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Simultaneous determination of nitrazepam and its metabolites in urine by micellar electrokinetic capillary chromatography

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ABSTRACT

We applied micellar electrokinetic capillary chromatography to simultaneous separation and determination of nitrazepam and its major metabolites, 7-aminonitrazepam and 7-acetamidonitrazepam, in spiked urine. Prior to electrophoresis, the three compounds were successfully extracted from the spiked urine with commercial disposable solid-phase cartridges. The optimum running buffer for the separation was prepared by combining 85 parts of 60 m*M* sodium dodecyl sulphate–6 m*M* phosphate–borate, adjusted to pH 8.5, with 15 parts of methanol. The separation order, completed within 25 min, was 7-aminonitrazepam > 7-acetamidonitrazepam > nitrazepam, at an applied potential of 20 kV. We obtained reproducible electropherograms in successive repetitions, and few other peaks or interferences appeared in the electropherogram. The detection limits of the three compounds were 50–100 pg (0.1–0.2 μg/ml of analyte in spiked urine), and the recoveries were 78.9–100.8% for 1 μg/ml and 84.1–100.3% for 5 μg/ml. The application of this method to forensic or clinical samples is demonstrated.

INTRODUCTION

Screening and confirmation of drugs and their metabolites in body fluids is important to clarify their therapeutic and toxic effects. Nitrazepam, a benzodiazepine derivative, is a widely used hypnotic and is frequently encountered in clinical and forensic analytical samples. Its major metabolites are 7-aminonitrazepam and 7-acetamidonitrazepam. Several attempts, including thin-layer chromatography [1], gas–liquid chromatography (GLC) [2], radioimmunoassay (RIA) [3] and high-performance liquid chromatography (HPLC) [4], have been made to determine the parent compound and its metabolites [5]. However, there are few practical analytical methods that are sensitive enough for simultaneous determination of the parent drug and its two major metabolites.

Since Terabe *et al.* [6] introduced micellar electrokinetic capillary chromatography (MECC) for separating neutral molecules, the method has been attractive for the analysis of drugs in body fluids [7–13]. This technique involves the addition of surfactant ions above their critical micelle concentration (CMC) to the mobile phase in order to control or adjust solute migration.

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The objective of the work described here was to investigate the suitability of using MECC for the analysis of nitrazepam and its two major metabolites in urine.

EXPERIMENTAL

Chemicals

Nitrazepam was obtained from Wako Chemicals (Osaka, Japan). 7-Aminonitrazepam and 7-acetamidonitrazepam were prepared from nitrazepam. All other reagents were of analytical-reagent grade. Disposable cartridges packed with ODS-silica (Sep-Pak® C_{18}) were obtained from Waters Assoc. (Milford, MA, USA).

Apparatus and conditions

Electrophoresis was performed with an automated CE system, Model 270A (Applied Biosystems, Foster City, CA, USA). For each run, a fused-silica capillary (72 cm × 50 μm I.D.) was washed with 1.0 *M* NaOH for 3 min and then reconditioned with a running buffer for 4 min. The sample was introduced by applying a precisely controlled vacuum of 16.7 MPa, for 4 s. This resulted in 12 nl of the sample being introduced into the column. Separation was performed at a voltage of 20 kV at 37°C. The cathode was on the detector side. Electrophoresed components were detected with an on-column UV detector (220 nm), and the signals were transmitted to an integrator (Chromatocorder 12, SIC, Tokyo, Japan).

Preparation of standard solution

Stock solutions (1 mg/ml) were prepared by weighing nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam, then dissolving them in absolute methanol and storing them at 4°C in Eppendorf 1.5-ml polypropylene microtubes. A working solution and spiked urine samples were prepared by diluting the stock solution with distilled water and human urine, respectively.

Optimization for MECC

Known aliquots of the working solution were dried under nitrogen gas at 50°C, then the residues were reconstituted in 50 μl of the running buffer, followed by filtration through a filter unit (0.45 μm) for MECC analysis. The electroosmotic flow time (t_0) of the bulk solution was determined from the migration time of mesityl oxide, which is insoluble in the micellar phase.

