 min at 300°C. Column programming was as follows: initial temperature 150°C, rising by 40°C min^{-1} to 230°C, hold time 2 min, then rinsing by 5°C min^{-1} to 250°C, hold time 1 min, finally rising by 15°C min^{-1} to 300°C with a hold time for 3 min.

2.8. Validation of the method

The benzodiazepines were determined from standard curves based on measurements of peak area. Prazepam was used as the internal standard (1 μmol l^{-1}) in the validation. For preparation of the standard curves, aliquots of 1500 μl buffered urine and 3000 μl plasma were spiked to obtain concentrations ranging from 0.5 to 3.0 μmol l^{-1} of oxazepam, flunitrazepam or alprazolam, or 0.1–3.0 μmol l^{-1} diazepam or nordiazepam, respectively. Samples spiked to 0.5, 1.0 and 3.0 μmol l^{-1} of all the drugs were analysed for within- and between-day validation data ($n = 6$). The limit of detection was determined at a signal to noise ratio of 3 ($S/N = 3$).

3. Experimental design

The pH of the urine (4, 5 and 6), the absorption time (1.0, 5.5 and 10.0 min), the desorption time (5.0, 7.5 and 10.0 min), addition of NaCl (0.00, 0.15 and 0.30 g ml^{-1}), the volume of the sample (1.50, 3.25 and 5.00 ml) and the immobilisation time in Octanol (0, 2 and 4 min), were included as variables in the screening design. A fractional factorial design (1/4 2^6) was chosen for the evaluation of these variable influence on the analyse recovery after performing 19 experiments on three different fibres. The settings and design are listed in Table 2.

The most significant factors found (absorption time, immobilisation of Octanol, concentration of NaCl and the pH) in the screening were selected for further investigation in a multifactorial response surface. By bringing the 'star points' into the face of the cube, a 'face centred central com-

Table 2
Factors and their levels during the screening experiment

Factor	Low level	Medium level	High level
Absorption time (min)	1.00	5.50	10.00
Desorption time at 250°C (min)	5.00	7.50	10.00
Immobilisation of Octanol (min)	0.00	2.00	4.00
pH	4.00	5.00	6.00
Concentration of NaCl (g ml^{-1})	0.00	0.15	0.30
Volume of the urine sample (ml)	1.50	3.25	5.00

posite is produced'. The addition of extra points around the method value allows better estimation of the effects in the area of interest and can provide higher confidence in the result [23]. The response surface experiment (RSM) with the levels of the factors is listed in Table 3.

Multiple linear regression analysis was used to determine if linear quadratic or interaction terms of the factors were significant for the effect. Both screening and RSM were carried out in a randomised order. The analyte peak areas for the model compounds were used as the response variable in all experiments. For each of the drugs, a second-order regression model (Eq. (1)) was developed by means of multiple linear regression.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_1x_2 + b_6x_1x_3 + b_7x_1x_4 + b_8x_2x_3 + b_9x_2x_4 + b_{10}x_3x_4 + b_{11}x_1^2 + b_{12}x_2^2 + b_{13}x_3^2 + b_{14}x_4^2 \quad (1)$$

where Y is the analyte recovery, $b_0 \ldots b_{14}$ represents the regression coefficients and $x_1 \ldots x_4$ are the coded levels for the absorption time, the concentration of NaCl, the immobilisation time in Octanol and the pH, respectively in Table 3. Non significant regression coefficients ($\alpha = 0.05$) were excluded from the models. The programme MODDE version 3.0 from Umetri AB (Umeå, Sweden) was used for design and evaluation of the chemometric studies.

4. Results and discussion

4.1. Evaluation of fibre coating

The choice of an appropriate fibre coating is essential for the SPME method development. The sensitivity of the fibre towards an analyte is, among others, depending on the molecular weight and polarity of the analytes to be extracted [2]. In the screening study, the three most frequently used fibre coatings all from Supelco were chosen in order to examine the extraction recovery of the benzodiazepines. The chemical structures of the coatings are shown in Fig. 1. The highest recoveries obtained in the screening are shown in Fig. 2. From this figure it can be seen that the recovery plays an important role for oxazepam, flunitrazepam and alprazolam, since these benzodiazepines show a low recovery on all the fibres. In all of these cases, the PDMS fibre gives the lowest recovery. Based on this figure one would probably prefer CAX to PA as fibre coating with the over all highest recovery. However, we observed that, compared to the CAX, the PA fibre was much more robust in biological matrices. This is probably due to the absorption of molecules in the biological matrices, which causes a solid layer onto the CAX fibre at high temperatures. The layer was found difficult to wash off between analysis and dissolved into the sample after some injections. The problem was not observed in aqueous solutions. The PA coating can be used at higher injector temperatures than the CAX fibre as recommended by the manufacturer. These results in lower desorption time and less chance for carry-over between analysis. Therefore, SPME on PA fibres was chosen to be further investigated.

