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Solid phase micro extraction coupled with semi-microcolumn high performance liquid chromatography for the analysis of benzodiazepines in human urine

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Abstract

SPME/semi-microcolumn HPLC (SPME/LC) was investigated to analyze benzodiazepines in human urine samples. SPME conditions such as extraction time, extraction temperature, salt concentration and pH of matrix, flush volume and desorption time were optimized by extracting various drugs from a prepared water matrix. Combination of adding saturated salts to the matrix and controlling pH ranged from neutral to weakly alkaline conditions makes the increase of extraction efficiency. Under optimal condition SPME/LC is more sensitive than direct HPLC analysis without the SPME process. The limits of detection (LODs) was several ppb level and the relative standard deviation (RSD) was $< 15\%$ when human urine samples were analyzed by this analytical system. The system is very useful and is enough to assay benzodiazepines in a human urine sample without tedious and complex analytical procedures. In this paper the applicability of SPME/LC to the analysis of benzodiazepines in human urine samples was reported. In addition, the extension to the evaluation of SPME/LC/MS system was also described.

Keywords: Solid phase micro extraction (SPME); Semi-microcolumn high performance liquid chromatography; SPME/LC; Benzodiazepines; Human urine

1. Introduction

Benzodiazepines are widely used as anticonvulsant, hypnotic, anxiolytic and muscle relaxant drugs and they are important in treating a variety of medical disorders and are subject to abuse. To extract and identify benzodiazepines in human fluids is very important to the analysis of these drugs for toxicological, pharmaceutical and forensic purposes. Gas chromatography/mass spectrometry (GC/MS) methods of analysis of benzodiazepines have already been reported [1–5]. However, GC is unsuitable for thermally unstable compounds, highly polar compounds and non-volatile compounds, since a high temperature is required to elute them but it induces the decomposition in the separation processes. In most

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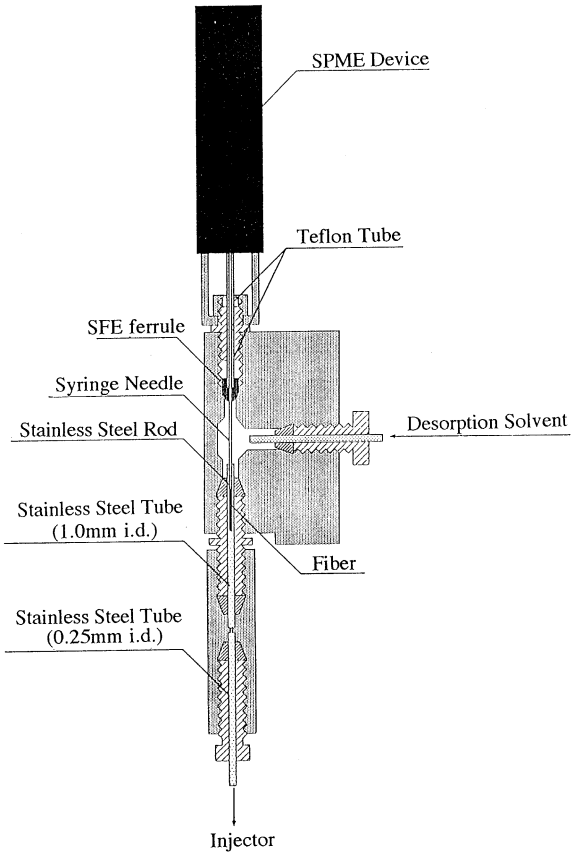

Fig. 1. Schematic of the desorption device developed in this study.

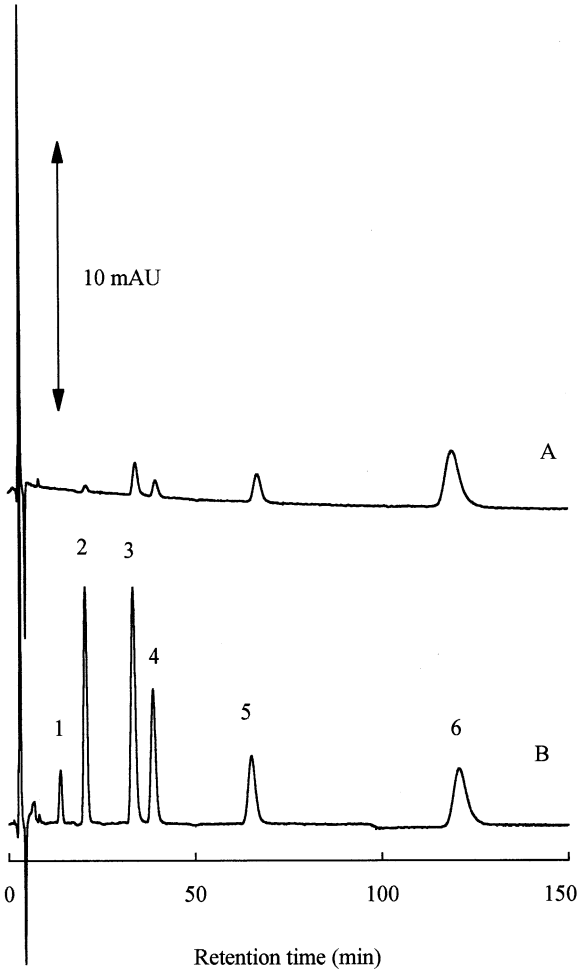

Fig. 2. Chromatograms for several drugs extracted by the SPME/LC system using two different fiber coatings: (A) PDMS, df = 100 μm ; (B) PA, df = 85 μm. LC conditions as described in the text. SPME conditions: extraction time, 60 min; extraction temperature, 60°C; salt concentration, 0.27 g ml^{-1}; pH of matrix, not adjusting; desorption time, 30 min; desorption solvent, acetonitrile (30 μl); flushing solvent and amount, acetonitrile (6 μl). The concentration of each drug is 100 ppb. Peaks: 1, nitrazepam; 2, flunitraxepam; 3, fludiazepam; 4, diazepam; 5, clotiazepam; 6, medazepam.