Sample preparation procedures

We carried out a solid-phase extraction procedure similar to that for GC analysis reported by Suzuki *et al.* [14]. Briefly, to 2 ml of human urine containing the compounds were added 6 ml of carbonate buffer solution (pH 9.8). A Sep-Pak C_{18} cartridge was activated by passing methanol (5 ml), water (5 ml) and carbonate buffer (2 ml) sequentially through it, the sample solution was poured into the cartridge. Then the cartridge was washed with water (5 ml), 20% acetonitrile (1 ml) and *n*-hexane (1 ml), and dried under full vacuum for 1 min. Next, the analytes were eluted from the column by passing 4 ml of methylene chloride through the cartridge. The liquid collected was evaporated to dryness under nitrogen gas at 50°C. The residue was dissolved in 50 μl of the running buffer, and this sample solution was filtered through a 0.45-μm pore filter unit before injection.

Precision

For the calculation of the day-to-day precision, four blank human urine samples were spiked with the analytes (0.1 and 0.5 μg/ml), and the samples were processed as described above on four separate days, with one analysis each day. For the within-run precision, human urine samples ($n = 7$) spiked with the analytes (0.1 and 0.5 μg/ml) were analysed within the same day.

Recovery

The recovery after sample pretreatment was determined by comparing MECC peak areas after extraction with peak areas obtained by direct injection of equal amounts of the compounds in buffer.

RESULTS AND DISCUSSION

Optimization for MECC

Preliminary experiments using samples from a working solution were first conducted at pH 8.5 without sodium dodecyl sulphate (SDS) in the running buffer. Because MECC of illicit drugs has been carried out in buffers of concentration below 10 m*M* [9,10,13], we chose 6 m*M* phosphate-borate as the running buffer. As a result, a single peak was observed for the three compounds, indicating that the electrophoretic medium in the absence of micelles did not provide sufficient separation of these compounds. In the presence of an anionic surfactant, *i.e.* SDS, the three compounds in the working solution could be separated on the basis of relative affinity for the micellar environment against the bulk aqueous phase (Fig. 1). Nitrazepam, which is more hydrophobic, tended to be strongly associated with the micelles and thus was eluted later. The migration times for the compounds increased in the order 7-aminonitrazepam < 7-acetamidonitrazepam < nitrazepam. The net mobility of the analyte is the sum of the electrophoretic and electroosmotic mobilities, and certain parameters should be incorporated in the optimization to achieve an adequate separation of the mixtured compounds [15,16].