4.1.1. Extraction time profile

SPME is a process in which the analytes partition between the sample matrix and the polymeric stationary phase. The time to reach equilibrium

Table 3
Factors and their levels in the response surface experiment

Factor	Low level	Medium level	High level
Absorption time (min)	5.00	10.00	15.00
Immobilisation of Octanol (min)	0.00	2.00	4.00
pH	4.00	6.00	8.00
Concentration of NaCl (g ml^{-1})	0.20	0.30	0.40

depends, among other factors, upon the distribution constant between the sample and the coating, and is usually up to several hours for analytes as the benzodiazepines [9]. To keep the absorption time within a reasonable time frame with respect to the GC analysis, the absorption time chosen to be investigated ranged from 1 to 10 min. Increasing the extraction time from 1 to 10 min was found to be highly significant for the extraction recovery and was further investigated up to 15 min in the RSM.

4.1.2. Desorption time

The fibre containing the analytes is transferred to the injector port of the GC. The analytes desorbs from the coating into the stream of carrier gas. The desorption process is inverse to the absorption from a well-agitated solution. The initial concentration in the gas should be zero and there should be a high linear flow rate around the fibre. This can be obtained by using a narrow insert in the injector. Desorption times at 250 and 300°C were investigated. Increasing the desorption time from 5 to 10 min at 250°C was not found significant. It is possible to use the PA coated fibre under higher temperatures as 300°C. At 300°C, full desorption of the analytes were obtained within 1 min, and desorption time was kept constant in further investigations.

4.1.3. Addition of salt

The addition of salt into the sample matrix decreases the solubility of the target analytes [2], which results in an increase in the amount of the analyses extracted by the fibre coating. In this way, the sensitivity can be significantly increased for polar analyses. It is observed that the largest amount extracted is under saturated salt conditions in aqueous solution. From the screening, it appeared that the addition of salt was significant for the extraction recovery.

4.1.4. Effect of pH

Since most of the benzodiazepines have two pK_a values, near 1 and 12, the partitioning is strongly affected by the pH. The effect of pH on the extraction of the benzodiazepines was examined in a range 4–6. This range is chosen based on previously published papers [8,9]. Increasing the pH from 4 to 6 was significant for the analyte recovery.

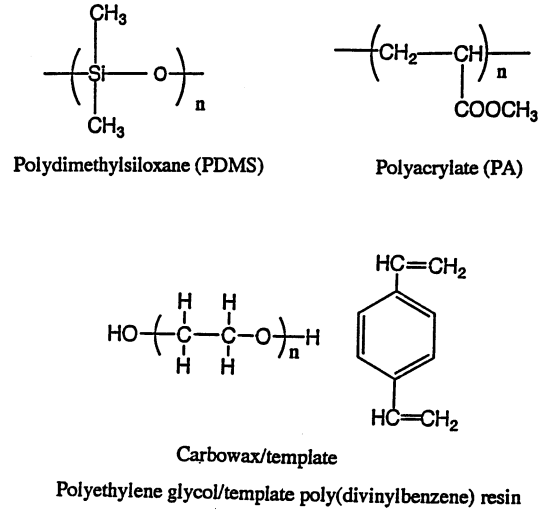


Fig. 1. Structures of polymeric materials for SPME coating chosen to be investigated.

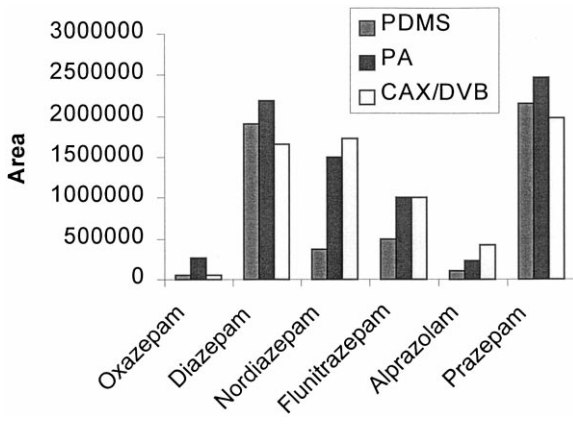


Fig. 2. Extractions of benzodiazepines by direct SPME in urine with PDMS, PA and CAX fibre. Results from screening.

