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Application of capillary electrophoresis to the simultaneous screening and quantitation of benzodiazepines

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Abstract

Capillary electrophoresis (CE) is an attractive approach for the analysis of drugs in body fluids. We made a simultaneous analysis of nitrazepam, diazepam, estazolam, bromazepam, triazolam and flurazepam using CE with on-column detection at 200 nm. We obtained the best electropherograms under a condition of 5 m*M* phosphate–borate (pH 8.5) containing 50 m*M* SDS and 15% methanol. We examined the effect of the sample solvent matrix on the electropherograms obtained, indicating that increasing the methanol content in the sample solvent or the injection volume above a certain threshold limit decreased the resolution. We then focused on application of the CE to the analysis of the drugs in spiked serum, being appropriate for an analysis within 25 min. Linearity, the detection limit, accuracy and reproducibility were established using this method. The calibration curve was linear up to 1 mg/l of serum concentration. The lower limit of detection was 5 pg per injection and 0.025 mg/l of the serum concentration for all the compounds except for flurazepam, for which they were 40 pg/injection and 0.2 mg/l. The detection limits obtained allowed toxicological and pharmacological determinations for nitrazepam, diazepam, estazolam and bromazepam, but not for triazolam and flurazepam. Only toxic blood levels for the latter two benzodiazepines could be quantified by this method. We concluded that the CE could at least be applicable to simultaneous screening for toxic levels of benzodiazepines. We suggest that this technique may offer criminal toxicologists a rapid, simple and adaptable approach for the estimation of many other drugs in body fluids.

Keywords: Benzodiazepines; Nitrazepam; Diazepam; Estazolam; Bromazepam; Triazolam; Flurazepam

1. Introduction

The benzodiazepines have become the most commonly used drugs for the treatment of anxiety and sleep disturbance and are widely prescribed throughout the world. However, they are frequently involved in cases of drug intoxication, are a contributory factor in traffic accidents and are also used by criminals to incapacitate their victims. Therefore, a rapid, sensitive and trace analysis of these drugs in body fluids is important for the forensic toxicologist. Furthermore, as the variety in types of benzodiazepines has increased and more patients receive multiple drug therapy, a reliable simultaneous method for the identification of several benzodiazepines is required for medical jurisdictional purposes. A number of methods for the analysis of these drugs in body fluids have been reported, including thin-layer chromatography (TLC) [1], gas chromatography (GC) [2,3], high-performance liquid chromatography

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(HPLC) [4–6], gas chromatography–mass spectrometry (GC–MS) [7] and the enzyme immunoassay [8]. Recently, Borggaard and Joergensen [9] and Ferrara et al. [10] reported results of comparisons of immunochemical and chromatographic techniques for the determination of benzodiazepines in urine.

Capillary electrophoresis (CE) has been found to be an attractive approach for the analysis of drugs in body fluids. This approach, which is fully automatable, is probably the most rapidly expanding analytical technique that has appeared in this decade. Using appropriate extraction procedures, the compounds extracted have been well identified by their retention behavior. Weinberger and Lurie [11], in comparing CE and HPLC, found CE to be superior to HPLC in efficiency, selectivity, peak symmetry and speed. Wernly and Thormann [12,13] demonstrated that most common drugs of abuse, including the benzodiazepines, could be confirmed in human urine using stepwise solid-phase extraction and CE with an on-column detector. Evenson and Wiktorowicz [14] described the possible broad applications of CE to therapeutic drug monitoring in clinical laboratories including monitoring of several of the benzodiazepines. We previously reported a CE analysis for nitrazepam and its metabolites in human urine [15]. The possibility of varying the selectivity of separation by changing the conditions of the buffer solutions is one of the valuable properties of this method.

In this paper, we present a rapid and simple procedure for simultaneous screening of nitrazepam, diazepam, estazolam, bromazepam, triazolam and flurazepam using capillary electrophoresis. The chemical structures are shown in Fig. 1. Efforts were first focused on the optimization of analytical conditions, i.e. on the effects of buffer concentration, modifier concentration and buffer pH on the separation. We also examined the effect of the sample solvent matrix, especially the effect of methanol in the sample solution, on the resolution. Next, the CE method was applied to the screening of benzodiazepines in spiked human serum samples. This also included examining method validation and sample clean-up procedures.

