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Article

Evaluation of Diazepam-Molecularly Imprinted Microspheres for the Separation of Diazepam and its Main Metabolite from Body Fluid Samples

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Molecularly imprinted microspheres (MIMs) for the drug diazepam and its main metabolite (nordiazepam) were prepared and used to separate the two species from urine and serum samples via molecularly imprinted solid-phase extraction. The specific binding capacity for diazepam was determined to be 1.97 mg/g, resulting in an imprinting factor of 5.8. The MIMs exhibit highly selective binding affinity for tricyclic benzodiazepines. Water–acetonitrile–acetone mixtures were used as the washing solvent and resulted in complete baseline separation, with a recovery of >87% for diazepam and of 88% for nordiazepam. The limits of detection are 21.5 and 24.5 ng/mL, respectively.

Introduction

Molecular imprinting is a technique used for preparing polymeric materials that have a prearranged structure and specific molecular recognition ability. The molecularly imprinted polymers (MIPs) are synthesized by co-polymerizing their functional monomers and cross-linkers in the presence of template molecules. After removal of a template, the resultant cavities, which complement a template in size, shape and arrangement of functional groups (from the functional monomer), are allowed to rebind to the template molecules (1, 2).

Bulk polymerization has traditionally been used for preparing MIPs. To generate particles with an appropriate size for use, the bulk MIPs must be crushed, ground and sieved. Commonly, the particles produced in this tedious and time-consuming process are irregular both in size and in shape, which ultimately results in significant yield losses. To overcome these technical issues, researchers have developed a variety of methods, such as suspension polymerization (3), precipitation polymerization (4, 5), surface imprinting on silica spheres (6–8) and sacrificial support imprinting (9), to make spherical MIPs. In particular, as an alternative to the tedious bulk methods, precipitation polymerization has been proposed as a simple and easily implemented strategy for rapidly obtaining molecularly imprinted microspheres (MIMs). It has been shown that this method does not require addition of surfactants (10), provides uniform and porous polymers with small particle diameters and large specific surface areas, and possesses rapid mass transfer characteristics.

Diazepam is widely used as an ataractic medication. However, overuse of diazepam can lead to exanimation, mydriasis, shock or even death; thus, it is important to identify a viable method for the qualitative and quantitative analysis of diazepam in biomedical samples. Several determination methods have been reported, such as stir bar sorptive extraction–thermal desorption–capillary gas chromatography–mass spectrometry (11), fluorimetric screening (12), electrochemical simulation (13) and solid-phase extraction (SPE) (14–16). Usually, analysis of pharmaceutical compounds in biological matrices, such as serum and urine, requires pretreatment of the samples to purify them before chromatographic separation, which is commonly achieved by SPE (17–20). Compared with liquid–liquid extraction (LLE), SPE is much more widely accepted for its convenience, simplicity and enrichment capability. The application of molecularly imprinted solid-phase extraction (MISPE) for pretreatment of biological samples before performing high-performance liquid chromatography (HPLC) (21, 22) is a new development in the field, because MIPs can selectively extract analytes by specific recognition, thereby avoiding co-extractions that could potentially interfere with the quantification of the analytes. Recently, a great deal of research has investigated the use of MISPE for the enrichment and separation of analytes from complex matrices, such as plants (23, 24), foods (25), environmental samples (26–28) and biological samples (29–32).

So far, diazepam-imprinted polymers have been reported to be commonly prepared by bulk polymerization (33–36). Ariffin (36) prepared diazepam-MIP by bulk polymerization in organic solvent and achieved optimum retention of diazepam on MISPE cartridges from postmortem hair samples in an organic environment, yielding cleaner diazepam extracts than those from conventional SPE.

In this research, diazepam-MIMs were prepared by precipitation polymerization in organic porogen, and their specific adsorption capability was evaluated in a water-based environment. Furthermore, the MISPE procedure was optimized for analysis of diazepam and its main metabolite in urine and serum samples.