cases, therefore, GC methods require derivatization to change the drugs to be more volatile compounds. High performance liquid chromatography (HPLC) was developed for the assays of benzodiazepines, although it rather lacks the sensitivity required for measuring drugs concentration in plasma which is the lowest among biological fluids [6–8]. Since in human fluid analysis a sample preparation technique is often necessary to extract and concentrate organic compounds of interest from the matrix, several sample preparation techniques have been developed. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) are the most common techniques. These methods, however, are time-consuming, require complicated procedures, are difficult to be automated, and need the use of large amounts of organic solvents except modern SPE which consumes a little smaller amount of solvents.

Solid phase micro extraction (SPME) which is fast, solvent-free and an excellent performance technique has been recently developed [9]. SPME has two processes, the extraction process involv-

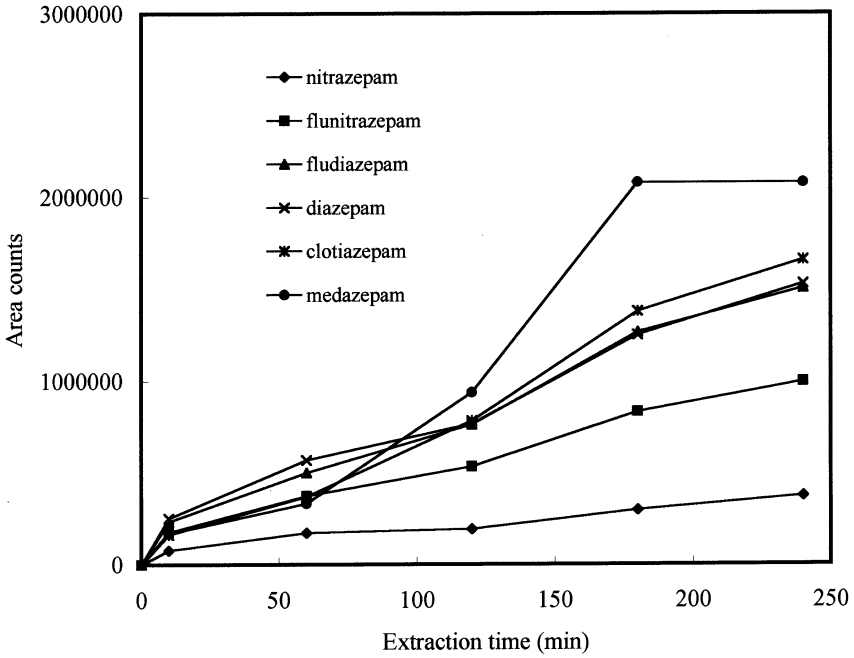

Fig. 3. The effect of extraction time on extraction efficiency. LC conditions as described in text. SPME conditions: extraction temperature, 60°C; salt concentration, 0.27 g ml^{-1}; pH of matrix, not adjusting; desorption time, 30 min; desorption solvent, acetonitrile (30 μl); flushing solvent and amount, acetonitrile (6 μl). The concentration of each drug is 100 ppb.