2. Experimental

2.1. Chemicals

Nitrazepam and diazepam were obtained from Wako Pure Chemistry (Osaka, Japan). Bromazepam, estazolam, triazolam and flurazepam were generously supplied by Kodama (Tokyo, Japan), Takeda Chem. Ind. (Osaka, Japan), Upjohn (Kalamazoo, MI, USA), and Roche Products (Welwyn, Garden City,

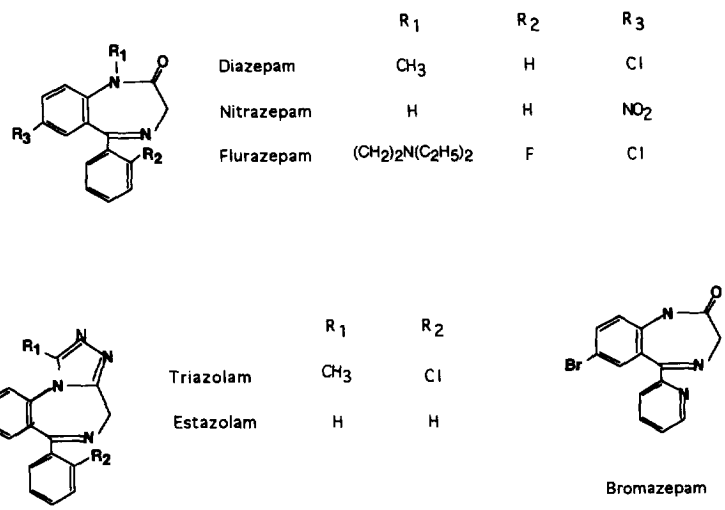

Fig. 1. Chemical structures of six benzodiazepines examined.

UK), respectively. Sodium dodecyl sulphate (SDS), mesityloxide and Sudan III were purchased from Nacalai Tesque (Kyoto, Japan). Disposable cartridges packed with ODS-silica (Sep-Pak C_{18}) were obtained from Waters Assoc. (Milford, MA, USA). All other reagents were of analytical-reagent grade.

2.2. Apparatus and conditions

Electrophoresis was performed with automated CE systems, Model 270A (Applied Biosystems, Foster City, CA, USA) and P/ACE 5000 (Beckman, Palo Alto, CA, USA). For each run, a fused-silica capillary was washed with 0.1 *M* NaOH for 4 min and then reconditioned with a running buffer for 6 min. A phosphate–borate buffer at pH 8.5 containing 50 m*M* SDS and 15% methanol was used as an aqueous run buffer unless otherwise noted. The sample was introduced by applying a precisely controlled vacuum. Separation was performed at a voltage of 25 kV at 25°C, the cathode being on the detector side. Electrophoresed components were detected with an on-column UV detector (200 nm).

2.3. Preparation of standard solution

Stock solutions (10 mg/ml) were prepared by weighing the drugs, then dissolving them in absolute methanol and storing them at 4°C. Working solution and spiked serum samples were prepared by diluting the stock solution with distilled water and human serum, respectively.

2.4. Clean-up procedures for spiked serum samples

We compared a solid-phase extraction with an organic solvent extractions. The solid-phase extraction using Sep-Pak C_{18} has been previously reported [16]. Briefly, a sample solution was prepared by adding 3 ml of 0.1 *M* borate buffer (pH 9.0) to 1 ml of spiked human serum. After activating a Sep-Pak C_{18} cartridge by sequentially passing methanol, water and borate buffer through it, the sample solution was poured into the cartridge. Then the cartridge was washed with borate buffer (3 ml), water (3 × 2 ml), 5% acetonitrile (1 ml) and *n*-hexane (1 ml) and dried under a full vacuum. 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. The residue was dissolved in 15% methanol, and then filtered through a filter unit of 0.45 μm pore size before injection. The benzene extraction was as follows: 0.5 ml of the spiked human serum was diluted three-fold with 0.1 *M* borate buffer. Then 4.0 ml of benzene were added and the mixture was shaken vigorously for 5 min. The supernatant obtained by centrifugation (3000 rpm for 5 min) was evaporated to dryness, following the sample preparation described above.

3. Results and discussion

Prior to the application of CE to the separation of benzodiazepines extracted from serum, the factors affecting the separation were studied in order to obtain optimum conditions. As described elsewhere [17–19], a probable important factor is the concentration of electrolyte, SDS and organic modifier in the running buffer. The pH of the buffer is also a main factor affecting the resolution. Changes in the concentrations of borate and phosphate ions had an effect on the migration times of the drugs, but not on the capacity factor. The higher the concentrations of the ions, the later the migration times for each component, probably because the electroosmotic flow decreases with an increase in ionic strength [20]. The migration times of six benzodiazepines increased with increasing SDS concentration, due to the solubilization of the solutes into the micellar phase [21,22]. Organic modifier was also effective in changing the capacity factors and migration times as described by Nishi et al. [23]. The capacity factors for each benzodiazepine decreased with increase in the methanol concentration. On the other hand, their migration times increased with increase in the methanol concentration because of the reduction of the electroosmotic flow. To decide the optimum pH value, we examined the value from 8 to 9, and found that the migration times had a tendency to increase at lower pH, but had no marked effect over this pH range. We obtained the best electropherograms under a condition of 5 m*M* phosphate-borate (pH 8.5) containing 50 m*M* SDS and 15% methanol.

