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On-line sample preconcentration on a packed-inlet capillary for improving the sensitivity of capillary electrophoretic analysis of pharmaceuticals

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ABSTRACT

An on-line preconcentration method is reported that improves the sensitivity of capillary electrophoretic analyses by at least two orders of magnitude. The method uses a concentrator capillary with a 75 μm I.D. and a l-mm length packed bed at the inlet end that can concentrate samples using the principles of liquid chromatography. Using low-molecular-mass pharmaceutical standards as examples, the parameters used in developing a preconcentration sensitivity enhancement method were optimized. The optimized method was then used to evaluate the quantitative aspects of capillaries of this type, including run to run and capillary to capillary reproducibility, linearity, and efficiency and resolution. In addition, the analysis of a urine sample spiked with doxepin at the 500 ppb level is reported.

INTRODUCTION

One of the most important issues facing the technology of capillary electrophoresis (CE) is sensitivity. In general, when compared with other analytical techniques such as liquid chromatography (LC), CE is a less sensitive technique. This difference lies primarily in two areas. The first is detector path length. In LC the path length of the detector cell is generally between 5 and 10 mm. However, in CE, since detection is performed on-column, the path length of the detector flow cell is determined by the internal diameter of the capillary, resulting in a detector cell path length that is about 100 times less than that of LC. Because absorbance is related to path length in a linear fashion according to Beer's law, sensitivity greatly suffers. The second difference is the ability to analyze low concentration samples. In LC, large volumes of a sample at a low concentration can be loaded and concentrated at the head of a column. Sample is later eluted with a gradient giving greatly enhanced sensitivity. However, in CE, the application of large sample volumes results in broader peaks and diminishing resolution.

Several approaches to improving the sensitivity of CE analyses have been devised. The use of different detection schemes such as fluorescence [1], and electrochemistry [2] have been reported that enhance sensitivity for compounds that are amenable to these types of selective detection. Extended path-length detector cells [3–5] have also been employed, however they provide only a 5-15 fold sensitivity enhancement, while compromising resolution and efficiency. Isotachophoretic sample loading (stacking) and field amplification have also been employed as on column concentration methods [6–8], however most of this work has been limited to low-molecular-mass organic acids or inorganic species. Guzman *et al.* [9] reported the use of both multiple capillaries arranged in bundles, and the use of concentrator capillaries containing an antibody covalently bound to a solid support material to increase sample loading, and hence sensitivity. This off-line method resulted in poor recovery, however, and no reproducibility or quantitative information was re-

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ported. In addition, capillaries of this type are not currently commercially available. This work describes the use of on-line preconcentration capillaries that improve the sensitivity of capillary electrophoretic analyses of some common pharmaceuticals by utilizing the principles of reversed-phase LC, first reported in 1990 for the analysis of peptides [10]. There are several advantages to this technique as opposed to off-line preconcentration. These include the ability to automate the assay, leading to better reproducibility, as well as the ability to address sample limited situations. Off-line preconcentration techniques can also add significant levels of impurities. It is for this reason that ion chromatographic trace enrichment is almost exclusively performed on-line. Using on-line concentration capillaries, we have been able to extend the detection limits over two orders of magnitude lower than those obtained with conventional capillaries without compromising resolution, efficiency, or reproducibility and quantitative capabilities.

EXPERIMENTAL

Chemicals

Doxepin and propranolol (Fig. 1) were purchased from Sigma (St. Louis, MO, USA), citric acid and citric acid trisodium salt dihydrate from Aldrich (Milwaukee, WI, USA), and LC-grade acetonitrile (ACN) and methanol from J. T. Baker (Phillipsburgh, NJ, USA). All were obtained in the highest purity available and used without further purification. Run buffers were prepared with Milli-Q water (Millipore, Marlborough, MA, USA) using the appropriate proportions of 0.025 ***M*** concentrations of citric acid and the sodium salt to a pH of 4.0. Eluent buffers were prepared by the addition of the appropriate amount of ACN to the run buffer. Both run and eluent buffers were filtered and degassed under vacuum daily. Sep-Pak C_{18} cartridges were obtained from Millipore.

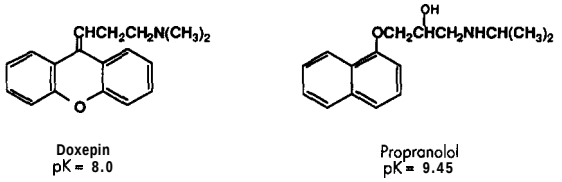

Fig. 1. Structure of doxepin and propranolol test compounds.

