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Micellar electrokinetic capillary chromatography of benzodiazepine antiepileptics and their desmethyl metabolites in blood[1]

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Abstract

A fast and reliable method for the MEKC separation and determination of benzodiazepine antipileptics and their active metabolites in serum has been developed, using a separation buffer composed of borate (pH 9.5), SDS (18 mM), and acetonitrile (14%) as an organic modifier. The method is sensitive enough to be used clinically with a precision of less than 3% for the analysis of nitrazepam, clonazepam, clobazam, diazepam and their desmethyl metabolites in serum. © 1997 Elsevier Science B.V.

Keywords: Micellar electrokinetic chromatography; Benzodiazepines; Antiepileptics; Human serum; Electrophoresis; Sample stacking; Sodium dodecylsulphate; Serum

1. Introduction

Clinical monitoring of the concentration of benzodiazepines in blood or other body fluids is important for establishment of better dosage guidelines for these drugs. A number of methods for the determination of benzodiazepines in plasma or serum have been published previously [1–6]. Due to the high resolution and the short analysis time, capillary electrophoresis (CE) constitutes an attractive approach for the separation and determination of such compounds in blood samples [7,8]. The aim of this work was to develop an analytical method for the four major benzodiazepine antiepileptics (i.e. nitrazepam, clonazepam, clobazam and diazepam; Fig. 1) and their pharmacologically active metabolites (i.e. desmethylclobazam and desmethyldiazepam) in human serum using CE with on-column UV detection. Though most benzodiazepines have weak basic and sometimes acidic character, clobazam and desmethylclobazam are electrically neutral compounds and cannot be separated on the basis of charge-to-size ratios by CE. Among the principal modes of CE, micellar electrokinetic chromatography (MEKC) developed by Terabe et al. [9] to extend the application range of CE to

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neutral molecules, appears to be useful for the analysis of this class of compound. However, with sodium dodecyl sulfate (SDS), the most popular surfactant in MEKC, the capacity factors (k') of benzodiazepines are high, due to their high hydrophobicity. Therefore, these drugs migrate close to the SDS micelle and resolution is limited. An organic solvent miscible with water can be used as an additive to the micellar solution to manipulate the capacity factors or selectivity [6,10,11]. The SDS-MEKC method with acetonitrile as an organic modifier has been chosen for the complete separation of the seven benzodiazepines, including the internal standard (I.S., flunitrazepam).

Another way to reduce k' values is to replace SDS with a less solubilizing surfactant, such as bile salt [12,13]. This bile salt-MEKC method was not selected in the present study because splitting occurred for the clobazam peak, possibly due to enantiomeric recognition by the bile salt. The addition of γ-cyclodextrin (γ-CD) to the SDS solution was found to be effective for the separation of these highly hydrophobic compounds, as reported previously [14]. The CD-SDS-MEKC method could not, however, be applied to the serum sample in the present work because a large interfering peak originating in serum overlapped with some of the benzodiazepine peaks when optimum amount of γ-CD was used.

A simple and rapid method is reported for the determination of benzodiazepine antiepileptics and their active metabolites in human serum by MEKC, combined with liquid–liquid sample extraction. The method was sensitive enough to be used for quantitation of therapeutic levels of these drugs in serum. The authors believe this to be the first report on the separation of clobazam or diazepam and their desmethylated metabolites in body fluids by CE, although other investigators have separated a similar combination of such drugs by GC [1] and HPLC [2–4]. The benzodiazepine antiepileptics are rarely administered simultaneously, but it is very useful to have a single analytical method for monitoring the serum levels of all these benzodiazepines in epileptic patients, whereby all samples containing any of these drugs can be assayed. This method can also be useful for screening when drug abuse is suspected.

2. Experimental

2.1. Chemicals

Sodium dodecyl sulfate (SDS), boric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol were HPLC grade (Kanto Chemical, Toyko, Japan). Ethyl acetate, *n*-pentane, CAPSO, Sudan III, nitrazepam and diazepam were obtained from Wako Pure Chemical (Osaka, Japan). Clonazepam and flunitrazepam were gifts from Nippon Roche (Tokyo); clobazam and desmethylclobazam were from Nippon Shoji (Osaka); clorazepate was from Dainippon Pharmaceutical (Osaka). Desmethyldiazepam was prepared by quantitative decarboxylation of clorazepate [15]. Human serum was purchased from Cosmo Bio (Tokyo).