Experimental

Materials

Reagents

Diazepam was provided by Changzhou No. 4 Pharmaceutical Factory (Jiangsu, China). Methacrylic acid (MAA) was obtained from Guangzhou Xingang Reagent Factory (Gongdong, China). Ethylene glycol dimethacrylate (EGDMA) was purchased from

Shanghai Shanhu Chemical Plant (Shanghai, China) and 2,2′-azobisisobutyronitrile (AIBN) was purchased from Shanghai Sihei Chemical Reagent Company (Shanghai, China). Methyl alcohol and HPLC-grade acetic acid were purchased from Kermel Chemical Reagents Development Center (Tianjin, China). Chloroform, acetonitrile and acetone were purchased from Guangzhou Chemical Reagent Factory (Guangdong, China). Barbital was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All reagents were analytical grade or better.

Apparatus

The HPLC system consisted of a Shimadzu LC-10AD pump (Kyoto, Japan) and a Shimadzu SPD-10A ultraviolet-visible (UV-Vis) detector. All separations were performed on an analytical, reverse-phased Symmetry C18 (4.6 mm i.d. × 15 cm length, 5 μm) column (Dikema) at a mobile phase flow rate of 1.0 mL/min, under isocratic conditions at room temperature. The mobile phase used for the HPLC experiments was a mixture of methanol–water–tetramethylethylenediamine–acetic acid (700:300:4.4:3.2 *v*/*v*/*v*/*v*), and was passed through a 0.45 μm-filter before use. Supelco provided the SPE installation. The scanning electron microscope (SEM) micrographs were generated on a Hitachi S-520 (Kyoto, Japan). Fourier transform infrared (FT-IR) spectra were recorded using a Spectrum One FT-IR spectrometer (PerkinElmer, Foster City, CA). The average pore diameter and surface area of the sorbents were determined by nitrogen adsorption using an ASAP2020 Surface Area and Porosity Analyzer (Micromeritics, Norcross City, GA).

Preparation of MIMs

We prepared the MIMs by precipitation polymerization. Diazepam (0.25 mmol) and MAA (1 mmol) were dissolved in 20 mL of toluene and allowed to prearrange for 4 h. Subsequently, EGDMA (5 mmol) and AIBN (1.9 mmol) were added into the solution. Oxygen was eliminated by purging the mixture with nitrogen for 15 min before we transferred it into a glass tube (25 mL). The tube was sealed and thermostated at 60 °C in a water bath to initiate the polymerization process, and was allowed to react for 24 h. The MIMs were vacuum dried at 60 °C for 8 h. Thereafter, the template was removed by Soxhlet extraction with a methanol–acetic acid (9:1 *v*/*v*) mixture until no template was detected in the washing solution. Non-imprinted microspheres (NIMs) were prepared in the same manner as the MIMs, but in the absence of the template.

MISPE

Fifty milligrams of dry MIMs or NIMs were suspended in methanol and packed into two separate 1-mL empty polypropylene SPE cartridges between two frits and two 0.45-μm filtration membranes were added between frits to secure the packing and outlet stopcocks. The pressure of SPE was 0.055 MPa.

Before extraction, the cartridges were activated with 3 × 1 mL of methanol–acetic acid (7:3 *v*/*v*) and washed with 3 × 1 mL of methanol to remove the residual acetic acid. Subsequently, we continuously passed the loading solvent through the cartridges to equilibrate them. We then loaded the solution of analytes into the MISPE cartridge. The cartridge was washed and eluted, maintaining a 0.5 mL/min flow rate for all solutions. The collected eluate was completely evaporated under a vacuum and redissolved in 200 μL of methanol for further HPLC analysis.