Capillary electrophoresis system

A Waters Quanta 4000 capillary electrophoresis system was used throughout (Millipore Waters Chromatography, Marlboro, MA, USA). Separations were performed using both standard capillaries (60 cm × 75 μm I.D.), and concentrator capillaries as described below. All analyses were performed with UV detection at 214 nm, electromigration injections of 5 kV, and an applied voltage of 250 V/cm. Data was collected and processed on a Model 845 chromatography data workstation (Millipore) at 10 points per second. The data workstation system suitability software was used to calculate reproducibility.

Concentrator capillaries

Concentrator capillaries were obtained from Millipore. These commercially available capillaries consist of a 1.0 mm packed bed of a polymeric reversed-phase chromatographic packing material at the injection end of the capillary, held in place with a glass frit on both ends. Capillaries were equilibrated prior to use by a manual purge of neat ACN using a syringe. This insured adequate wetting of the packing material. This was followed by an instrument purge of ACN for 15 min, water for 15 min, and run buffer for 15 min. Under continuous use this equilibration procedure was replaced with a between run instrument purge of run buffer only. The capillaries were stored in ACN when not in use. Samples were concentrated using these capillaries by performing an electromigration sample injection varying in time from 20 s to over 16 min in length. The sample was retained by the packing material. while the sample solution gradually filled the capillary. Following the injection, the capillary was purged, refilling it with the separation buffer. The sample was eluted from the packing using an injection of a small volume of an aqueous ACN mixture, again using electromigration. From this point the electrophoretic separation proceeded in a normal fashion.

Sample preparation

Standards were prepared in Milli-Q water at the 1 .O mg/ml level and diluted with water to the desired concentrations. Spiked urine samples were prepared by adding 1 .O ml 5.0 ppm doxepin in water to 9.0 ml urine filtered prior to use with a Millex-HA

filter (Millipore). Sample preparation was performed by loading the entire 10-ml spiked urine sample onto a prepared C_{18} Sep-Pak cartridge, and washing the cartridge with a two-step procedure first with water, followed by methanol-water (60:40). The doxepin was eluted from the cartridge in a 10-ml fraction of methanol-water (75:25), which was collected, concentrated by drying, and reconstituted to 1.0 ml with water, and injected.

RESULTS AND DISCUSSION

Method development

At low pH, both doxepin and propranolol (Fig. 1) are positively charged and can be separated by conventional free zone CE using a standard capillary as shown in Fig. 4a. Lower pH values were evaluated, and while providing increased resolution, a significant decrease in the electroosmotic flow (EOF) is obtained. This results in poor sample loading using the electrokinetic injection mode employed with the concentrator capillary. During this stage of research, doxepin alone was used to develop the concentrator capillary elution conditions as outlined below. The method development consisted of determining the ACN concentrations and elution time necessary to elute the sample from the packing once concentrated. As a starting point, an arbitrary figure of three times the packed bed volume was used as an eluent volume. This was determined by measuring the EOF velocity, accomplished by measuring the time for a neutral compound (ACN solvent) to migrate the length of the capillary according to the formula:

$$V = L/N,$$

where V = EOF velocity in mm/s; N_1 = migration time of ACN in s; L = length of capillary in mm. Using this formula, the EOF velocity was found to be 0.253 mm/s. The reciprocal of velocity, 3.95 s/mm, indicates that for a l.O-mm packed bed, an elution time of 12 s should be used.

Next, the effect of different eluent ACN concentrations were measured as a way of assessing the recovery of doxepin from the packing. Since different concentrations of ACN in the eluent can affect the background current and subsequently affect the migration time, and hence the area, areas normalized for migration time were calculated and plotted *versus* the percent ACN in the eluent. This graph is presented in Fig. 2. As can be seen, ACN concentrations in excess of 50% result in a plateau in the normalized area. These data, in conjunction with subsequent blank injections, offers proof that 100% recovery is obtained using ACN concentrations in electrolyte above 50%.

Next, elution time was varied to determine if the 12-s injection of eluent is sufficient. As seen in Fig. 3. after 10 s a plateau in the normalized area is reached that, in combination with subsequent blank injections, again indicates 100% recovery. All additional work was therefore carried out at eluent ACN concentrations above 50%, with elution times exceeding 10 seconds.

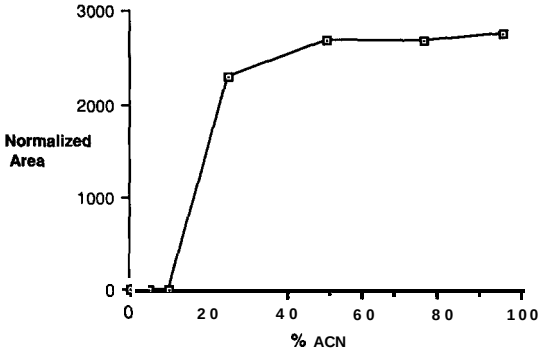

Fig. 2. Plot of normalized area *versus* %ACN used in eluent. Conditions as described in experimental. A 1 .O-ppm solution of doxepin was injected with a 5-min concentration step and a 12-s elution both at 5 kV. Normalized area is area/migration time.