2.2. Capillary electrophoresis

Capillary electrophoresis was performed with a Beckman P/ACE 5010 CE System (Beckman, Fullerton, CA, USA) equipped with a sample cooling option and a filter UV detector set at 214 nm, where the peak of clonazepam, whose therapeutic level is the lowest, revealed the optimum S/N detection sensitivity. A fused-silica capillary (Supelco, Bellefonte, PA, USA) of 47 cm length (40 cm to the detector) and 50 μm I.D. was used. Data acquisition and processing was performed using the Beckman System Gold software. The migration time-normalized peak-area ratios of the analytes to the internal standard (I.S.) were calculated to generate standard calibration curves of normalised peak-area ratio versus drug concentration. Running buffer containing organic modifier was prepared by mixing buffer with a range of volumes of organic solvent while maintaining the

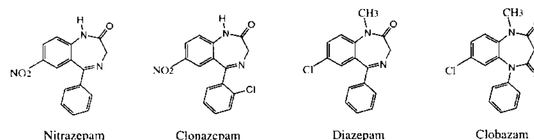

Fig. 1. Structures of the benzodiazepine antiepileptics.

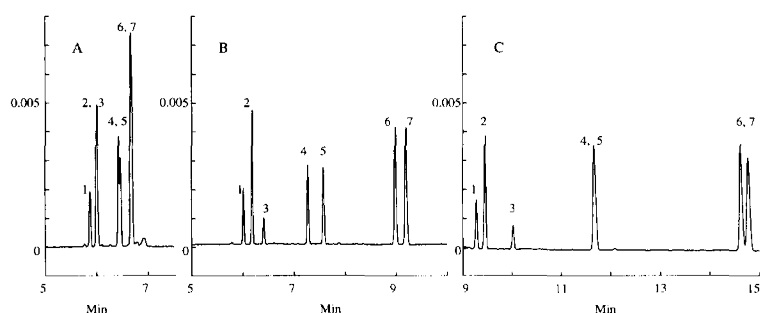

Fig. 2. Effect of the addition of organic modifers to the mobile phase. Electropherogram A: no organic modifiers to the mobile phase; Electropherogram B: 14% acetonitrile by volume. Electropherogram C: 18% methanol by volume. The running solutions contained 18 mM SDS and 50 mM borate (pH 9.5), with different organic modifier concentrations. Peaks: 1, nitrazepam (1.9 μg ml^{-1}); 2, flunitrazepam (3.8 μg ml^{-1}, I.S.); 3, clonazepam (0.75 μg ml^{-1}); 4, desmethylclobazam (3.1 μg ml^{-1}); 5, clobazam (3.1 μg ml^{-1}); 6, desmethyldiazepam (3.8 μg ml^{-1}); and 7, diazepam (3.8 μg ml^{-1}).

buffer and SDS concentrations constant; pH values were measured before addition of the organic modifier. Unless otherwise stated, a sodium borate buffer (pH 9.5; 50 mM boric acid) containing 18 mM SDS with 14% v/v acetonitrile was used as the run buffer to afford optimum separation of the benzodiazepines. Sample were prepared in 50 mM borate (pH 9.5) containing 4.5 mM SDS and introduced pneumatically by application of 0.5 psi pressure for 10 s. A constant voltage of 20 kV was applied; the capillary temperature was set at 25°C; the autosampler was cooled moderately at a coolant temperature around 18°C. The coolant temperature was determined to be a few degrees higher than the Krafft temperature of SDS. At the beginning of each working day and after each sample analysis, the capillary was conditioned by successive washings with 0.1 M sodium hydroxide, water and the running buffer.