Urine sample

Preparation of urine samples

The urine samples were prepared according to the method previously described by Leenheer (37). We added 4 mL of urine sample with specific concentrations of diazepam and nordiazepam into a 50-mL amber centrifuge tube. The urine sample was buffered with 8 mL of 0.2 M sodium acetate buffer (pH 4.5) prior to adding 11630 U of β-glucuronidase. The tube was shaken vigorously and subsequently incubated at 56 °C for 2 h. After centrifugation at 1,500 rpm for 10 min, we transferred the supernatant to another 50-mL amber centrifuge tube. We then added 40 μL of 1M sodium hydroxide and 8 mL phosphate buffer (pH 6.8), containing 2 mL of acetonitrile, to the tube before performing SPE.

MISPE of urine samples

The MISPE cartridge was conditioned with 5 × 1 mL of methanol followed by 3 × 1 mL of acetonitrile–phosphate buffer (pH 6.8, 1:9 *v*/*v*). We then loaded 5 × 1 mL of prepared urine sample onto the cartridge. Air was passed through the cartridge for 10 min, and the cartridge was washed with 2 × 1 mL of acetonitrile–phosphate buffer (pH 6.8, 1:9 *v*/*v*) and 3 × 1 mL water–acetonitrile–acetone (87:10:3 *v*/*v*), and eluted with 3 mL of methanol–acetic acid (7:3 *v*/*v*). We also added 2 μg of the alprazolam internal standard for control, and then the collected eluate was completely evaporated under a vacuum. The residues were redissolved in 200 μL of acetonitrile before analysis by HPLC. Experiments were performed in triplicate.

Real urine samples

Urine samples were collected from a healthy female volunteer 6 h after she took two tablets of diazepam (5 mg). These urine samples were pretreated as described previously, but no diazepam or nordiazepam was added during the protocol.

Serum samples

We added acetonitrile to serum samples from a healthy volunteer to give serum–acetonitrile (9:1 v/v). We then analyzed 1 mL of spiked sample by the MISPE process and HPLC, as described previously.

Results and Discussion

Characteristics of polymers

To verify the successful preparation of MIMs, we inspected FT-IR spectra of the MIM sample. The observed features at 3,600, 3,000, 1,750 and 1,160 cm^{-1} indicate the –O–H in the carboxyl of MAA, and =C–H, –C = O, and –C–O– stretching vibrations, respectively. The distorting vibrations of =C–H are reflected around 1450 – 1300 cm^{-1}. These results suggest that

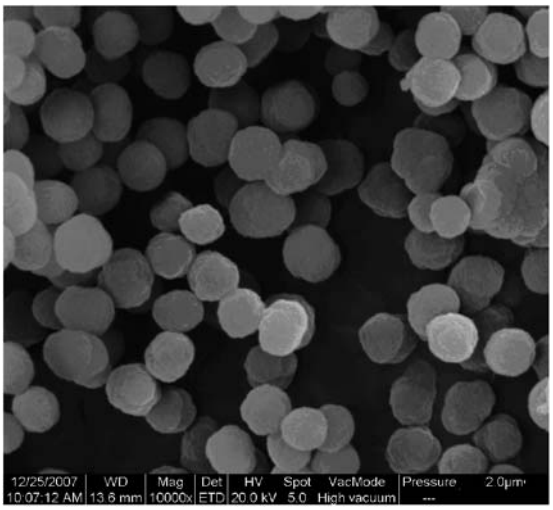

Figure 1. SEM image of MIMs.

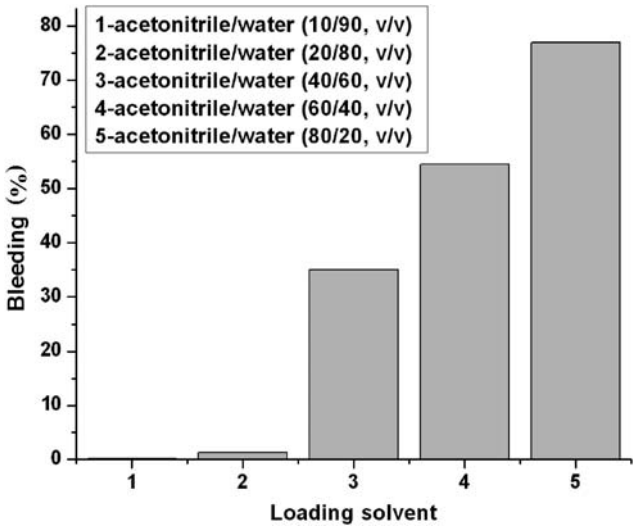

Figure 2. Effect of water content in loading solution.

