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Capillary electrophoresis with on-line sample pretreatment for the analysis of biological samples with direct injection

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ABSTRACT

A method of capillary electrophoresis (CE) using on-line sample pretreatment was developed. The system utilized on-line solid-phase extraction and capillary column switching. To explore the advantages of the method, propranolol in serum was determined by direct injection. The pretreatment part of the system consisted of an injection capillary including a small bed of protein-coated ODS. The protein-coated ODS concentrated the analyte by hydrophobic interactions, and the serum proteins were allowed to flow into a drain capillary. As samples up to *ca.* 1 μl could be loaded without a decrease in resolution, the detection limit was improved by two orders of magnitude (0.15 μg/ml). Propranolol was detected in a separation capillary by UV absorbance measurement without any interfering peaks. A linear response was obtained between 100 fg and 2 ng per injection. This method provides novel possibilities for extending the application of capillary electrophoresis.

INTRODUCTION

Capillary electrophoresis (CE) techniques that use electrical force to drive separations in capillary tubes have been applied in many areas of analysis [1–3]. Compared with high-performance liquid chromatography (HPLC), the practical use of CE has been focused on the analysis of highly purified samples (ranging from small molecules to macromolecules), and few papers have described the application of CE to substances in a mixture of complex matrices. Often the analyte of interest is present at a low concentration in a complex matrix, which may cause problems owing to the presence of proteins that may adhere to the wall of the capillary [4].

There are various approaches by which these difficulties may be overcome. One way is to use traditional sample pretreatment such as liquid–liquid or solid-phase extraction prior to analysis by capillary zone electrophoresis (CZE), which

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has become one of the high-performance separation techniques for the analysis of complex mixtures because of its very high resolution [5,6]. The traditional sample pretreatments serve for clean-up (deproteinization) and preconcentration of analytes. However, the methods are not combined "on-line" with the CZE analysis.

Compared with ordinary HPLC, CZE requires much smaller volumes of samples. However, the detection of analytes at lower concentrations is difficult. Some instrumental devices have been reported for performing on-capillary peak concentration to enhance the detectability in CZE, *e.g.*, the use of multiple capillaries arranged in bundles and combined into a single capillary through a glass connector [7] or coupling of isotachophoresis and CZE [8]. These techniques contributed to improved detection limits by increasing the amounts of sample loaded into the capillary.

Micellar electrokinetic chromatography (MEKC), which was introduced by Terabe and co-workers in 1984 [24], is performed with the same instrument as in CZE and uses an ionic surfactant solution at a concentration above the critical micellar concentration (CMC). MEKC has many attractive advantages in addition to the capability of electrophoretic separation of electrically neutral substances. In its application to pharmaceutical analysis, plasma samples were injected directly without any sample pretreatment prior to analysis [9,10]. In the MEKC with sodium dodecyl sulphate (SDS), plasma proteins were probably solubilized by the anionic SDS micelles, and it eliminated the adsorption of plasma proteins on the capillary wall. Consequently, similarly to micellar HPLC, MEKC permitted direct plasma sample injection without any sample pretreatment such as deproteinization or extraction.

In HPLC, various approaches have been developed to streamline sample pretreatment [11,12], including the use of column switching, specially designed HPLC columns or a micellar mobile phase with conventional reversed-phase HPLC. Morita and co-workers have reported HPLC methods for the analysis of biological samples with direct injection [13–19]. The development of protein-coated ODS, which was designed to partition small molecules but to elute proteins in one peak with high recovery, allowed the direct injection of plasma samples without sample pretreatment such as protein precipitation, and the determination of drugs (procainamide, propranolol, doxorubicin, etc.) or endogenous components (tryptophan and its metabolites) in biological fluids using a precolumn packed with protein-coated ODS has been reported. These techniques have contributed the automation of analyses with high precision.

The aim of this paper is to present a new concept of CE with the advantages of both liquid chromatography and electrophoresis. This modified CE method, which was designed to include on-line sample pretreatment and column-switching techniques, provides possibilities for introducing multi-separation fields with a single injection of sample by using different separation modes coupled with solid-phase extraction. This paper reports the development of CE with sample pretreatment for the determination of drugs in biological fluids with direct injection and demonstrates some of its features with the separation of propranolol in serum.