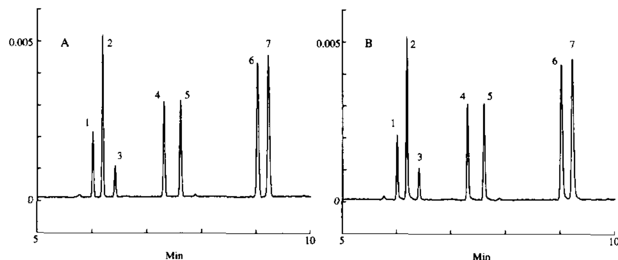

Fig. 3. Effect of cooling of the inlet buffer vial during analysis. Analysis was performed (A) with or (B) without cooling (18°C) of the autosampler. Sample concentrations and peak assignment as in Fig. 2.

2.3. Extraction procedure

To 500 μl of human serum containing flunitrazepam (300 ng ml^{-1}) as I.S. were added 250 μl of 0.8 M CAPSO buffer (pH 10.0; 0.8 M) and 4.0 ml of a mixture of *n*-pentane/ethyl acetate (3:1, v/v). The mixture was vortexed for 1 min and centrifuged at $1500 \times g$ for 5 min at 25°C. The organic layer was separated and evaporated to dryness in a centrifugal evaporator at 28°C. The residue was reconstituted in 40 μl of the sample buffer and filtered through a 0.2-μm pore syringe filter (Nihon Millipore, Tokyo, Japan); 20 μl were transferred into microvials for pressure injection.

3. Results and discussion

3.1. Optimization of electrophoretic conditions

For the determination of benzodiazepines by MEKC, the factors affecting the separation of the seven drugs including I.S. were examined. The effect of organic modifiers in the running buffer on the electrophoretic separation is shown in Fig. 2. The organic solvents were expected to contribute to the improvement of resolution due to the expansion of the migration time window and alteration of selectivity [11]. The addition of ace-

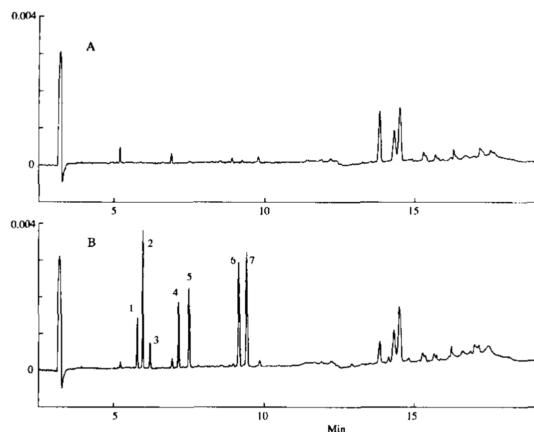

Fig. 4. Electropherograms of (A) extracted blank human serum, (B) extracted human serum spiked with therapeutic levels of benzodiazepine antiepileptics and their active metabolites. Peaks: 1, nitrazepam (150 ng ml^{-1}); 2, flunitrazepam (I.S.); 3, clonazepam (60 ng ml^{-1}); 4, desmethylclobazam (250 ng ml^{-1}); 5, clobazam (250 ng ml^{-1}); 6, desmethyldiazepam (300 ng ml^{-1}); and 7, diazepam (300 ng ml^{-1}).

tonitrile (6–22%) to the buffer improved the separation markedly; by adding the optimum concentration (14%) of acetonitrile, a complete baseline separation of these compounds was obtained. On the other hand, the pairs of the parent drug and the desmethylated metabolites; clobazam–desmethylclobazam, diazepam–desmethyldiazepam, were incompletely resolved at various concentrations (5–25%) of methanol.

The influence of SDS concentration (10–50 mM) on migration times and separation efficiencies was also investigated. In order to separate these hydrophobic compounds in a short time, a relatively low concentration (18 mM) of SDS was used in the running buffer, in combination with 50 mM borate (pH 9.5) as the background electrolyte. Consequently, a migration time of less than 10 min could be obtained even for the most strongly retained compound. Under these optimized conditions (Fig. 2B), the migration time of electroosmotic flow (t_0), and that of the micelle (t_{mc}) were 3.2, and 18.1 min, respectively, and the calculated k' values (1.2–4.0) for the analytes were close to the optimum for maximum peak resolution [16]. High separation efficiency was also observed with this MEKC system (250 000–300 000 theoretical plates), which was reduced considerably if the SDS concentration was increased. Bechet et al. reported low values (20–30 mM) for SDS concentrations that yielded maximum efficiencies of the separation of benzodiazepines by MEKC [10].