4.1.5. Immobilisation of Octanol

Immobilisation of an organic solvent onto the fibre has been shown to increase the extraction recovery from a PA fibre for diazepam [8], By immobilising Octanol, a hydrophobic layer is created on the fibre. Hydrophobic drugs are then more easily extracted onto the fibre. The effect of immobilisation of Octanol onto the fibre was found to be significant in the screening and it was chosen to further investigate this within a time frame of up to 4 min.

4.1.6. Multiple linear analysis of the screening

Multiple linear analysis of the screening experiment showed significant terms for the absorption time, pH, immobilisation of Octanol and addition of NaCl. This was valid for all model compounds with the exception of alprazolam. Changing the pH from 4 to 6 was not found significant for the extraction of alprazolam. Non-significant terms included the volume of the sample and the desorption time. The statistical analysis of a regression model containing the significant terms gave R^2 values ranging from 0.929 to 0.984 and Q^2 values ranging from 0.846 to 0.979. The regression models were significant for all experiments. ($P < 0.001$). The significant terms were chosen for further investigation in a response surface experiment.

4.1.7. Response surface method (RSM)

A central composite face centred design was used to examine the influence of the variables on the extraction recovery. The pH was varied from 4 to 8, the absorption time from 5 to 15 min, the concentration of NaCl from 0.2 to 0.4 g ml^{-1} and the immobilisation time in Octanol from 0 to 4 min. The terms found non-significant according to ANOVA were excluded from the second order regression models with a backward elimination mode. The terms found least significant for the recovery were eliminated, and the model was refitted. The process was repeated until the regression model for the drugs only contained significant terms, or terms which was not allowed to exclude in order to enforce hierarchy of the model terms. The multiple linear regression analysis of the final models obtained (Table 4) gave an acceptable summary of fit R^2 ranging from 0.844 to 0.980 and Q^2 ranging from 0.745 to 0.972. All models were significant ($P < 0.001$). For all of the drugs, the linear terms of the absorption time and the immobilisation time in Octanol had a positive effect on the extraction recovery. The second order term for addition of NaCl was also found significant implying that there must be an optimum NaCl concentration. The interaction term absorption time x immobilisation time in Octanol was found significant for all the drugs except for flunitrazepam. The interaction term indicated that when the immobilisation time in Octanol was high, a steeper increase in the analyte recovery with increasing absorption time was observed, compared to low immobilisation time in Octanol. Based on the predicted equations (Table 4), 3D contour plots for the analyte recoveries of the model compounds were constructed. The 3D contour plot for nordiazepam is shown in Fig. 3. The plot shows the predicted values for the SPME recoveries of various combinations of variables. From the response surface for all of the drugs, the final analysis conditions were chosen to be the immobilisation of Octanol on to the fibre for 4 min, absorption in the sample for 15 min, and the addition of 0.3 g ml^{-1} NaCl at a pH of 6.0. Since the final analysis conditions chosen are under non-equilibrium conditions, it can be expected