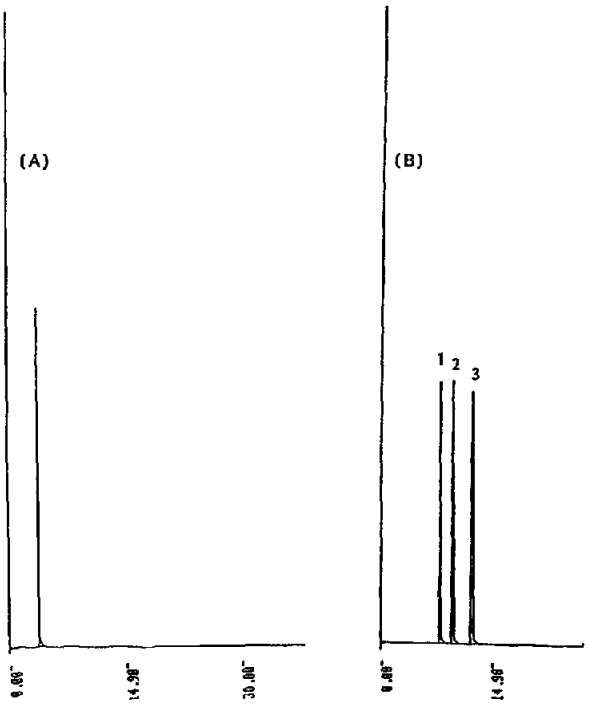

Fig. 1. Electropherogram obtained from a standard mixture using a running buffer of 6 m*M* phosphate–borate (pH 8.5) containing (A) no SDS or (B) 30 m*M* SDS. Separation tube, 72 cm × 50 μm I.D. fused-silica capillary; detection wavelength, 220 nm; voltage, 20 kV; temperature, 37°C. Peaks: 1 = 7-aminonitrazepam (5 μg/ml); 2 = 7-acetamidonitrazepam (5 μg/ml); 3 = nitrazepam (5 μg/ml).

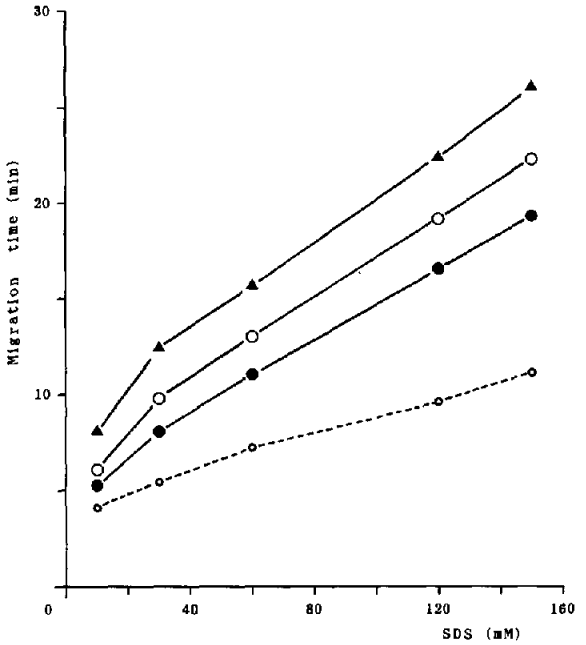

Fig. 2. Effect of SDS concentration on the electroosmotic flow time (t_0; ○---○) and the migration times of 7-aminonitrazepam (●), 7-acetamidonitrazepam (○) and nitrazepam (▲). Running buffer, 6 m*M* phosphate–borate (pH 8.5); other conditions as in Fig. 1.

The important parameters are micelle concentration, organic modifier composition and pH. As shown in Fig. 2, there was an increase in the migration times of the compounds when the SDS concentration in the running buffer (6 m*M* phosphate–borate, pH 8.5) increased. This increase in the migration times can be accounted for by the fact that, at higher SDS concentrations, the phase ratio of the micelle to the aqueous phase would be larger, thus solubilization of the compounds by the micelles would be higher, resulting

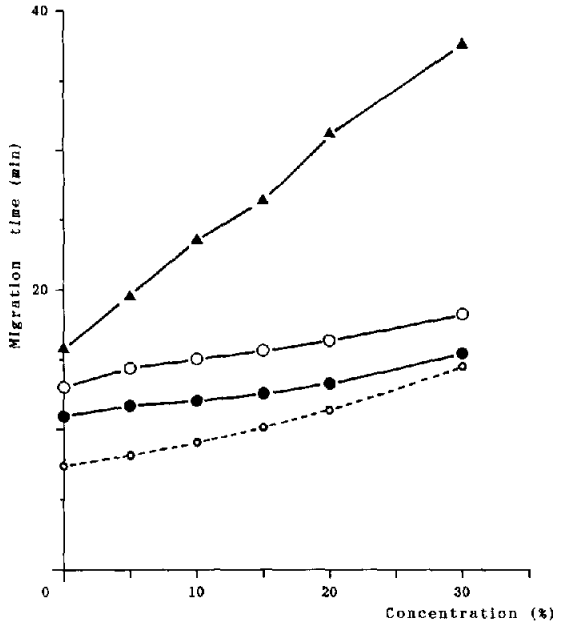

Fig. 3. Effect of methanol concentration on the electroosmotic flow time (t_0, ○---○) and the migration times of three compounds. Running buffer, 6m*M* phosphate–borate (pH 8.5) containing 60 m*M* SDS; other conditions and symbols as in Figs. 1 and 2.