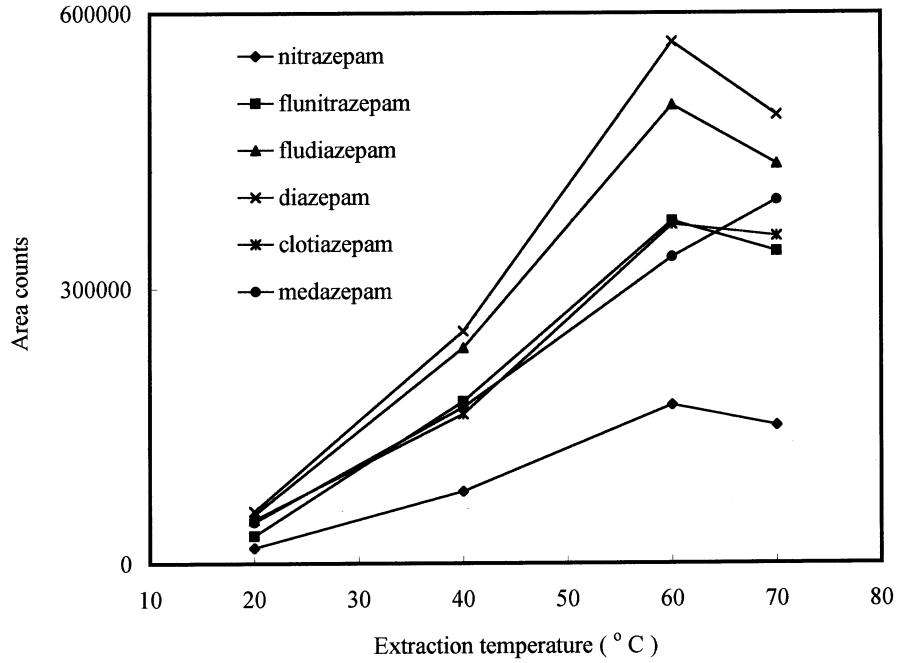

Fig. 4. The effect of temperature on the extraction efficiency of six drugs. LC conditions as described in the text. SPME conditions: extraction time, 60 min; salt concentration, 0.27 g ml^{-1}; pH of matrix, not adjusting; desorption time, 30 min; desorption solvent, acetonitrile (30 μl); flushing solvent and amount, acetonitrile (6 μl). The concentration of each drug is 100 ppb.

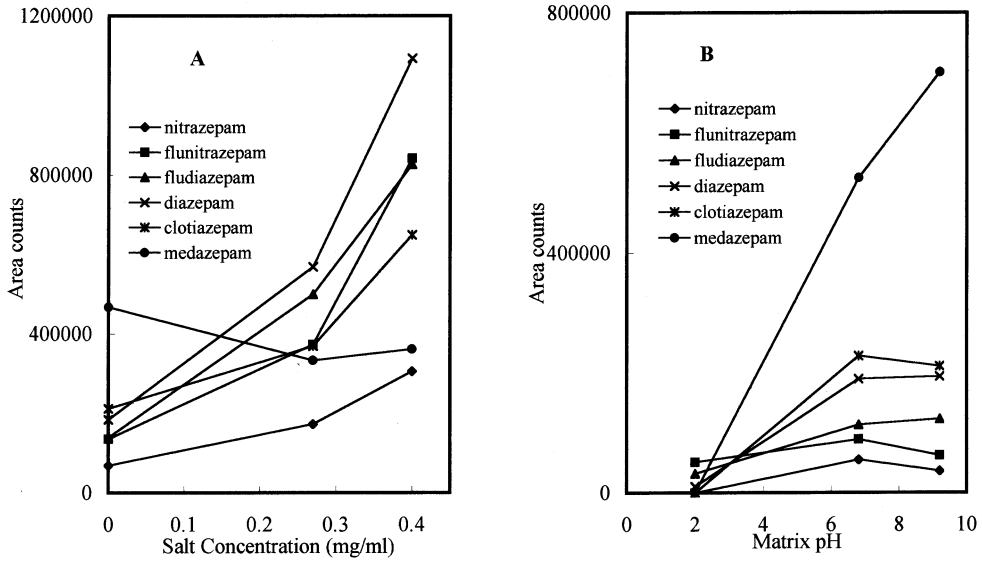

Fig. 5. The effect of salt concentration (A) and pH of matrix (B) on the extraction efficiency of six drugs. LC conditions as described in the text. SPME conditions: extraction time, 60 min; extraction temperature, 60°C; salt concentration, not adding for (B); pH of matrix, not adjusting for (A); desorption time, 30 min; desorption solvent, acetonitrile (30 μl); flushing solvent and amount, acetonitrile (6 μl). The concentration of each drug is 100 ppb.

ing equilibrium between sample analytes and the fiber coating and the desorption process from the fiber coating to further analytical processes. SPME coupled with GC has been successfully applied to the analysis of polycyclic aromatic hydrocarbons (PAHs), phenols and fatty acids [10–14]. However, most organic compounds can not be analyzed with GC because they are non-volatile or semivolatile and are thermally unstable. In order to strengthen the advantages of the SPME method, SPME coupled with HPLC has been developed to analyze samples which are non-volatile and thermally unstable, where it has been used to analyze diluted surfactants, pesticides and PAHs [15–17].

In this paper the analysis of benzodiazepines in human urine using SPME/semi microcolumn HPLC (SPME/LC) is described. The semi-microcolumn HPLC needs less solvents as mobile phase than conventional HPLC, and SPME is a solvent free sample preparation technique, and therefore if one can couple these two methods effectively, the solvent free analytical method would be realized and feasible. This is one of the most urgent and important points in analytical chemistry, if you are concerned with the ecosystem, zero-emission and saving energy on our planet.

2. Experimental

2.1. Materials and reagents

The holder and the assembly of the SPME device for manual sampling were purchased from Supelco (Bellefonte, PA). The 1.0 cm long fibers used were coated with 85 μm thick polyacrylate (PA) and 100 μm thick polydimethylsiloxane (PDMS) which were also obtained from Supelco. The new fibers were conditioned by immersing them into the solvent until interfering peaks were disappeared in LC chromatograms.

All solvents were reagent grade purchased from Kishida Chemical (Osaka, Japan) and deionized water obtained from a Milli-Q water system (Millipore, Tokyo, Japan). Water samples were prepared by spiking 150 μl of the standard into 15 ml Milli-Q water in a 20 ml sample vial with a cylindrical-shaped stirrer bar (4 × 6 mm).