Moderate on-capillary concentration of sample molecules was achieved in the present MEKC method by means of field-amplified sample stacking [17]. The stacking process was accomplished by dissolving the drugs in 50 mM borate containing 4.5 mM SDS and injecting the sample solution for 10 s. This method provided an approximately 4-fold enhancement of peak heights compared with the injection of the sample dissolved in the running buffer for 2 s. On the other hand, the injection of the latter sample solution for 10 s resulted in only serious peak broadening. The critical micelle concentration (CMC) of SDS has been determined in this sample buffer following the method of Jacquier et al. [18], with a value of 2.8 mM. It was suggested that the best stacking efficiency was obtained when the micelle concentration was slightly higher than the CMC [17].

Though capillaries were employed with a straight-edged inlet, to avoid peak distortion caused by slanted edge capillaries [19], tailing of the eluted peaks has often been observed when the autosampler was not cooled by circulator during the analysis (Fig. 3B). This peak distortion was augmented as the injection time was decreased from 10 s (Fig. 3B) to a shorter time (data not shown). As shown in Fig. 3, a significant improvement in peak shape was obtained, when the analysis was performed while the inlet buffer vial was being cooled moderately with circulator. This can be explained by the occurrence of convection currents in the buffer vial, which arise from the temperature difference between the buffer and the cooled parts of the vial walls. The convection currents would descent around the walls of the vial and rise in the centre near the capillary inlet and thus prevent delayed entry of the residual sample on the outside surface of the capillary from above. Preheating of the inlet buffer above the ambient temperature of samples in the vial tray before the analysis also gave a higher peak efficiency without tailing, even in the absence of

Table 1
Intra-day and inter-day precision of the method at representative therapeutic concentrations and limits of quantitation of the benzodiazepine antiepileptics as well as their active metabolites

Drug	Spiked concentration (ng ml^{-1})	R.S.D. (%)		LOQ (ng ml^{-1})
		Intra-day ($n = 6$)	Inter-day ($n = 6$)	
Nitrazepam	150	1.5	2.4	10
Clonazepam	60	2.1	2.1	10
Desmethylclobazam	250	1.3	1.5	15
Clobazam	250	1.1	2.7	15
Desmethyldiazepam	300	1.3	1.3	20
Diazepam	300	1.9	1.7	20

The LOQ values were calculated as ten times the signal-to-noise [21] and analysis of samples at the LOQ gave R.S.D. values of 10% or better.

the coolant circulation, probably due to the occurrence of the similar convection currents, while the buffer was being cooled to the ambient temperature. Lux et al. [20] demonstrated that peak distortion was eliminated by rinsing the outside surface of the capillary after introduction of the sample. Cooling of the buffer tray may be another convenient choice in eliminating such peak tailing.

3.2. Determination of benzodiazepines in human serum

The benzodiazepines were recovered from 0.5 ml of serum using single-step liquid–liquid extraction. Recovery yields were 93% for nitrazepam and clonazepam, 96% for flunitrazepam, 90% for desmethylclobazam, and 94% for clobazam, desmethyldiazepam and diazepam. Fig. 4 shows typical electropherograms of blank human serum and serum containing drugs spiked at therepeutic levels. Good separation of the analytes and the I.S. from the matrix of the serum was achieved by the selected analytical conditions. It was also found that the migration times of the most common antiepileptic drugs (i.e. carbamezepine, phenytoin, ethosuximide, primidone, phenobarbital, valproic acid, zonisamide) were shorter than for the nitrazepam (peak 1 in Fig. 4) and that these drugs did not interfere with the present assay.

The inter-day (between-day) and intra-day (within-day) precision at representative therapeutic concentrations and the limit of quantitation (LOQ) of the method are summarized in Table 1. The low relative standard deviation (R.S.D.), which ranged from 1.1 to 2.7%, demonstrated the good precision of the method. LOQ with an acceptable precision (R.S.D. < 10%) ranged from 10 ng ml^{-1} to 20 ng ml^{-1}. Because the lowest therapeutic level of benzodiazepine antiepileptics is around 10 ng ml^{-1} for clonazepam, this method should be capable of determining the drugs in a clinical sample with sufficient sensitivity.

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