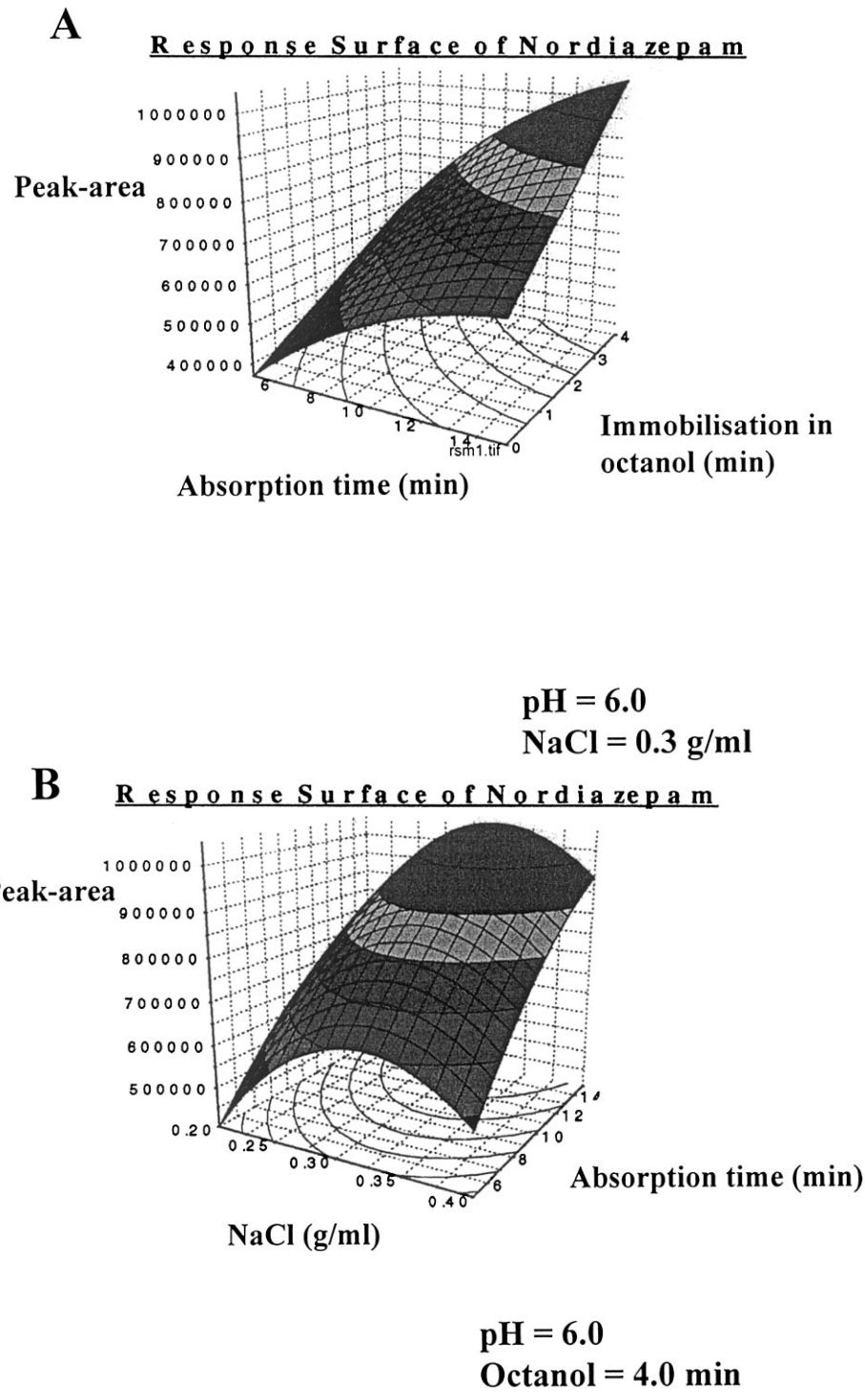


Fig. 3. Response surfaces of nordiazepam according to the central composite face-centred design. (A) The analyte recovery (peak-area) as a function of the absorption time (min) and the immobilisation time in Octanol (min). Addition of sodium chloride, 0.3 g ml^{-1}; pH, 6.0; (B) The analyte recovery (peak-area) as a function of addition of sodium chloride (g ml^{-1}) and the absorption time (min). Immobilisation time in Octanol, 4.0 min; pH, 6.0.

Table 4
Predicting equations for the analyte recovery of the benzodiazepines based on coded influence variables[a]