2.2. Semi-microcolumn HPLC system

Semi-microcolumn HPLC (LC) was performed with an Nanospace SI-1 (Shiseido, Tokyo, Japan), which consists of a pump, a UV-Vis detector, a column oven and a degasser. Superiorex ODS column (25 cm × 1.5 mm i.d) and Rheodyne 7125 injector (Cotati, CA) with a 1 μl loop were used. The flow rate was 100 μl min^{-1} and the column temperature was controlled at 35°C. The mobile phase was acetonitrile–water (35–65) and the detection wavelength was at 220 nm. Borwin chromatography software (Jason, Tokyo, Japan) was used for data acquisition and handling.

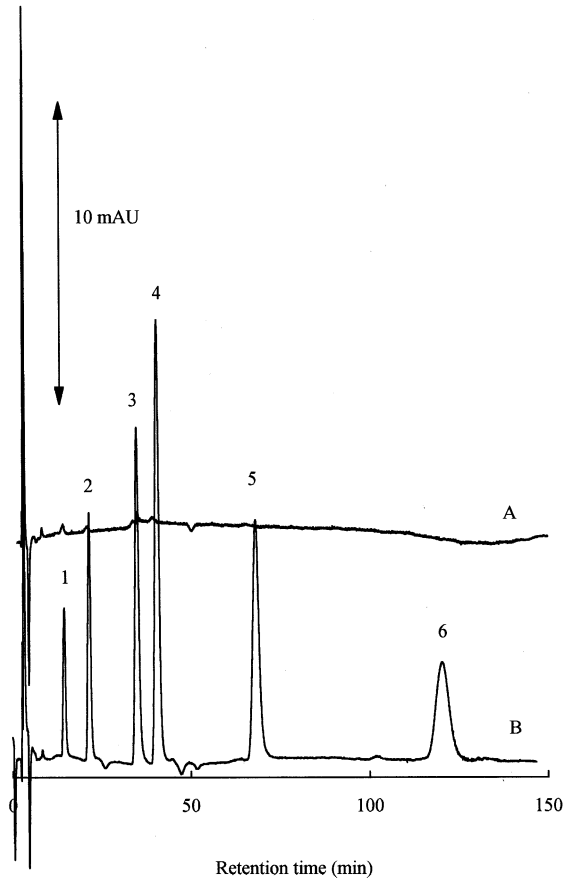

Fig. 6. Chromatograms of drugs mixture by SPME/LC and direct LC analysis. (A) Chromatogram by direct injection of the sample. (B) Chromatogram by SPME/LC. LC conditions as described in the text. SPME conditions: extraction time, 60 min; extraction temperature, 60°C; salt concentration, saturated; pH of matrix, neutral to weakly alkaline; desorption time, 30 min; desorption solvent, acetonitrile (30 μl); flushing solvent and amount, acetonitrile (6 μl). The concentration of each drug is 100 ppb. Peaks: 1, nitrazepam; 2, flunitrazepam; 3, fludiazepam; 4, diazepam; 5, clotiazepam; 6, medazepam.

2.3. LC/ESI-MS system

Jasco PU-980 pump, Jasco UV-970 detector set at 220 nm, Rheodyne 7125 injector with a 1 μl sample loop, Superiorex ODS column (25 cm × 1.5 mm i.d.), and Jason 865-CO oven for controlling column temperature at 35°C were used for LC/MS analysis.

The LC/MS instrument used was VG Biotech Platform equipped with the electrospray interface (Jasco, Tokyo, Japan). The MS conditions were as follows: detection, positive and negative ion; MS range, 50 to 450; capillary, 3.2 kV for positive and 2.8 kV for negative; HV lens, 0.2 kV; corn, 40 V.

2.4. SPME/LC procedure

SPME has two processes which are equilibrium between analytes and the fiber coating, and desorption to the mobile phase. In SPME/GC the fiber is transferred to the GC injector as soon as extraction is finished and the analytes are thermally desorbed, separated on the column, and quantified by the detector. Although the LC extraction procedure is similar to that used for GC analysis, the desorption procedure is different for SPME coupled with LC. For SPME/LC the solvent desorption was previously proposed to desorb the analytes from the fiber coating [17]. Therefore, we have constructed the interface to SPME coupled with LC. Previously our group made a similar interfacing device to analyze pesticides in environmental water by the SPME/LC system [15]. In this investigation the interface was constructed to have smaller volume than that one for increasing extraction efficiency and sensitivity. The interface used here has only < 30 μl as volume. The interface consists of stainless-steel tee, connecting fittings, stainless-steel tubing, PTFE tubing and ferrules, which are parts normally used for GC, LC and SFE. The schematic diagram of this interface is demonstrated in Fig. 1. This interface is connected to regular six-port

injection valve which are connected to injection loop when the injection valve is located on load position. After the extraction the SPME fiber is withdrawn and inserted into the desorption device filled with acetonitrile as the desorption solvent. After the desorption process in certain amount of time, the injection valve is changed to the load position and a certain amount of the solvent is flushed through the interface. Immediately the sample loop is filled with the desorption solvent containing the sample analytes, the injection valve is changed to the injection position. Thus analytes are introduced to the chromatographic system.