As few medicines are generally soluble in water,

organic additives such as methanol are necessary for making their sample solutions. Although the sample volume represents a small portion of the total capillary volume, the sample matrix plays an important role in CE. In general, plate number and peak height can be improved by using lower current conducting conditions for the samples [24]. Shihabi [25] reported that acetonitrile deproteinization could increase the plate number and peak height for some compounds by a special stacking effect in addition to removing proteins. We examined the effect of the sample solvent matrix, especially the effect of methanol in the sample solution on the separation. Fig. 2 shows the effect of methanol in the sample solution on the electropherograms obtained when the injection time was 4 s. At high concentrations of the sample methanol, the peaks were wide and sometimes high and split, especially when the concentration was above 30%. On the contrary, no effect was observed on some other variables such as migration times and capacity factor. As illustrated in Fig. 3, a longer injection time also resulted in poor selective electropherograms. These data show that peak height, peak width and its separation were influenced by the organic additives in the sample matrix.

The method described above was applied to the separation of six benzodiazepines extracted from spiked serum. A typical electropherogram obtained under our optimum conditions is shown in Fig. 4. In this trial, we used a shorter capillary column, 50 cm length, for complete analysis within a shorter time, the full separation requiring less than 25 min. There was no marked difference between the electropherograms of the samples obtained by using the Sep-Pak C_{18} column and benzene. Both extractions of each drug from spiked serum, followed by capillary electrophoresis, gave us linear plots for the peak area against concentrations up to 1 mg/l (Table 1). Other solvents such as acetonitrile and ethyl ether were also investigated, showing relatively impure extracts with interfering compounds in the electropherograms (data not shown). Next, we compared the two extraction methods with regard to recovery and precision. The extraction recovery was determined by comparing the representative peak areas of extracted serum with those of the methanolic standard of the same concentration. The relative re-

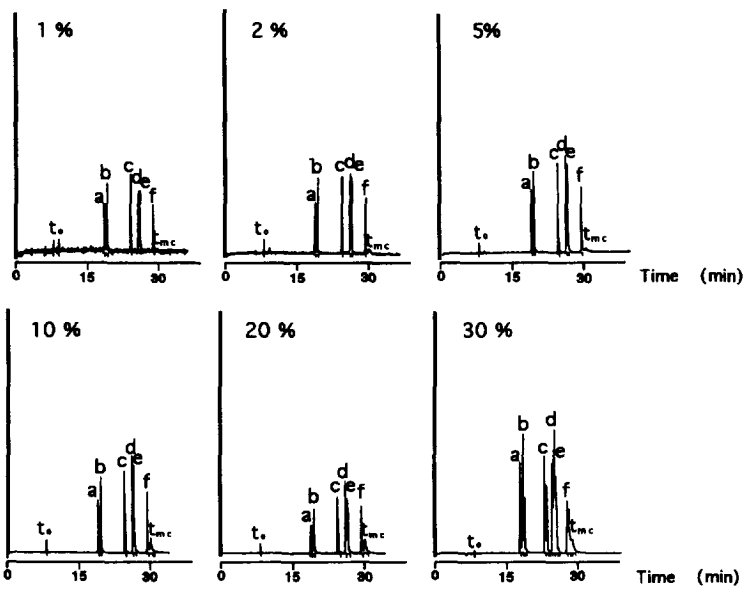

Fig. 2. Effect of the methanol concentration in the sample solution on the electropherograms. Peaks: a, bromazepam; b, nitrazepam; c, estazolam; d, triazolam; e, diazepam; f, flurazepam. Conditions: capillary, 72 cm long (50 cm to detector) fused silica, I.D. 50 μm; voltage, 25 kV; detection, UV at 200 nm; 4 s injection; run buffer, phosphate–borate, pH 8.5, with 50 m*M* SDS and 15% methanol.