Analyte	Equation	R^2	Q^2
Oxazepam	$y = 0.18\times10^6 + x_1\ 0.04\times10^6 - x_2\ 0.02\times10^6 + x_3\ 0.04\times10^6 - x_2^2\ 0.16\times10^6 + x_1x_3\ 0.02\times10^6$	0.844	0.745
Diazepam	$y = 1.22\times10^6 + x_1\ 0.50\times10^6 + x_2\ 0.06\times10^6 + x_3\ 0.26\times10^6 - x_2^2\ 0.16\times10^6 + x_1x_3\ 0.10\times10^6$	0.983	0.972
Nordiazepam	$y = 0.73\times10^6 + x_1\ 0.16\times10^6 + x_2\ 0.07\times10^6 + x_3\ 0.17\times10^6 + x_4\ 0.06\times10^6 - x_1^2\ 0.05\times10^6 - x_2^2\ 0.18\times10^6 + x_1x_3\ 0.03\times10^6 + x_2x_4\ 0.04\times10^6$	0.980	0.949
Flunitrazepam	$y = 0.37\times10^6 + x_1\ 0.11\times10^6 + x_2\ 0.01\times10^6 + x_3\ 0.10\times10^6 - x_4\ 0.01\times10^6 - x_2^2\ 0.07\times10^6 + x_1x_3\ 0.03\times10^6 + x_2x_4\ 0\ 04\times10^6$	0.882	0.759
Alprazolam	$y = 0.25\times10^6 + x_1\ 0.06\times10^6 + x_2\ 0.00\times10^6 + x_3\ 0.04\times10^6 - x_4\ 0.02\times10^6 - x_2^2\ 0.11\times10^6 + x_1x_3\ 0.02\times10^6$	0.969	0.948
Prazepam	$y = 1.32\times10^6 + x_1\ 0.49\times10^6 + \mathrm{x}_2\ 0.02\times10^6 + x_3\ 0.30\times10^6\ - x_2^2\ 0.32\times10^6 + x_1x_3\ 0.13\times10^6$	0.970	0.944

[a] The fraction of variation of the analyte recovery explained by the model (R^2), and the fraction of variation of the response predicted by the model (Q^2). All models were significant. ($\alpha = 0.05$), $P_{reg} < 0.001$, x_1, absorption time; x_2, sodium chloride; x_3, immobilisation time in Octanol, x_4, pH.

that small variations in the absorption time and immobilisation time in Octanol will affect the robustness of the method. In quantitative analysis under non-equilibrium conditions addition of an appropriate internal standard and exact timing of all sampling step must be taken care of.

4.2. Extrapolation of the results obtained in urine to plasma

Benzodiazepines are strongly bound to plasma proteins and must be released prior to analysis to ensure a high extraction recovery. Release is mainly carried out in two ways. One is dilution of the plasma with a buffer. Here the drawback is a decreased sensitivity caused by the dilution and the competition of the proteins with the drugs for absorption to the SPME fibres. This approach yields a very low recovery and a high background in the chromatogram. The second approach is precipitation of the proteins followed by centrifugation and further analysis of the supernatant. 1 M HCl in glycerol was added firstly to plasma in order to release the drugs from the proteins. In addition, TCA was added to precipitate the proteins. After centrifugation the supernatant obtained is highly acidic and must be buffered to pH 6 with a much more concentrated buffer (5 M) than used in the urine samples. Addition of the same amount of salt as in the urine samples to the buffered supernatant decreased the recovery. This is due to the high ionic strength in the supernatant and further addition of salt might even precipitate out the drugs of interest. High salt concentration can form an ionic layer around the fibre causing repulsing of the charged analytes. Addition of salt to plasma was therefore excluded. Absorption time, immobilisation of Octanol, desorption time and the actual pH were the same as for urine analysis.

5. Validation

This method was found linear in the concentration range 0.5–3 μmol l^{-1} for oxazepam, alprazolam and flunitrazepam respectively and in the range 0.1–3 μmol l^{-1} for diazepam and nordiazepam. The correlation coefficients obtained where 0.991 or better. The within-day and between-day validation data are shown in Tables 5 and 6. The limit of detection in urine at a signal to noise ratio of 3 ($S/N = 3$) were 0.45, 0.02, 0.01, 0.09 and 0.35 μmol l^{-1} for oxazepam, nordiazepam, diazepam, flunitrazepam and alprazolam, respectively. The corresponding limits of detection in plasma were determined to be 0.37, 0.02, 0.01, 0.10 and 0.48 μmol l^{-1}.

5.1. Analysis of clinical samples

Two volunteers were administered a single dose of 1 mg flunitrazepam and 5 mg diazepam, respec-

Table 5
Within -day validation data for the determination of benzodiazepines in human urine and plasma[a,b]