3. Results and discussion

3.1. Evaluation of fiber coating

For a polar coating to extract polar compounds from a water matrix, it must have that much stronger affinity with the analytes than water does. For example, PDMS, a nonpolar coating, extracts nonpolar compounds such as BTEX (benzene, toluene, ethylbenzene, and xylene isomers) and PAHs (polyaromatic hydrocarbons) from water very effectively but cannot extract polar compounds such as phenol and its derivatives. PA, the more polar coating, extracts phenol and its derivatives quite well but dose not provide high sensitivity when extracting BTEX compounds [18]. The PDMS and PA coatings were compared for benzodiazepines using the SPME/LC system. The fiber was immersed directly into a 15 ml water sample which contained six drugs (nitrazepam, flunitrazepam, fludiazepam, diazepam, clotiazepam and medazepam), salt and a cylinder-shaped magnetic stirrer bar. Results (see chromatograms shown in Fig. 2) suggest that the PA coating fiber is better than the PDMS coating except for medazepam which has the highest hydrophobicity of the six drugs. It seems that the higher the hydrophobicity the better the extraction efficiency obtained by PDMS. Due to these above results the PA coated fiber is used to the further works in their investigation.

3.2. Optimization of SPME conditions

In order to determine the most optimal extraction time for the drugs, a fresh solution is used for each time interval. The speed of extraction is controlled by mass transfer from the sample matrix to the fiber coating. In SPME sampling, the mass transfer rate is determined by means of the diffusion of analytes in the coating if the sample matrix is perfectly agitated. Fig. 3 shows the time profile of the extraction for the drugs. So far it has been reported that the shorter extraction time, which is of the order of minutes, is one of the advantages of the SPME method. For nitrazepam, flunitrazepam, fludiazepam, diazepam and clotiazepam, however, the extraction time of over 180 min was required to reach the equilibrium when PA coating was used. This problem can be improved to some extent by using the thinner coating, but it is not available at present. Although the equilibrium is not reached at 180 min for nitrazepam, flunitrazepam, fludiazepam, diazepam, and clotiazepam, the extraction time was set at 180 min for subsequent experiments for practical convenience.

Table 1
Linear calibration range, limits of detection (LODs) and relative standard deviation (RSD)

Drug	Calibration range (ppb)	r^2	LODs (ppb)	RSD
Nitrazepam	5–1000	0.997	4	9.5
Flunitrazepam	5–400	0.993	2	13.8
Fludiazepam	5–1000	0.986	1	8.3
Diazepam	5–400	0.990	1	13.0
Clotiazepam	5–1000	0.979	2	11.0
Medazepam	20–400	0.996	6	13.3

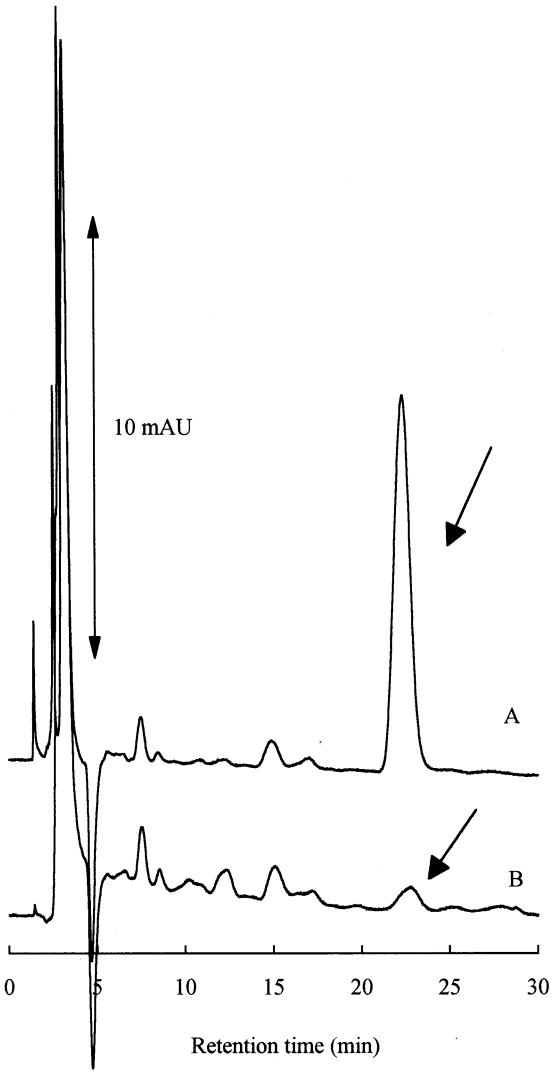

Fig. 7. Chromatograms obtained for urine sample by SPME/LC. (A) Flunitrazepam (100 ppb) spiked. (B) Original urine sample. LC conditions as described in the text. SPME conditions as in Fig. 6.

Then the temperature effect on the extraction efficiency was studied. The temperature is thought to be a very important parameter for the extraction process [19]. The plots for the temperature versus the peak area counts shown in Fig. 4 suggest that 60°C is the best temperature and elevated temperatures higher than 60°C decreased efficiency for the extraction of the drugs except for medazepam. As the extraction is controlled by mass transfer from the sample matrix to the fiber coating, the mass transfer resistance is reduced in term of the increasing temperature as well as agitation by the magnetic stirrer bar. Although the higher temperature gave better efficiency, benzodiazepines are probably decomposed by over 60°C. The temperature was set at 60°C for the subsequent experiments.

Salting can enhance the extraction of some compounds from water [10]. Fig. 5A shows the effect of sodium chloride concentration on the extraction efficiency of examined drugs. All of the drugs except for medazepam have a maximum extraction efficiency at saturated salt concentration. Because medazepam has a higher pK_a than that of other drugs used in this study, it seems that medazepam protonates in water matrix. Saturated salt concentration was selected for the subsequent experiments.