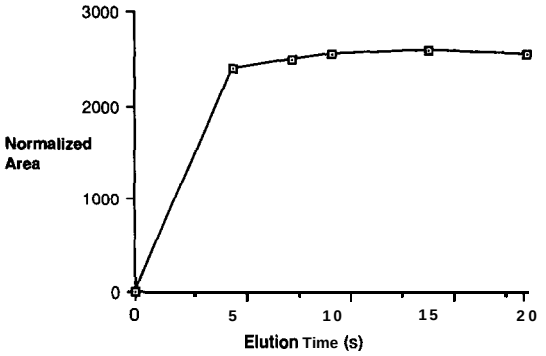

Fig. 3. Plot of normalized area versus elution time in s. Conditions as described in Experimental. A 1 .O-ppm solution of doxepin was injected with a 5-min concentration step at 5 kV. A 75% solution of ACN in run buffer was used as eluent.

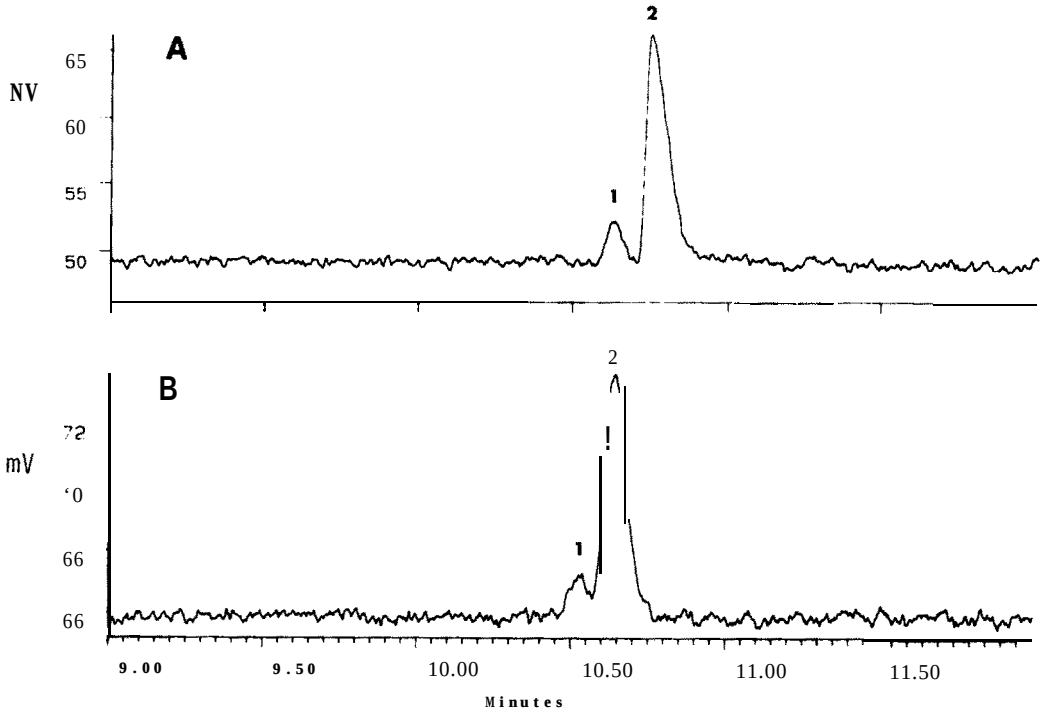

Fig. 4. Comparison electropherogram of (A) standard capillary and (B) concentrator capillary. Conditions as described in Experimental. Peaks: 1 = doxepin, 2 = propranolol. For (A) a 10-s electromigration injection at 5 kV was used. Peaks represent doxepin and propranolol concentrations of 10 (signal-to-noise ratio of 3) and 60 ppm respectively. For(B) a 16.7-min concentration step and a 15-s elution step both at 5 kV was used, with an eluent concentration of 75% ACN in run buffer. Peaks represent doxepin and propranolol concentrations of 100 (signal-to noise ratio of 3) and 600 ppb respectively.