Added conc. (μmol l^{-1})	Measured conc.			RSD (%)	Accuracy (%)
	Mean ± SD, $n = 6$ (μmol l^{-1})				
Oxazepam					
Urine					
0.5	0.55	±	0.03	5.45	110.00
1.0	1.05	±	0.03	3.08	105.19
3.0	2.95	±	0.28	9.44	98.29
Plasma					
0.5	0.47	±	0.04	8.51	94.00
1.0	1.02	±	0.03	2.94	102.00
3.0	2.90	±	0.07	2.41	96.67
Diazepam					
Urine					
0.5	0.53	±	0.01	1.89	106.00
1.0	1.02	±	0.10	9.80	102.00
3.0	2.91	±	0.08	2.75	97.00
Plasma					
0.5	0.51	±	0.01	1.96	102.00
1.0	1.04	±	0.03	2.88	104.00
3.0	2.98	±	0.11	3.69	99.33
Nordiazepam					
Urine					
0.5	0.49	±	0.01	2.04	98.00
1.0	0.95	±	0.04	4.33	94.98
3.0	2.80	±	0.18	6.54	93.27
Plasma					
0.5	0.52	±	0.01	1.92	104.00
1.0	0.99	±	0.01	1.01	99.00
3.0	2.85	±	0.13	4.56	95.00
Flunitrazepam					
Urine					
0.5	0.51	±	0.02	3.92	102.00
1.0	1.01	±	0.08	7.92	101.00
3.0	3.27	±	0.15	4.59	109.00
Plasma					
0.5	0.54	±	0.02	3.70	108.00
1.0	1.05	±	0.10	9.52	105.00
3.0	3.10	±	0.24	7.74	103.33
Alprazolam					
Urine					
0.5	0.54	±	0.02	3.70	108.00
1.0	0.93	±	0.05	4.84	93.00
3.0	3.02	±	0.12	3.97	100.67
Plasma					
0.5	0.47	±	0.03	6.38	94.00
1.0	0.93	±	0.05	4.84	93.00
3.0	3.02	±	0.12	3.97	100.67

[a] Determined with solvent-modified SPME and gas chromatography.
[b] SD, Standard deviation; RSD, Relative standard deviation.

Table 6
Between-day validation data for the determination of benzodiazepines in human urine and plasma[a,b]

Added conc.	Measured conc. (μmol l^{-1})			RSD (%)	Accuracy (%)
	Mean ± SD, $n = 6$, (μmol l^{-1})				
Oxazepam					
Urine					
0.5	0.52	±	0.05	9.62	104.0
1.0	0.96	±	0.09	9.38	96.0
3.0	2.78	±	0.34	12.23	92.7
Plasma					
0.5	0.40	±	0.06	15.00	80.0
1.0	1.01	±	0.04	3.96	101.0
3.0	3.05	±	0.09	2.95	101.7
Diazepam					
Urine					
0.5	0.54	±	0.02	3.70	108.0
1.0	1.01	±	0.03	2.97	100.7
3.0	2.83	±	0.29	10.31	94.4
Plasma					
0.5	0.49	±	0.02	3.92	98.0
1.0	1.03	±	0.05	4.85	103.0
3.0	2.96	±	0.14	4.73	98.7
Nordiazepam					
Urine					
0.5	0.46	±	0.01	2.17	94.0
1.0	0.95	±	0.01	1.01	99.0
3.0	2.85	±	0.13	4.56	95.0
Plasma					
0.5	0.47	±	0.02	4.26	94.0
1.0	0.98	±	0.03	3.06	98.0
3.0	2.80	±	0.27	9.64	93.3
Flunitrazepam					
Urine					
0.5	0.47	±	0.07	14.89	94.00
1.0	1.01	±	0.07	6.93	101.00
3.0	3.02	±	0.23	7.51	100.70
Plasma					
0.5	0.55	±	0.06	10.91	110.00
1.0	1.11	±	0.13	11.31	110.50
3 0	3.33	±	0.34	10.21	111.00
Alprazolam					
Urine					
0.5	0.55	±	0.07	12.73	110.00
1.0	1.04	±	0.06	5.77	104.00
3.0	2.98	±	0.14	4.70	99.33
Plasma					
0.5	0.54	±	0.05	9.26	108.00
1.0	0.97	±	0.07	7.22	97.00
3.0	2.98	±	0.14	4.70	99.33

[a] Determined with solvent-modified SPME and gas chromatography.
[b] SD, Standard deviation, RSD, Relative standard deviation.