In order to extract benzodiazepines by LLE, pH has been adjusted to a value at while the drugs are neutral form. In SPME it has been shown that adjusting the pH of a matrix solution will alter K (dissociation constant) for dissociable species, assuming that only the undissociated form of the acid and base can be extracted by the coating [20]. The effect of the matrix pH on the extraction efficiency of the drugs were examined by using three different pH buffers (100 mM sodium phosphate for pH 2.1 and 6.8 and 100 mM sodium borate for pH 9.1) and the comparison of the amount of drugs extracted by using SPME/LC is shown in Fig. 5B. Most benzodiazepines which are weak bases, are undissociated forms at a pH of neutral and alkaline such as 6.8 and 9.1, resulting in a higher extraction efficiency.

The distribution of drugs in acetonitrile in the desorption device is suspected to be non-homogeneous. The optimum flushing volume was determined for SPME/LC. In this experiment 100 ppb drugs in water was extracted at 60°C. After desorption a certain amount of acetonitrile was flushed into the desorption device using a micro syringe, LC analysis was performed, and the amounts injected into the column were determined for each drug. It is found that when 6 μl of acetonitrile was flushed, the highest peak area was

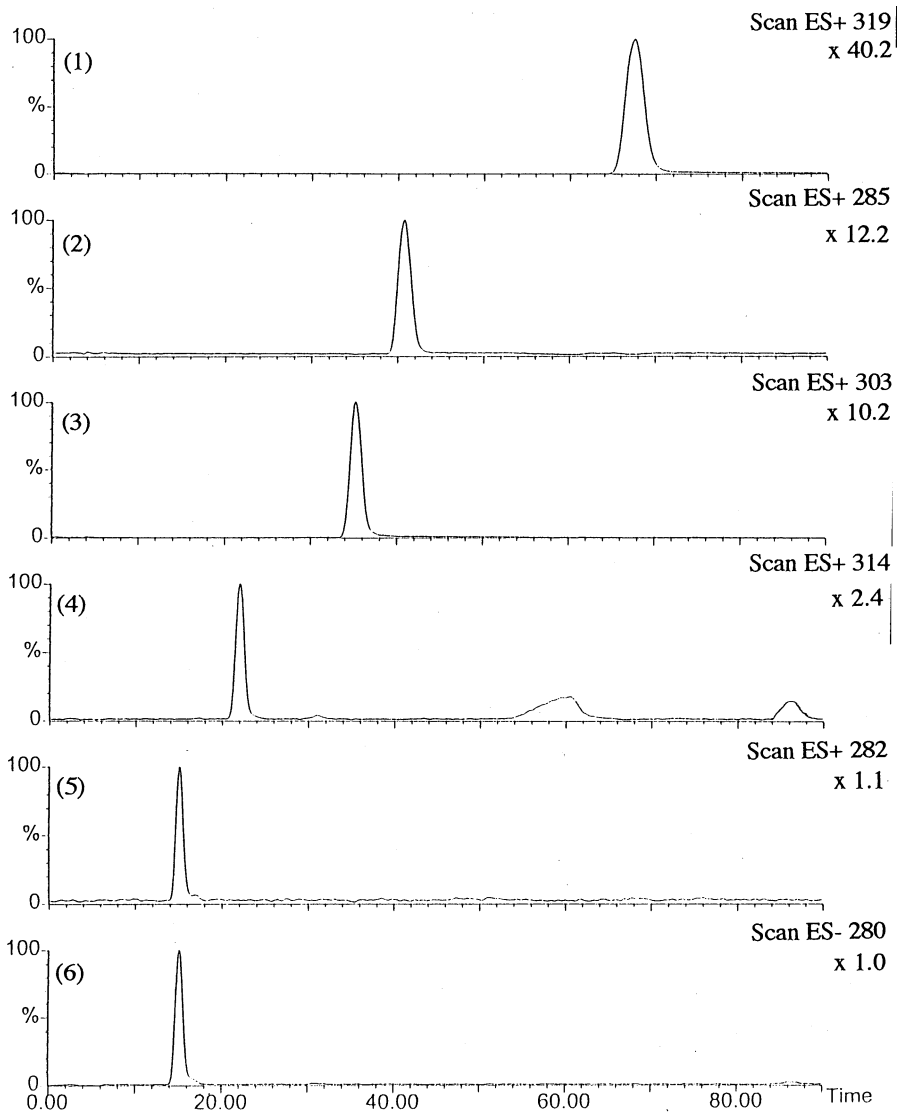

Fig. 8. Chromatograms of several drugs sample by SPME/LC/ESI-MS analysis (1) SIM chromatogram for m/z 319, clotiazepam; (2) SIM chromatogram for m/z 285, diazepam; (3) SIM chromatogram for m/z 303, fludiazepam; (4) SIM chromatogram for m/z 314, flunitrazepam; (5) SIM chromatogram for m/z 282, nitrazepam; (6) SIM chromatogram for m/z 280, nitrazepam. LC conditions as described in the text. SPME conditions as in Fig. 6. Each scaling factor is defined as normalization by the scale of m/z 280.

obtained for almost all drugs. Therefore, 6 μl volume is selected for flushing of the acetonitrile solution.