EXPERIMENTAL

Reagents and samples

Propranolol (Nacalai Tesque, Kyoto, Japan) was used as a model compound. Human serum was purchased from Sigma (St. Louis, MO, USA). A stock solution of propranolol (0.01 *M*, 2.9 mg/ml as hydrochloride salt) was prepared with distilled water and stored at −20°C. Addi-

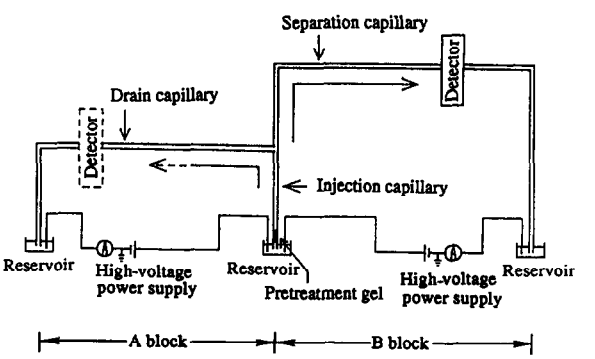

Fig. 1. Schematic diagram of the apparatus.

tional sample solutions were prepared by appropriate dilution of the standard propranolol solution in human serum (serum sample) or in phosphate buffer (buffer sample). The electrophoretic separation was performed using phosphate buffer (sodium salt); 50 m*M* phosphate buffer (pH 7.0) was used as the running buffer and acetonitrile–20 m*M* phosphate buffer (pH 7.0) (75:25) was used as the elution buffer. The buffers were passed through a 0.45-μm membrane filter and degassed by ultrasonication before use. Acetone (1%) was used as a neutral marker solute (no electrophoretic mobility) to obtain the electroosmotic flow-rate (V_{eo}). All chemicals were of analytical-reagent grade.

Apparatus

A schematic illustration of the system used in this study is shown in Fig. 1 [20]. A Model 890-CE high-voltage power supply was obtained from Jasco (Tokyo, Japan). Detection of separated solutes was monitored by on-column measurement of UV absorption at 290 nm using a Model 875-CE instrument (Jasco). A Chromatopac C-R6A (Shimadzu, Kyoto, Japan) was used for data processing. Untreated fused-silica capillary tubes were obtained from GL Sciences (Tokyo, Japan), and various lengths were used.

The system consisted of three pieces of capillary tubing connected to each other through a T-type connector; one was an injection capillary (5 cm × 50 μm I.D.). The injection capillary was tipped with a short capillary that contained a small bed of gel (protein-coated ODS). The fabrication of the injection capillary with gel was carried out as follows. The polyimide coating outside the capillary at the inlet side of the capillary (50 μm I.D. and 370 μm O.D.) was removed by burning, then the capillary was inserted into a capillary of 350 μm I.D. and 470 μm O.D., inside which a small bed of protein-coated ODS gel (particle size 20–32 μm, prepared as described [15,21]) was held by two porous glass frits. The other two capillaries were drain and separation capillaries (50 μm I.D., 35 and 60 cm long, respectively). The drain and separation capillaries were connected with the injection capillary using a T-type connector.

The system was operated by applying a high voltage between two capillaries (the injection capillary was placed in the anodic reservoir and the drain or the separation capillary was placed in the cathodic reservoir; A or B block in Fig. 1) alternately to produce an electric field as a driving force.

Procedures

Typical working procedures are given in Table I. The injection capillary (capillary with gel) was first conditioned by passing 100% organic solvent (acetonitrile). All three pieces of capillary tubes were filled with phosphate buffer (pH 7.0, 50 m*M*) by using a 50-μl microsyringe and the ends of the capillaries were dipped into the phosphate

TABLE I

PROCEDURES FOR DIRECT INJECTION ANALYSIS OF DRUG

Injection capillary with protein-coated ODS, 5 cm × 50 μm I.D. Drain capillary, 50 μm I.D. × 35 cm long (effective length 17 cm). Separation capillary, 50 μm I.D. × 60 cm long (effective length 40 cm).

Step	Operation	Procedure		Electric field	Conditions		
		Pretreatment	Separation		Anodic reservoir	Voltage (kV)	Time
1	Sample injection	Concentration	–	A block	Sample	15	2.5–50 s
2	Clean-up	Deproteinization	–	A block	Running buffer[a]	15	10 min
3	Elution	–	–	B block	Elution buffer[b]	10	15 s
4	Separation	–	+	B block	Running buffer[a]	15	10 min

[a] Running buffer: 50 m*M* phosphate buffer (pH 7.0).