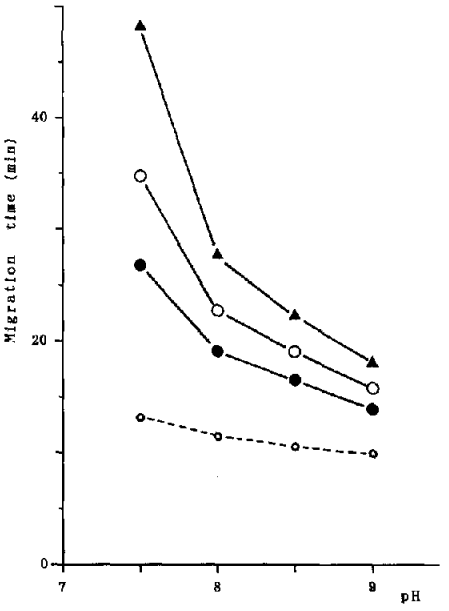

Fig. 4. Effect of pH on the electroosmotic flow time (t_0, ○---○) and the migration times of three compounds. Running buffer, 6 m*M* phosphate–borate (pH 8.5) containing 120 m*M* SDS; other conditions and symbols as in Figs. 1 and 2.

in an increase in the migration times, as described elsewhere [6]. Additionally, the electroosmotic flow decreased as the concentration of SDS increased. The effect of methanol on the migration time of three compounds in 6 m*M* phosphate–borate (pH 8.5) containing 60 m*M* SDS is shown in Fig. 3, which shows that the migration times of the analytes increased with increase in the methanol concentration. This effect is, in part, attributed to the decrease in the electroosmotic flow, as shown by an increase in t_0. In addition, it is known that the migration time is directly proportional to viscosity, and that the viscosity of methanol solution increased up to 50% [16]. The effects of pH on the migration times of three compounds and on elctroosmotic flow time (t_0) were also investigated. The pH was varied from 7.5 to 9.0 with 6 m*M* phosphate–borate containing 120 m*M* SDS. Although there was no change in the migration order, the migration times increased at lower pH (Fig. 4), probably owing to the changes in some factors with varying pH, *i.e.* changes in the electroosmotic flow, the ionization of the solute and the micelle–aqueous phase partition. As can be seen in Fig. 4, as the pH is lowered, the t_0 value increases, owing to the decrease in the electroosmotic flow. It is known that electroosmotic mobility increases with increase in pH [17].

Application to urine samples

The method described here was applied to the determination of the three analytes in spiked urine samples. On the basis of the results described above, the optimum running buffer was selected as 6 m*M* phosphate–borate buffer (pH 8.5) containing 60 m*M* SDS modified with 15% (v/v) methanol. A typical electropherogram obtained under our optimum experimental conditions is shown in Fig. 5. 7-Aminonitrazepam, 7-acetamidonitrazepam and nitrazepam showed peaks at *ca.* 11.3, 13.8 and 23.5 min, respectively. Here, we successfully used disposable cartridges of ODS-silica (Sep-Pak C_{18}) for the extraction and the clean-up. We obtained a better electropherogram by washing the cartridge with 1 ml of *n*-hexane before drying under full vacuum. Meth-