The relationship between desorption time and carryover (which is the ratio of the amount of drugs remaining on the fiber after the first desorption to the amount of total drugs desorbed) was examined. All drugs showed a decrease of the carryover when the desorption was done in more than 30 min. After 30 min desorption was done,

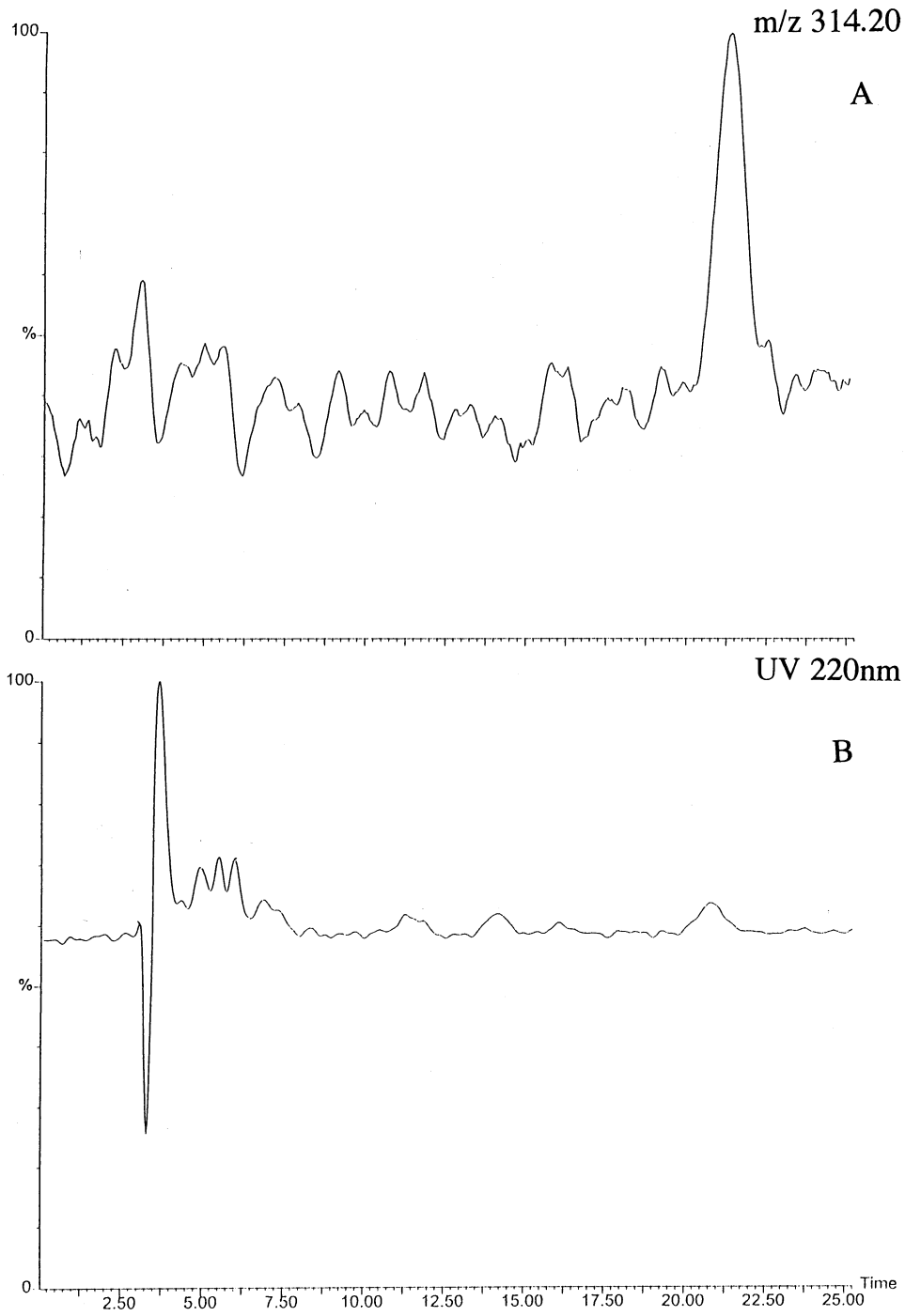

Fig. 9. Comparison of SIM chromatogram (A) and UV chromatogram (B) of original urine sample using SPME/LC/ESI-MS analysis. LC conditions as described in the text. SPME conditions as the same in Fig. 6.