[b] Elution buffer: acetonitrile–20 m*M* phosphate buffer (pH 7.0) (75:25).

buffer (running buffer) solutions whose surfaces were kept at the same level. The anodic reservoir at the injection capillary side was changed according to the procedures. Samples were electrokinetically injected into the injection capillary for 2.5–50 s at 15 kV using the A block (step 1). After injection, a high voltage (+15 kV at the sample introduction side and 0 V at the end of the drain capillary, A block) was applied (step 2). Sample pretreatment included the retention of the analyte on the protein-coated ODS packed in the injection capillary and the drawing of serum proteins and others into the drain capillary. The analyte was eluted from the protein-coated ODS with the elution buffer [acetonitrile–20 m*M* phosphate buffer (pH 7.0) (75:25)] by applying a high voltage (10 kV at the end of the injection capillary and 0 V at the end of separation capillary, B block) for 15 s (step 3). By applying a high voltage (15 kV) to B block, the electric field was changed from A block to B block, and the analyte could be separated in the field of the separation capillary with the phosphate buffer (step 4).

For CZE, a Model CE-800 capillary electrophoresis system (Jasco) was used in the CZE mode. It was equipped with a 50 μm I.D. fused-silica capillary. Sample solution was electrokinetically injected into the anodic end of the capillary and CZE was performed with on-column measurement of the UV absorption at 290 nm. A longer capillary with gel (50 cm) was used to investigate the loading capacity of the injection capillary and in that case an elution step (at 10 kV for 15 s with elution buffer) was introduced before the CZE analysis.

RESULTS AND DISCUSSION

The system is based on the combination of packed-column capillary electrochromatography (CEK) [22,23] and CZE. These techniques differ from microcolumn HPLC in that the flow of effluent is driven by an electric field rather than pressure. Throughout this experiment, electroosmotic flows were used as a driving force in addition to electromigration of ionic substances.

Use of protein-coated ODS gel as an analyte concentrator

It is expected that the capillary containing ODS gel will have the advantage of a higher loading capacity than open-tubular capillaries. Prior to analysis, the loading capacity of the gel (protein-coated ODS) within the injection capillary was investigated by increasing the injection volume of aqueous propranolol (Fig. 2). Plots of peak height (area) *versus* volume injected were linear up to 500 s at 15 kV (1200 nl) in the CZE system using the capillary with the gel. The injected volume was found to increase linearly with time at the rate of 2.4 nl/s. In a capillary without gel (open-tubular capillary as reference), the plot of peak height *versus* volume injected

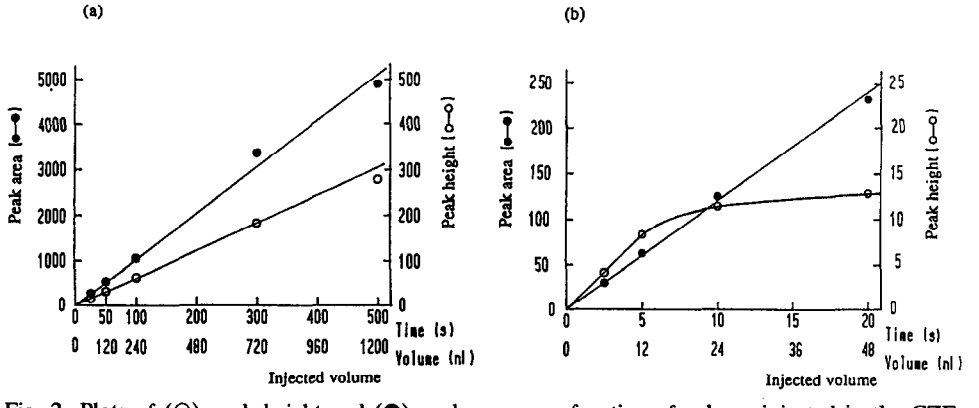

Fig. 2. Plots of (○) peak height and (●) peak area as a function of volume injected in the CZE mode using (a) a capillary containing gel and (b) a capillary without gel. Test compound, propranolol ($5 \cdot 10^{-5}$ *M*) in phosphate buffer (50 m*M*, pH 7.0). Capillary size, 50 μm I.D., 50 cm long (30 cm effective length). Injection was made electrokinetically at 15 kV for several seconds and electromigration was performed at 15 kV with the running buffer. For other conditions, see Experimental.

did not increase linearly over 5 s at 15 kV (12 nl). The capillary with the gel permitted concentration of propranolol, being based on solid-phase extraction, and the sensitivity with respect to sample concentration was increased by two orders of magnitude by permitting loading of larger sample volumes (detection limit 0.5 μM, 0.15 μg/ml).