the functional monomer (MAA) and cross-linker (EGDMA) were successfully grafted onto MIMs.

Figure 1 presents a microscopic image of the MIMs from SEM. As depicted in the figure, we obtained spherical particles with fairly similar diameters, ranging from $1 - 2$ μm.

To measure the surface condition of the MIMs, we undertook N2 adsorption experiments, which indicated that the MIMs had an average surface area of 5.94 m^2/g^{-1} with an average pore size of 4.4 nm.

Optimization of the MISPE procedure

MISPE cartridge loading

Because our objective was to use MIMs to extract diazepam from body fluid samples, we required the inclusion of a water-based loading solution. Although it is usually necessary to subside proteins in real samples, the loading solution contained polar organic solvent as acetonitrile. As a result, we used 1 mL of diazepam solution (5 μg/mL) from a range of acetonitrile–water ratios as loading solutions for investigation. We found that the percentage of diazepam bleeding decreased with the increase of water content in the loading solution (Figure 2), which indicated that the adsorption of diazepam on the MIM cartridge primarily resulted from hydrophobic interactions. Furthermore, we found that only a small amount of diazepam bled after loading a solution of diazepam in acetonitrile–water (1:9 *v*/*v*), so it was selected as the loading solvent for further experiments.

Table I
Effect of Loading Solutions with Different pHs

pH	Recovery (%)
3.7	87.4 ± 0.5
4.9	95.6 ± 0.7
6.8	98.8 ± 0.1
9.2	98.6 ± 0.3

We also investigated the effect of sample pH value on the loading step (Table I). Specifically, we used phosphate buffer in the 3.7 − 9.2 pH range. We found that the percentage of diazepam bleeding decreased with the increase of the pH of the loading solution; the highest percentage of bleeding (at pH 3.7) can potentially be explained by protonation of the diazepam molecules. In particular, these protonated molecules might not fit into the binding sites of the MIMs, and therefore could not be adsorbed by the uncharged MIMs. We found that high acidity of the loading solution also affected the hydrogen bonding interaction between the host and guest. By contrast, diazepam bled only ∼1.2% at pH 6.8, which was close to the pH value of body fluid. Accordingly, we chose acetonitrile–phosphate buffer at a pH 6.8 (1:9 *v*/*v*) as the loading solvent for the extraction of analytes from body fluid samples.

MISPE cartridge washing

We loaded 1 mL of diazepam solution (5 μg/mL) into each of three cartridges, and then washed the cartridges with 3×1 mL washing solvent. We found that the loss of diazepam increased with the augmentation of the content of acetonitrile (Figure 3A).

Acetone is less polar than acetonitrile. To remove some less polar impurities from the real urine samples and achieve a well-defined chromatographic trace, we chose water–acetonitrile–acetone as the washing solvent in these experiments. We found that the loss of diazepam was less than 10% (Figure 3B) when the cartridge was washed with water–acetonitrile–acetone (80:10:10 *v*/*v*), which was chosen as the washing solvent for subsequent experiments.

Elution of MISPE cartridges

Methanol and acetonitrile have generally been used as the eluents in the MISPE process. Occasionally, a small percentage of acid, such as trifluoroacetic or acetic acid, is also added to achieve a more efficient elution of the more strongly bound analytes (8, 38–39).