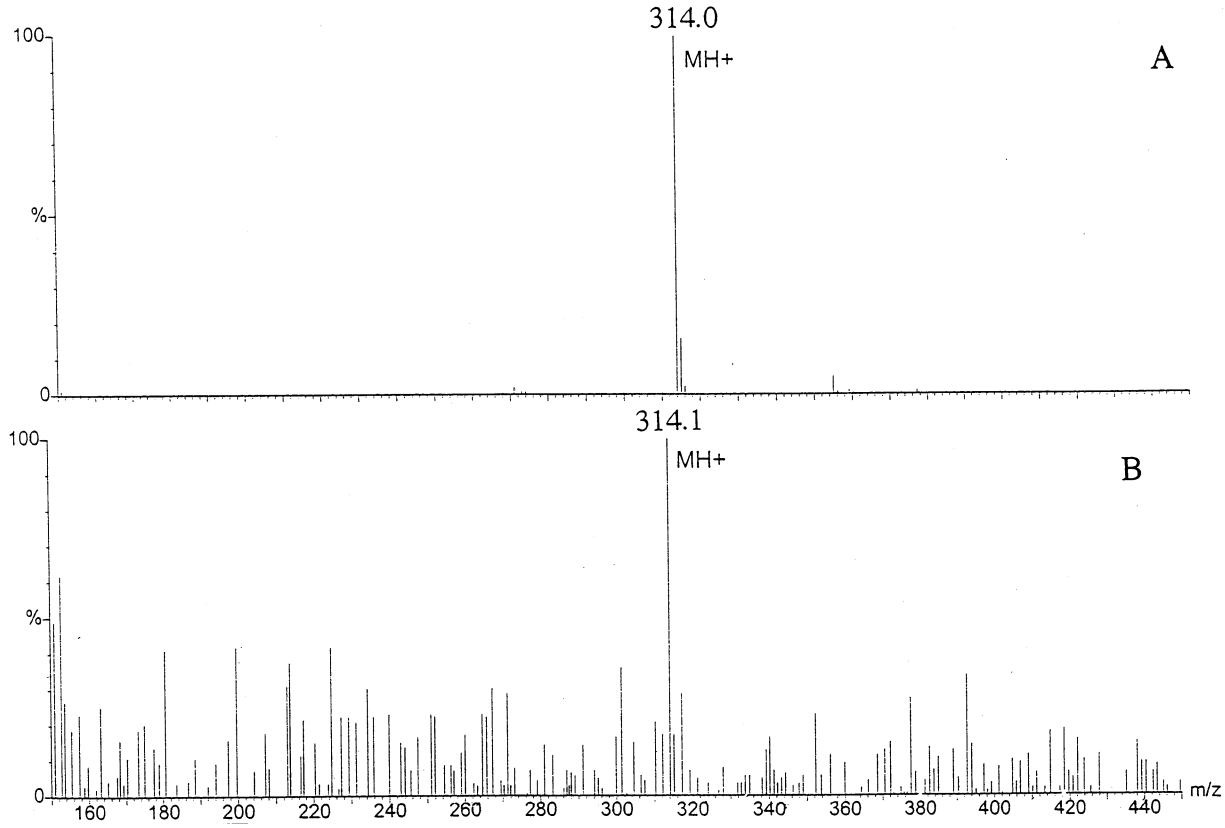

Fig. 10. MS spectra for the peak demonstrated in the chromatograms in Fig. 7(A) and (B). A for the peak in Fig. 7(A). B for the peak in Fig. 7(B).

the carryover was $< 5\%$ and this value indicates that there is no significant influence to the next extraction process.

The comparison of chromatograms in which standard drug sample was analyzed by SPME/LC and direct LC analysis within SPME process is shown in Fig. 6. This result demonstrates that under the optimal condition SPME/LC for drugs is much more effective than the direct LC analysis without the SPME process.

3.3. Measurement of benzodiazepines in urine sample

Linear calibration range, LOD and RSD value for the analysis of drugs are summarized in Table 1. A tendency to give higher RSD at 100 ppb concentration is seen, likely because only 1 μl out of 6 μl of acetonitrile containing drugs is manually introduced into the column, and increases of some variation in the desorption process. The linear calibration range for each drug was between 5 and 1000 ppb concentration and LOD was several ppb level at a signal to noise ratio of 3.

The SPME/LC under the optimal conditions was applied to the patient's urine sample which was diluted 10-fold with sodium borate (pH 9.1, 100 mM) to adjust matrix pH. In addition the same urine sample matrix spiked with 100 ppb flunitrazepam was analyzed as well. In the original urine sample a peak which has the same retention time with flunitrazepam was detected,

while many peaks were also detected as shown in Fig. 7. In order to confirm the identity of that peak, electrospray ionization-mass spectrometry (ESI-MS) is more useful than a photodiode array detector because UV spectra of benzodiazepines are very similar to each other. In addition SPME/LC coupled with ESI-MS is thought to be facilitative, since the semi-microcolumn LC system uses less solvent as the mobile phase. SIM chromatograms for five drugs by SPME/LC/ESI-MS are shown in Fig. 8. The standard flunitrazepam which has 313 as the molecular weight was detected at m/z 314 (M + H) on ESI positive mode. Then the original urine sample was analyzed by the same system and the obtained results are shown in Fig. 9, indicating the interest peak has the m/z value of 314. MS spectra obtained from two chromatograms are demonstrated in Fig. 10 and it is apparent that in two spectra the same parent peaks with different intensity are seen. The information of the retention time and the MS data suggest that the original urine contains flunitrazepam and the concentration determined is about 7 ppb by the analytical system.

4. Conclusion

SPME/LC can be used to analyze benzodiazepines in a human urine sample. The combination of semi-microcolumn HPLC and SPME, which requires less organic solvents, is important from an ecological and analytical view point. The total amount of solvents used by this system for one measurement was < 15 ml. In addition SPME/LC can be easily coupled to MS which provides useful information on unknown compounds. This system offers several ppb level as the detection limits for benzodiazepines. Much smaller diameter LC columns ~ 1 mm i.d. or less will give better performance and lower solvent consumption and combined with SPME will be a true solvent-less analytical system. Such work is in progress in our laboratory.

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