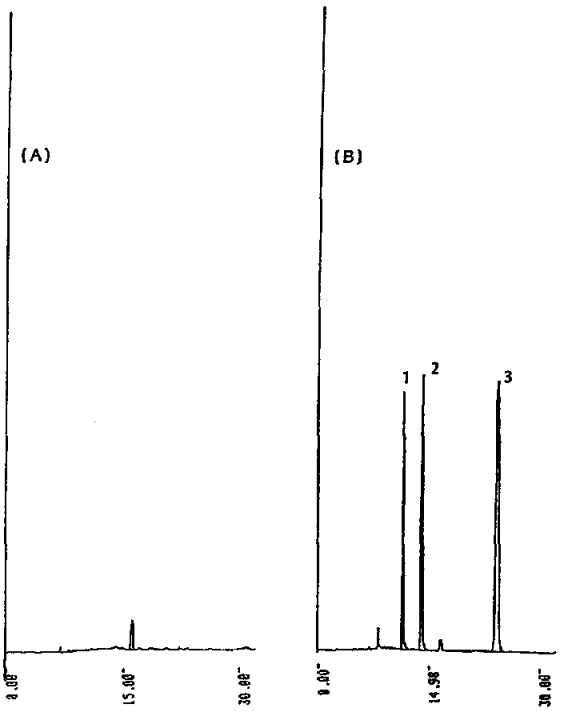

Fig. 5. Electropherogram of (A) blank human urine and (B) human urine spiked with three compounds (5 μg/ml for each analyte). Running buffer, 6 m*M* phosphate–borate (pH 8.5) containing 60 m*M* SDS modified with 15% (v/v) methanol; other conditions and peaks as in Fig. 1.

ylene chloride, not methanol or acetonitrile, removed interferences in the electropherogram.

Precision, recovery and linearity

The within-run and day-to-day precisions for the peak areas were tested at concentrations of 1.0 and 5.0 μg/ml (Table I). The precision obtained, expressed as a coefficient of variation (C.V.), was 1.7–8.0% for the within-run assay, and 2.0–7.7% for the day-to-day assay. As shown in Table II, the precision for migration time was 0.2–1.7% for the within-run assay, and 1.0–1.8% for the day-to-day assay. The relative recoveries of each compound ranged from 78.9% to 100.8% (1 μg/ml) and from 84.1% to 100.3% (5.0 μg/ml). The detection limits obtained were 50–100 pg for each compound per 12-nl injection, and the sensitivity was 100–200 ng/ml under our conditions (measured at a 2:1 signal-to-noise ratio). Extractions of each analyte from spiked urine samples, followed by MECC, gave linear plots for the peak area against concentration up to 10 μg/ml: $r = 0.9962$ for 7-aminonitrazepam, $r = 0.9956$ for 7-acetamidonitrazepam and $r = 0.9868$ for nitrazepam ($n = 10$).

Kangas [2] demonstrated a sensitive determination of nitrazepam and its two main metabolites in urine using GLC. The detection limits were 0.2 ng/ml for nitrazepam and 50 ng/ml for the metabolites, but the method required two different detectors for these three compounds: an electrochemical detector for nitrazepam and a nitrogen-selective detector for its two metabolites. In addition, the separation between nitrazepam and 7-aminonitrazepam was poor, and the peaks obtained were somewhat broad and tailing. The liquid chromatographic method reported by Kel-

TABLE I

WITHIN-RUN AND DAY-TO-DAY PRECISION OF PEAK AREA FOR 7-AMINONITRAZEPAM, 7-ACETAMIDONITRAZEPAM AND NITRAZEPAM

Concentration (μg/ml)	Coefficient of variation (%)					
	7-Aminonitrazepam		7-Acetamidonitrazepam		Nitrazepam	
	W[a]	D[a]	W	D	W	D
1.0	4.0	3.4	8.0	6.4	5.8	2.5
5.0	3.0	7.7	1.7	7.2	2.9	2.0

[a] W = within-run precision ($n = 7$); D = day-to-day precision ($n = 4$).