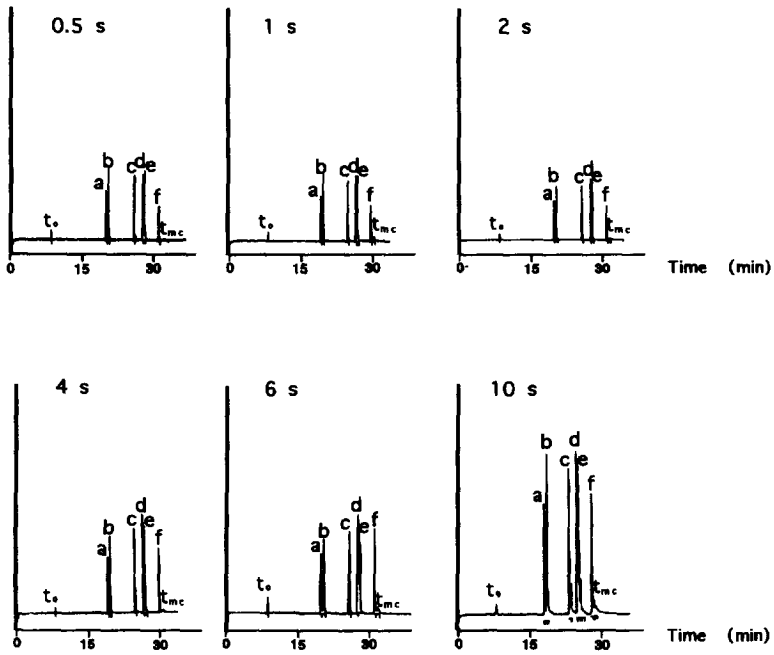

Fig. 3. Effect of the injection time (injection volume) on the electropherograms; 1 s equals about 3 nl. Peaks a–f and conditions as in Fig. 2 with the exception of the injection time.

coveries for each compound, except for flurazepam, ranged from 81.4 to 117.6% (0.1 mg/l) and from 83.4 to 91.5% (0.5 mg/l) for the Sep-Pak C_{18} extract, and from 71.5 to 96.6% (0.1 mg/l) and from 76.5 to 93.1% (0.5 mg/l) for the benzene extract. The recoveries for flurazepam (0.5 mg/l) were

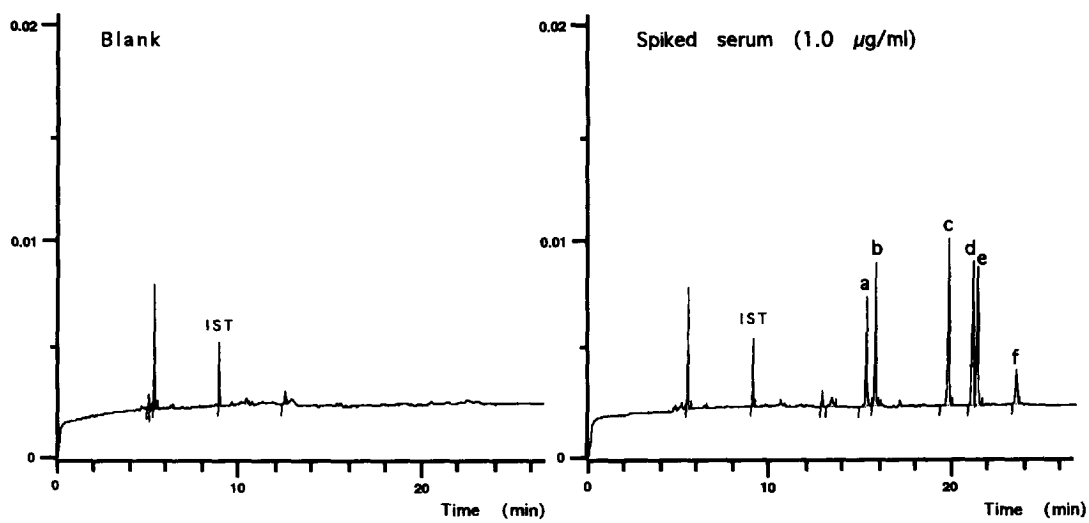

Fig. 4. Typical electropherograms for the Sep-Pak C_{18} extracts from human spiked serum. Peaks a–f as in Fig. 2. Conditions: capillary, 72 cm long (50 cm to detector) fused silica, I.D. 50 μm; voltage, 25 kV; detection, UV at 200 nm; sample, 15% methanol solution, 4 s injection; run buffer, 5 m*M* phosphate–borate, pH 8.5, with 50 m*M* SDS and 15% methanol, internal standard secobarbital.