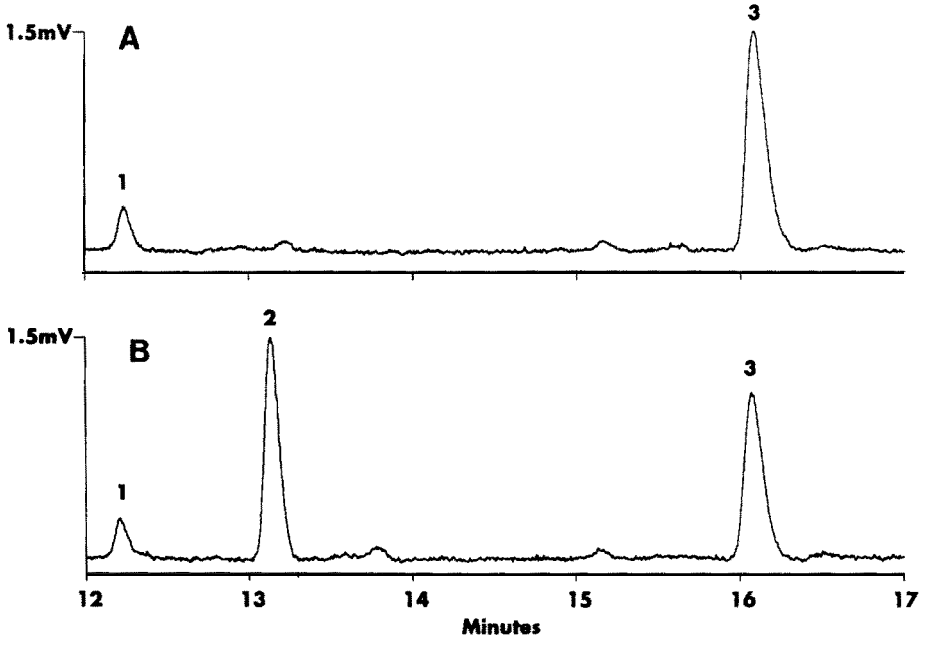

Fig. 5. Comparison electropherogram of (A) urine hiank, and (B) urine spiked with doxepm at the 0.5 ppm level. Conditions arc identical to those reported in Fig. 4B. Peaks: 1 = unknown. 2 = doxepin, 3 = unknown.

TABLE I

CONCENTRATOR CAPILLARY REPRODUCIBILITY

Conditions as reported in Fig. 4B. A 1.0-ppm solution of doxepin was used.

	R.S.D. (%)	
	Migration time	Area
Run to run (n = 5)	1.74	1.90
Capillary to capillary (n = 3)	7.87	4.18

Sensitivity enhancement

To determine the sensitivity enhancement that the concentrator capillary provides, a head to head comparison under identical separation conditions was made between a standard capillary and a concentrator capillary. The results are presented in Fig. 4. This comparison shows that under these conditions, the concentrator capillary can provide up to two orders of magnitude improvement in sensitivity as measured by the signal to noise ratio. The slight differences in migration times between the two capillaries is a result of batch to batch differences in capillary stock (see Reproducibility below).

Spiked urine sample

Following sample preparation, a urine blank and a spiked urine sample were run under identical conditions with the results shown in Fig. 5. Using the concentrator capillary a peak for doxepin at the 0.5 ppm level can easily be seen. This level is well into the therapeutic range for most small molecule pharmaceuticals and given the signal-to-noise level, could be easily quantitated. This level would be below the detection limits of a standard capillary using conventional CE techniques.

Concentrator capillary reproducibility

Both run to run ***(n = 5)*** and capillary to capillary ***(n =*** 3) reproducibility are presented in Table I. The relative standard deviation (R.S.D.) for both migration time and peak area are less than 2.0% for the run to run experiment which is typical of concentional CE techniques [11]. The R.S.D.s are higher for the capillary to capillary experiment reflecting the differences in lots of stock capillary material. From the authors' experience this error is similar to that obtained from lot to lot of standard capillaries.

Concentrator capillary linearity

Linearity was evaluated over three orders of magnitude from 10 ppb to 10 ppm. Excellent linearity with a correlation coefficient of 1.00 was obtained over the entire range. Above 10 ppm, a plateau was reached indicating detector photomultiplier saturation under the injection conditions employed. The solution to detector saturation is to inject less at higher ppm levels, and this is done by decreasing the concentration time. Decreasing the concentration time at higher concentrations can extend the linear range another two orders of magnitude. Therefore, under these conditions, using a combination of concentration times, linearity over five orders of magnitude, from 10 ppb to 1000 ppm can be obtained.

CONCLUSIONS

The use of concentrator capillaries of this type to perform on-line preconcentration improves the sensitivity of CE analyses by at least two orders of magnitude into the low ppb range. Run to run and capillary to capillary reproducibility, as well as linearity, is sufficient to allow quantitative use of these types of capillaries in the pharmaceutical laboratory. In conjunction with other CE techniques such as low wavelength detection and isotachophoretic sample loading, concentrator capillaries of this type may help to overcome the sensitivity limitations of CE to extend the range of applications to therapeutic drug monitoring.

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