TABLE II

WITHIN-RUN AND DAY-TO-DAY PRECISION OF MIGRATION TIME FOR 7-AMINONITRAZEPAM, 7-ACETAMIDONITRAZEPAM AND NITRAZEPAM

Concentration (μg/ml)	Coefficient of variation (%)					
	7-Aminonitrazepam		7-Acetamidonitrazepam		Nitrazepam	
	W[a]	D[a]	W	D	W	D
1.0	1.1	1.3	1.3	1.5	1.7	1.7
5.0	0.2	1.0	0.2	1.2	0.3	1.8

[a] W = within-run precision (n = 7); D = day-to-day precision (n = 4).

ly *et al.* [4] is also very sensitive, but has the disadvantage of a broad peak for 7-aminonitrazepam with a detection limit of 50 ng/ml. Dixon *et al.* [3] developed an RIA for assay of nitrazepam in plasma. The method required a small sample and no extraction and was very sensitive. However, it could detect only nitrazepam and not its metabolites simultaneously, while unchanged nitrazepam is excreted poorly via the kidneys (1.1% during 7 days) in comparison with its two metabolites (52.0% during 7 days) [2]. On the other hand, in two fatalities due to overdoses of nitrazepam, post-mortem urine concentrations of 5.9 and 6.0 μg/ml were reported [18].

CONCLUSION

The method presented here was based on a combination of two processes. One used the Sep-Pak cartridge to achieve high extraction efficiency and recovery of the parent drug and its metabolites from human urine. The second used the capillary electrophoresis system for MECC. The rapidity and specificity of this method are superior to those of previously described methods, and this method can be useful in practical forensic intoxication. The sensitivity obtained is somewhat compromised because the injection volume is limited to the nanolitre order. Therefore, further studies into solutions to this problem, for example on-column sample stacking, are necessary.

Urine analysis has become a useful screening method for clinical and forensic analysts. The MECC method combined with solid-phase extraction described here is highly effective for separating nitrazepam and its two metabolites in spiked human urine. Although the sensitivity is somewhat compromised, we confirm that the MECC method would be an alternative separation strategy for drug screening in future.

REFERENCES

1 H. Sawada and K. Shinohara, *Arch. Toxicol.*, 27 (1970) 71.
2 L. Kangas, *J. Chromatogr.*, 172 (1979) 273.
3 R. Dixon, R. Lucek, R. Young, R. Ning and A. Darragh, *Life Sci.*, 25 (1979) 311.
4 H. Kelly, A. Huggett and S. Dawling, *Clin. Chem.*, 28 (1982) 1478.
5 M. Japp and K. Garthwaite, *J. Chromatogr.*, 439 (1988) 317.
6 S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
7 W. Thormann, P. Meier, C. Marcolli and F. Binder, *J. Chromatogr.*, 545 (1991) 445.
8 M. Tomita, T. Okuyama, Y. Nigo, B. Uno and S. Kawai, *J. Chromatogr.*, 571 (1991) 324.
9 P. Wernly and W. Thormann, *Anal. Chem.*, 63 (1991) 2878.
10 P. Wernly and W. Thormann, *Anal. Chem.*, 64 (1992) 2155.
11 I. S. Lurie, *J. Chromatogr.*, 605 (1992) 269.
12 K.-J. Lee, G. S. Heo, N. J. Kim and D. C. Moon, *J. Chromatogr.*, 608 (1992) 243.
13 R. Weinberger and I. S. Lurie, *Anal. Chem.*, 63 (1991) 823.
14 O. Suzuki, H. Seno and T. Kumazawa, *J. Forensic Sci.*, 33 (1988) 1249.
15 M. G. Khaledi, S. C. Smith and J. K. Strasters, *Anal. Chem.*, 63 (1991) 1820.

16 G. M. McLaughlin, J. A. Nolan, J. L. Lindahl, R. H. Palmieri, K. W. Anderson, S. C. Morris, J. A. Morrison and T. J. Bronzert, *J. Liquid Chromatogr.*, 15 (1992) 961.
17 K. Otsuka and S. Terabe, *J. Microcol. Sep.*, 1 (1989) 150.
18 J. S. Oliver and H. Smith, *Forensic. Sci.*, 4 (1974) 183.