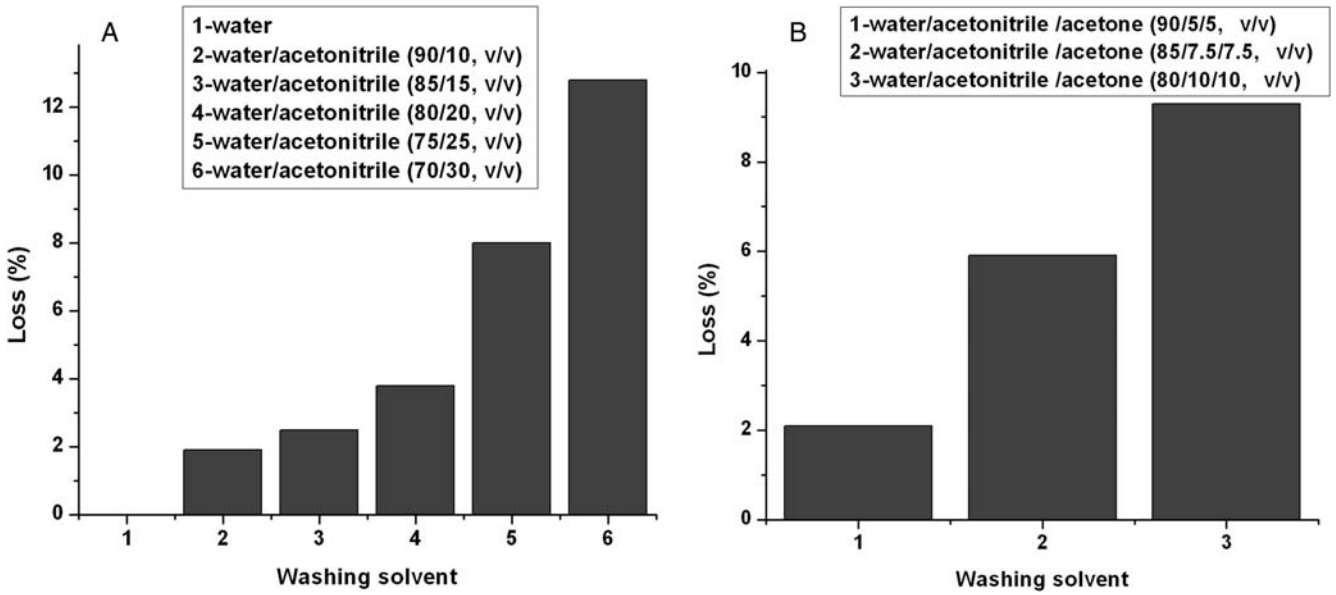

Figure 3. Effects of different washing solvents.

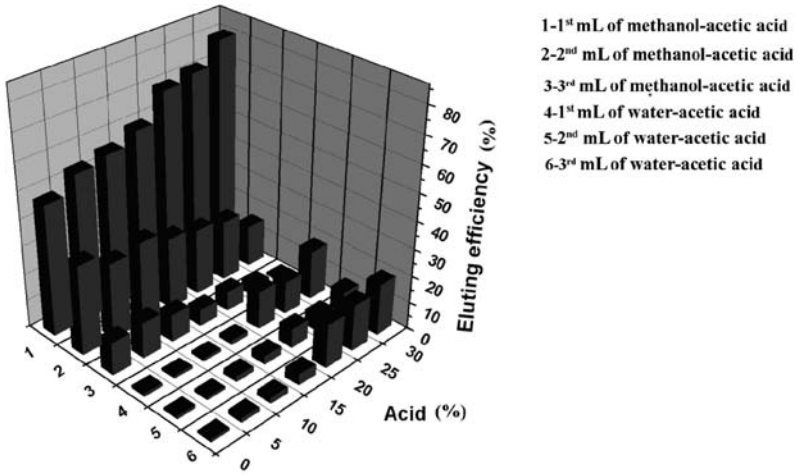

Figure 4. Effects of different eluents.