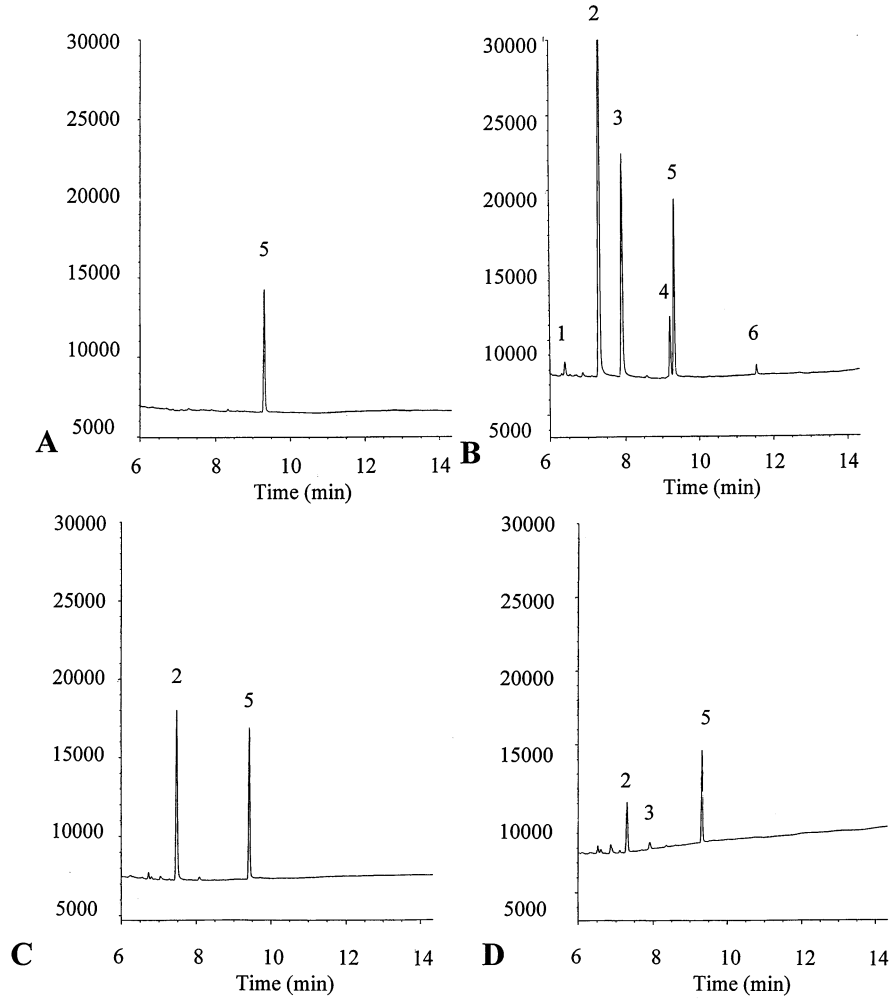


Fig. 4. Chromatograms of plasma samples. (A) Drug free human plasma added prazepam (I.S), 1 μmol l^{-1}; (B) Drug free human plasma added oxazepam, diazepam, nordiazepam, flunitrazepam, prazepam (I.S) and alprazolam, 1 μmol l^{-1}; (C) Chromatogram of human plasma 2 h after intake of 5 mg diazepam; diazepam, 0.33 μmol l^{-1}; prazepam (I.S), 1 μmol l^{-1}; (D) Chromatogram of human plasma 18 h after intake of 5 mg diazepam; diazepam, 0.14 μmol l^{-1}; nordiazepam, 0.09 μmol l^{-1}; prazepam (I.S), 1 μmol l^{-1}. Peaks: 1, oxazepam; 2, diazepam; 3, nordiazepam; 4, flunitrazepam; 5, prazepam (I.S); 6, alprazolam.

tively. Plasma samples were obtained 2 and 18 h after administration. Urine was sampled for 48 h. Diazepam and its metabolite nordiazepam could be detected in the plasma samples obtained. In urine a hydrolysis step was necessary prior to SPME sampling in order to release nordiazepam from its glucuronides. Chromatograms from the single dose experiment are shown in Figs. 4 and 5. Flunitrazepam could not be detected in neither plasma nor urine. These results imply that the SPME method has not yet sufficient sensitivity to detect single doses of low-dose benzodiazepines as flunitrazepam. Hydrolysis of urine can be useful to increase the sensitivity in urine analysis.