Electropherograms of an aqueous propranolol sample obtained by CZE using a capillary with or without gel are shown in Fig. 3. As the pK_a of propranolol is 9.45, under the neutral conditions employed in this analysis the analyte migrates towards the cathode faster than the electroosmotic flow. In Fig. 3a, a negative peak shows the migration position of a neutral compound (organic solvent used in the elution buffer). Compared with the capillary without gel, a slight decrease in electroosmotic flow-rate (V_{eo} = 0.85 mm/s) was observed in CZE using the capillary with gel (*ca.* 10%). This might be due to the suppression of electroosmotic flow by the change in the electrical double layer in the area of the packed gel in the capillary. The theoretical plate number of propranolol in CZE analysis using the capillary with gel was *ca.* 88 000/m, showing a similar chromatographic performance to the corresponding CZE analysis using an open-tubular capillary (reference capillary, *ca.* 91 000 plates/m). This means that it is possible to perform concentration of propranolol without a great decrease in plate number. The decrease in the theoretical plate number often encountered, although usually less than 10%, seemed to be due to the band broadening caused by the connection of the capillaries. Therefore, the manufacture of analyte concentrators is critical for optimum performance.

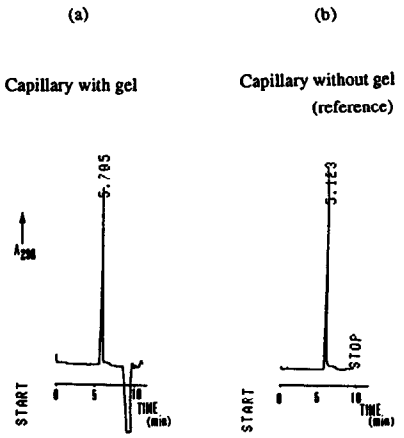

Fig. 3. Electropherograms of propranolol in CZE analysis using (a) a capillary containing gel and (b) a capillary without gel. Propranolol in phosphate buffer ($5 \cdot 10^{-4}$ M) was injected electrokinetically at 15 kV for 5 s. Other conditions as in Fig. 2.

Analysis of biological samples with direct injection

The protein-coated ODS columns were specially designed to partition small molecules and elute proteins in one peak with a high recovery for the analysis of biological samples with direct injection. It had characteristics of reversed-phase packings for small molecules owing to the presence of ODS on the internal surfaces of small pores, but had no longer affinity for plasma proteins [21]. Plasma proteins could not be adsorbed on the external surface of the protein-coated ODS gel, and deproteinization in the protein-coated ODS column was accompanied by size exclusion due to the small pores of the packing.

The pretreatment part of the CZE system consisted of a capillary with protein-coated ODS gel which extracted propranolol dynamically from directly injected samples and allowed serum proteins to flow out under electroosmotic flow in CZE, similarly to HPLC. However, even with the use of the protein-coated ODS gel-packed capillary, the problem would still remain that serum proteins might adhere to capillary wall and therefore cause a slower migration in the following run in CZE.

Column switching was used to avoid the problems caused by proteins. By changing the electrical force between the two blocks alternately, two separation fields were produced, one to drain out proteins and the other for the separation of an analyte. The operational procedures used are given in Table I. Fig. 4 shows typical electropherograms for propranolol. As acetone could not be retained on the protein-coated ODS, acetone was drawn into the drain capillary during the clean-up step under an electroosmotic flow, while propranolol was retained on the

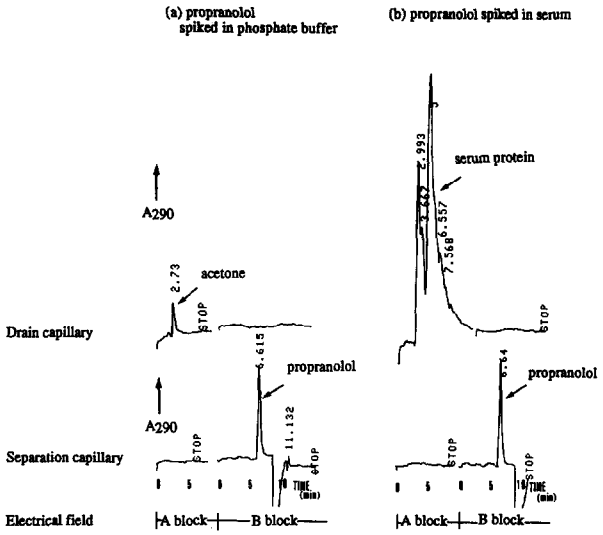

Fig. 4. Electropherograms of propranolol ($5 \cdot 10^{-4}$ *M*) added to (a) phosphate buffer (50 m*M*, pH 7.0) and (b) serum. For details, see Experimental.

protein-coated ODS in the injection capillary. The propranolol was then eluted from the protein-coated ODS by passage of a solution containing a high concentration of organic solvent (elution buffer) and electrophoresed in the field of the separation capillary. UV monitoring of the drain capillary in addition to the separation capillary proved that there was no leakage of the samples in the non-electrical field. The direct injection assay was validated for concentrations ranging from 15 to 300 μg/ml ($5 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ *M*) of propranolol in human serum by electrokinetic injection of samples (15 kV, 5 s) (Fig. 5). The calibration graph for propranolol in human plasma was linear.