We found that the desorption efficiency of diazepam decreased in the following order: methanol–acetic acid (7:3 v/v) > methanol >> water–acetic acid (Figure 4). The analyte was barely eluted by water, whereas the recovery of diazepam reached ~95% when it was eluted by 3 mL of methanol–acetic acid. Therefore, methanol–acetic acid was selected as the eluent. According to Snyder's solvent selectivity system (40), the proton-acceptor δa and proton-donor δh solubility parameters are large for acetic acid, while both are equal to 7.5 for methanol. These results suggest that a hydrogen bond interaction occurs between the MIMs and diazepam.

Imprinting effect

A high concentration of diazepam solution was continuously loaded into the MIMs and NIMs cartridges to make full use of their binding sites. Figure 5 indicates that 90% of diazepam was adsorbed by the MIMs cartridge after loading the first 1 mL of solution. The adsorption amounts of the MIMs and NIMs cartridges were determined to be 7.2 and 6.4 mg/g, respectively, after loading 6 mL of diazepam solution. The adsorption of MIMs included not only specific adsorption, but also non-specific adsorption; however, only non-specific adsorption was involved for the NIM sample.

Two methods are normally used for investigating the imprinting factor (IF) of MIPs. The first method is to compare the adsorption amounts of analyte for MIPs and NIPs in a loading step using a porogen as a loading solvent. The second method involves contrasting the residual amounts of analyte for MIPs and NIPs after thoroughly washing with a certain washing solvent. The diazepam-MIMs were prepared in

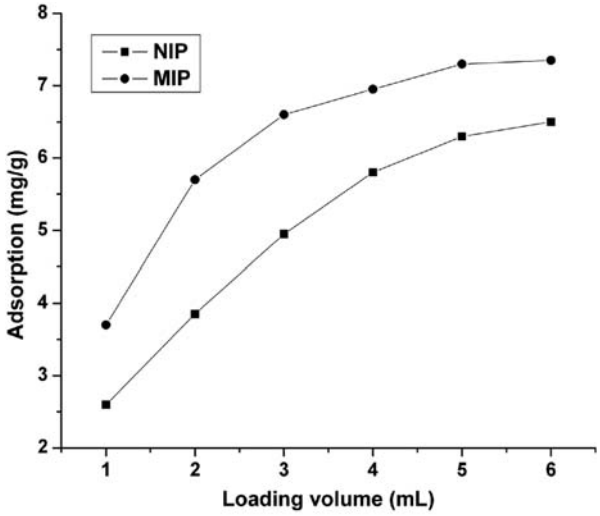

Figure 5. Adsorption of diazepam on cartridges after loading 6 × 1 mL of diazepam solution (200 μg/mL).

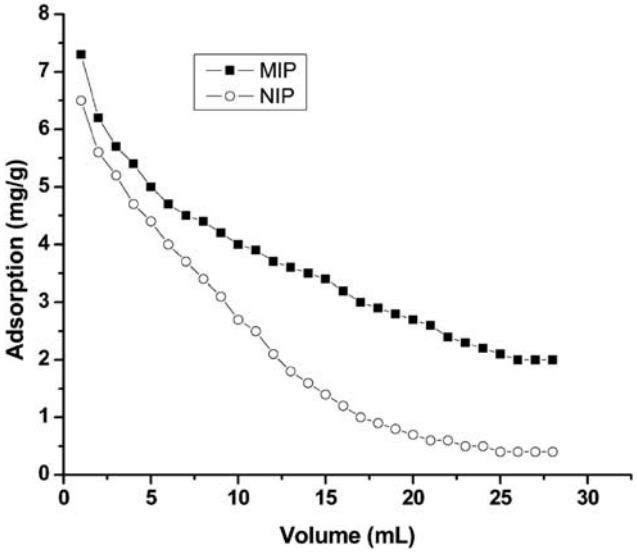

Figure 6. Adsorption of MIMs and NINs after washing with 28 × 1 mL of acetonitrile–acetone–water (10/10/80 *v*/*v*/*v*).