6. Conclusion

An SPME method for the determination of benzodiazepines in urine and plasma has been

developed with experimental design strategies, which showed to be a helpful tool to rationalise and systematically develop the final SPME procedure. Factors found to be significant for the recovery were the type of the fibre, its robustness in biological matrices and performance in the GC injector at high temperature. Increasing the absorption time and the immobilisation time of Octanol prior to sampling was found to positively effect the extraction recovery. The results indicated that there was an optimum NaCl concentration. A PA fiber was selected in the final conditions for SPME analysis of the benzodiazepines. Immobilisation of Octanol onto the fiber prior to enrichment was carried out for 4 min followed by absorption in the matrix for 15 min. The pH of the sample was kept at 6.0. Urine samples were added to 0.3 g ml^{-1} sodium chloride. In urine and plasma the method offers sufficient enrichment for bioanalysis after a single

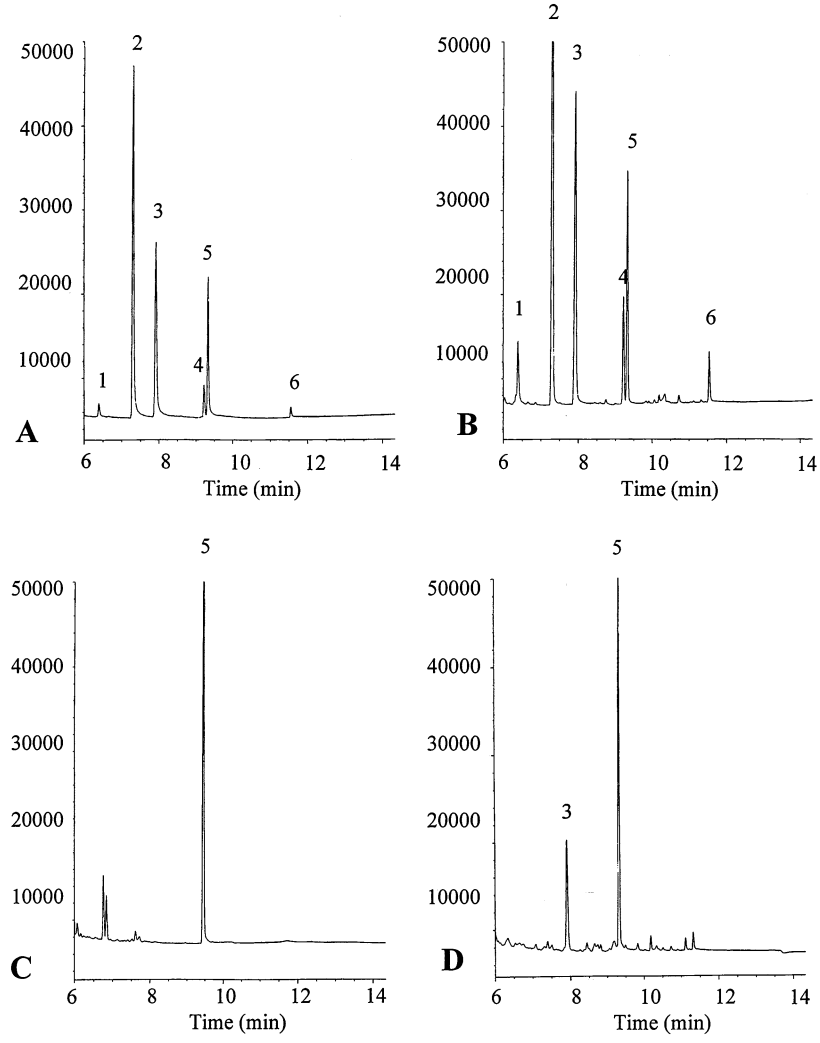


Fig. 5. Chromatograms of urine samples. (A) Drug free human urine added oxazepam, diazepam, nordiazepam, flunitrazepam, prazepam (I.S) and alprazolam, all 1 μmol l^{-1}; (B) Chromatogram of the same sample as in (A) after enzymatic hydrolysis; (C) Chromatogram of human urine 22 h after intake of 5 mg diazepam; prazepam (I.S), 1 μmol l^{-1}; (D) Chromatogram of the same sample as in (C) after enzymatic hydrolysis; nordiazepam, 0.18 μmol l^{-1}; prazepam (I.S), 1 μmol l^{-1}. Peaks: 1, oxazepam; 2, diazepam; 3, nordiazepam; 4, flunitrazepam; 5, prazepam (I.S); 6, alprazolam.

dose of high dose benzodiazepines as diazepam, but for low dose benzodiazepines as flunitrazepam further sensitivity is needed. Enzymatic hydrolysis of the urine samples increases the sensitivity of the method. In plasma protein precipitation prior to SPME extraction was found necessary in order to increase the analyte recovery and prolong the lifetime of the PA fibres.

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