Table 1
Correlation coefficient (r) for Sep-Pak C_{18} and benzene extract ($n = 3 - 4$)

Compound	Sep-Pak C_{18}	Benzene
Bromazepam	0.99977	0.99945
Nitrazepam	0.99924	0.99977
Estazolam	0.99989	0.99998
Triazolam	0.99944	0.99952
Diazepam	0.99980	0.99988
Flurazepam	0.99314	0.98913

45.0% (Sep-Pak C_{18}) and 65.6% (benzene), respectively. Table 2 and Table 3 show the reproducibility (C.V.) of the peak area at two concentrations obtained by this method: the within-run and between-run precisions of replicate assays using sera spiked with 0.5 and 1 mg/l of each drug. The precision obtained by Sep-Pak C_{18} was better than that by benzene with the exception of flurazepam.

Table 2
Within-run and between-run assay precision (C.V., %) for Sep-Pak C_{18} extract

Compound	Concentration (mg/l)			
	Within-run ($n = 3$)		Between-run ($n = 3$)	
	0.1 mg/l	0.5 mg/l	0.1 mg/l	0.5 mg/l
Bromazepam	1.4	0.5	5.5	2.2
Nitrazepam	1.5	1.3	1.8	2.3
Estazolam	2.2	1.2	3.2	2.7
Triazolam	0.7	1.4	3.2	1.2
Diazepam	1.0	0.5	4.5	3.3
Flurazepam	–	6.9	–	8.3

Table 3
Within-run and between-run assay precision (C.V., %) for benzene extract

Compound	Concentration (mg/l)			
	Within-run ($n = 3$)		Between-run ($n = 3$)	
	0.1 mg/l	0.5 mg/l	0.1 mg/l	0.5 mg/l
Bromazepam	2.8	4.0	6.3	6.4
Nitrazepam	5.7	1.6	4.9	5.3
Estazolam	4.6	2.5	5.0	4.4
Triazolam	2.7	3.1	7.1	2.9
Diazepam	1.9	2.2	6.1	4.2
Flurazepam	–	3.4	–	5.5

Flanagan et al. [26] published a review of drug-induced toxicological investigations. In that review, benzodiazepines (49%) and barbiturates (43%) were the drugs encountered most frequently. The plasma concentrations of benzodiazepines in patients who died due to an overdose of more than one pharmaceutical preparation, for example, were: nitrazepam, 4.6 mg/l; diazepam, 0.25 and 0.32 mg/l; flurazepam, 0.2 mg/l. Patients presenting in coma ($n = 22$) poisoned principally with benzodiazepines showed the following plasma levels: diazepam plus nordiazepam, 0.2–3.2 mg/l; nitrazepam, 0.6–7.0 mg/l; flurazepam plus desalkylflurazepam, 0.3–1.1 mg/l. After single doses of 5 and 10 mg nitrazepam, the plasma concentrations at 12 h were 0.04 and 0.05 mg/l, respectively [8]. After chronic daily oral dosing of 5 mg diazepam twice daily to 15 subjects, the steady-state plasma levels of diazepam were 0.09–0.37 mg/l [27]. In a review of 914 drug-related deaths involving diazepam poisoning, diazepam was the sole cause of death in only two cases, and the postmortem concentrations were 5 and 19 mg/l [28]. After single doses of 12 mg bromazepam and 9 mg daily, the peak and steady-state plasma concentrations were 0.11–0.17 mg/l and 0.08–0.15 mg/l, respectively [29]. The detection limit of our CE method was 5 pg per 12 nl injection and the minimum plasma level detectable was 0.025 mg/l (measured at a 2:1 signal-to-noise ratio) for all the compounds examined except for flurazepam. That for flurazepam was 40 pg per injection (0.2 mg/l of serum). These limits of detection were valuable for toxicokinetic and pharmacokinetic studies and monitoring drugs, except for triazolam and flurazepam, of which the serum levels in patients receiving the drugs are usually less than 5 μg/l [30,31]. Accordingly, this method is only valuable for toxicokinetic studies when assaying these two drugs.

In summary, we studied in detail the CE conditions for an analysis of benzodiazepines. The optimal running buffer was 5 mM phosphate-borate, pH 8.5, containing 50 mM SDS and 15% methanol. It is recommended that a lower methanol concentration be used in sample solution than that in the running buffer. We showed that the CE automatable system can be applied to simultaneous determination of benzodiazepines in spiked human serum. We believe that this strategy may be helpful for the

toxicological screening of benzodiazepines in the body fluids of victims.

Acknowledgments

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