toluene, and are intended to be applied to extract target molecules from water-based samples. Therefore, the IF was investigated in a washing step in this study. Figure 6 shows the results of this washing process. Major parts of non-specific adsorption on both cartridges with MIMs and NIMs for diazepam were removed with gradual washing, resulting in a high IF (IF = QMIMs/QNIMs) of 5.8. A specific adsorption amount of 1.97 mg/g was left on the MIM cartridge.

MISPE selectivity

The molecular structures of eight ataractics that we used to investigate the selectivity of MIMs in this study are presented in Figure 7.

Table II indicates that almost all of the benzodiazepine ataractics were retained by the cartridges after loading, except for alprazolam. However, the washing step caused the loss of some

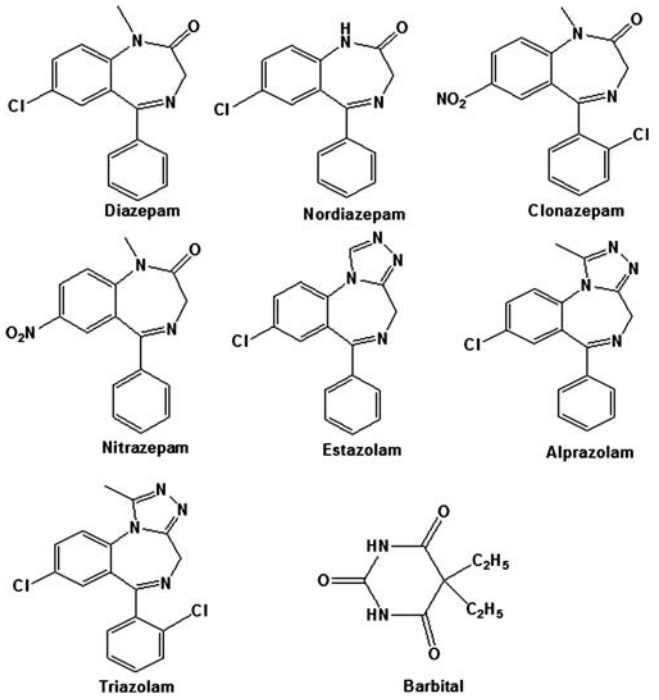

Figure 7. Structures of diazepam and its analogs.

Table II
The Recoveries of Eight Ataractics Studied by MISPE

Solute	Loading (%)	Washing (%)	Recovery (%)	Total recovery (%)
Diazepam	0.3 ± 0.3	9.2 ± 0.8	92.9 ± 2.6	102.4 ± 1.6
Nordiazepam	0.4 ± 0.2	14.3 ± 1.2	77.2 ± 5.2	91.9 ± 5.0
Nitrazepam	n.d.*	13.2 ± 2.2	88.4 ± 3.8	101.6 ± 5.0
Clonazepam	n.d.	27.4 ± 1.6	71.8 ± 4.2	99.2 ± 5.0
Alprazolam	7.6 ± 4.0	78.0 ± 5.7	7.0 ± 3.8	92.6 ± 4.6
Estazolam	1.2 ± 0.1	57.1 ± 5.7	40.8 ± 13.0	99.0 ± 7.7
Triazolam	n.d.	48.1 ± 3.2	41.8 ± 6.2	89.9 ± 4.0
Barbital	33.5 ± 7.9	59.0 ± 5.3	n.d.	92.5 ± 2.9

*Indicates that the measurement was not determined.