As a reference, Fig. 6 shows the separation profiles of serum spiked with propranolol obtained by CZE with direct injection. Propranolol migrated toward the cathode, eluting before serum proteins, but the elution position was close to that of serum proteins. With the proposed system, serum proteins were drawn into the drain capillary and easily separated from propranolol. Hence propranolol was detected as a single peak without any interfering peaks of biological components in the separation capillary tube.

As a preliminary study, the recovery of pro-

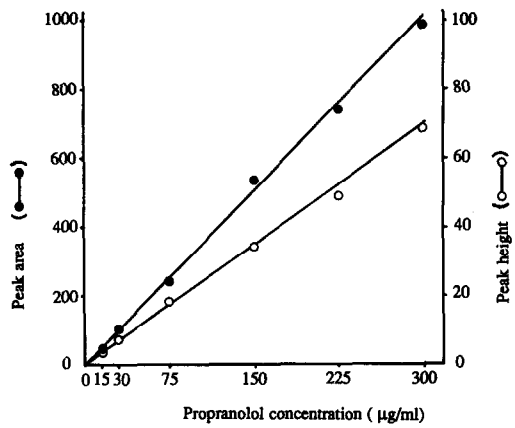

Fig. 5. Plots of (●) peak area and (○) peak height *vs.* propranolol concentration obtained from analysis of samples containing various amounts of propranolol added to normal human serum. Experimental conditions as in Fig. 4.

pranolol was determined by comparing the peak height of propranolol in serum with that of a standard sample. In CZE with direct injection, the average recovery of propranolol added to serum (150 μg/ml, $5 \cdot 10^{-4}$ *M*) was *ca.* 70% ($n = 5$). To decrease the protein content, serum was diluted 1:10 with phosphate buffer, and the

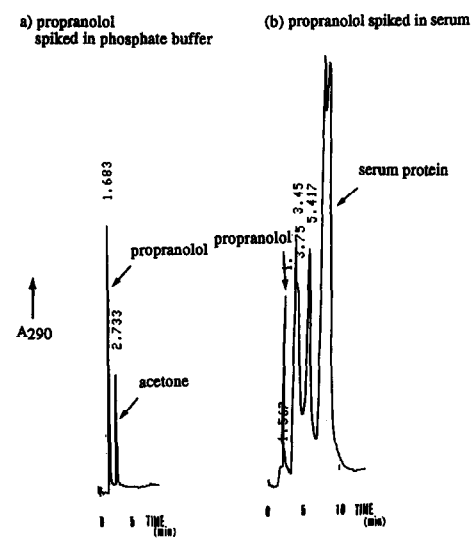

Fig. 6. Typical separation profiles in the CZE of propranolol ($5 \cdot 10^{-4}$ *M*) added to (a) phosphate buffer (50 m*M*, pH 7.0) and (b) serum. Injection was made electrokinetically at 15 kV for 5 s and electromigration was performed at 15 kV with the running buffer. Capillary size, 50 μm I.D., 45 cm long (18 cm effective length).

recovery in serum (1:10 dilution) was *ca.* 90% ($n = 5$). These results suggest that a higher protein binding of propranolol caused a slower release of propranolol from the proteins, which resulted in a decrease in recovery. With the modified system with direct injection, an almost quantitative recovery (100%) was obtained in both serum and diluted serum (1:10 dilution) samples ($n = 3$). Therefore, the total amount of both bound and unbound propranolol could be determined.

CONCLUSIONS

A modified method of CE including a sample pretreatment system and a capillary column-switching system has been developed. A capillary with protein-coated ODS gel at the inlet side served as an analyte concentrator to achieve an increased injection volume. Direct injection of serum samples without any sample pretreatment was applied to determine propranolol in serum. The sample pretreatment part of the system retained the analyte on the protein-coated ODS within the injection capillary while drawing serum proteins into the drain capillary. Therefore, an analyte separated from serum proteins could be detected as a single peak without any interfering peaks in the separation capillary. The protein-bound drug was released in a free form by partitioning to the hydrophobic phase on the protein-coated ODS. Hence it is assumed (but not proved) that the concentration of total drug could be determined. This method presents novel possibilities for extending the application of CE.

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