Table III
Recoveries of Diazepam and Nordiazepam Spiked in Urine Samples

C (μg/mL)	0.05	0.2	1.6
$Recovery_{diazepam}$ (%)	87.2 ± 2.4	87.7 ± 3.3	87.8 ± 5.3
$Recovery_{nordiazepam}$ (%)	88.6 ± 2.2	90.4 ± 4.7	89.4 ± 3.6

ataractics. We found that the recoveries in the elution step were more than 71.8% for tricyclic benzodiazepine, but less than 42% for tetracyclic benzodiazepine. Barbital, which is not a benzodiazepine, bled 33.5% in the loading step. The MIMs exhibited highly selective binding affinity for diazepam and some strictly structure-related ataractics, and could be applied to extract tricyclic benzodiazepines. These results also indicate that the absorption of tricyclic benzodiazepines was primarily due to the structural recognition of the MIMs.

Urine sample

The results from the MISPE process showed good reproducibility for urine samples and had a linear working range from 0.05 to 1.6 μg/mL of diazepam and nordiazepam. The correlation

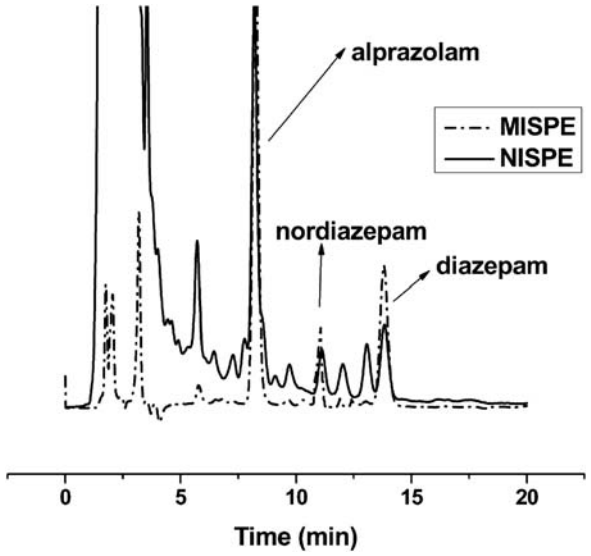

Figure 8. HPLC of serum samples with MISPE and NISPE protocols.

coefficients of the calibrations were 0.998 for both analysts. We calculated the limits of detection (LODs) as three times the standard deviation of the feasible lowest concentration. We found that the LODs for diazepam and nordiazepan were 21.5 and 24.5 ng/mL, respectively. These LODs were low enough to allow the body fluid samples to be analyzed for diazepam at realistic concentration levels.

The recoveries of diazepam and nordiazepam at different concentrations are provided in Table III. We found that 10% or more of diazepam or nordiazepam were lost during the MISPE process, possibly due to the addition of acetone into the washing solvent, through which well-defined chromatographic traces were obtained. However, some analytes were likely washed out, and the recoveries of diazepam and nordiazepam were reduced.

The concentrations of diazepam and nordiazepam in the real urine sample were determined to be 44.4 ± 0.18 and 30.8 ± 0.30 ng/mL, respectively, according to corresponding regression equations.

Serum samples

The chromatogram of serum samples spiked with diazepam and nordiazepam (1 μg/mL) is shown in Figure 8. The sample generated with the MISPE protocol showed a particularly well-defined chromatographic trace. The recoveries of diazepam and nordiazepam were $88.5 \pm 6.5\%$ and $91.1 \pm 4.8\%$, respectively.

Conclusion

In this study, we developed a systemic methodology to prepare MIMs in organic solvent and to apply the MIMs to clean up water-based samples. The MIMs showed high selectivity and specific adsorption capacity for diazepam. Adding acetone to the washing solvent decreased the non-specific adsorption of analytes on MIM cartridges, and improved its selectivity. Furthermore, we determined that it was favorable to eliminate some low-polar interfering substances in body fluid samples before their analysis, and thus we obtained a well-defined chromatographic trace. We conclude that the molecular imprinting technique combined with SPE-HPLC is a powerful approach for determining diazepam and other tricyclic benzodiazepine levels in body fluid samples.

Acknowledgments

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