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Continuous on-line concentration based on dynamic pH junction for trimethoprim and sulfamethoxazole by microfluidic capillary electrophoresis combined with flow injection analysis system

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Abstract

A novel, rapid and continuous on-line concentration approach based on dynamic pH junction for the analysis of trimethoprim (TMP) and sulfamethoxazole (SMZ) by microfluidic capillary electrophoresis (CE) combined with flow injection analysis is developed in this paper. Stacking is due to decreases in the velocity of analytes when migrating from the low-pH sample zone (sample was dissolved in 50 mM HCl) to a relatively high-pH buffer (30 mM phosphate buffer, pH 8.5) filled in the capillary. This results in 2.9–4.7-fold improvement in concentration sensitivity relative to conventional capillary electrophoresis methods. The separation could be achieved within 2 min and sample throughput rate can reach up to 38 h^{-1}.

Keywords: Microfluidic capillary electrophoresis; Flow injection; Dynamic pH junction; Trimethoprim; Sulfamethoxazole

1. Introduction

In the last decade, capillary electrophoresis (CE) has been proven to be an efficient technique for the separation of charged compounds [1–3]. Although CE provides advantages over other separation techniques, including rapidity, high resolving power, and small amounts of samples and reagents required, some aspects of the technique still remain weak. The discontinuous sample introduction mode and low concentration sensitivity primarily as a consequence of short optical path and small injection volumes are among those which most often demand further efforts for improvement [4].

On-line preconcentration methods based on electrophoresis represent one of the most facile ways for sample enrichment in CE, since the preconcentration step is performed within the same capillary used for analysis. Several on-line preconcentration techniques have been developed to improve the concentration sensitivity of CE, including field-amplified sample stacking [5–9], large-volume sample stacking [10,11], isotachophoresis [12,13], stacking of neutral analytes [14,15], the transient moving chemical reaction boundary method [16,17] and so on. Each method relies on specific modification of the composition of electrolyte relative to the background electrolyte (BGE) used for separation. Early reports have also indicated that dynamic pH junction may be a promising approach for on-line focusing of not only zwitterionic analytes but also any weakly acidic species that possess different velocities in the sample and the BGE zones. Changes in an analyte's velocity are caused by both pH difference and differential borate complexation in two segments of electrolyte in the capillary. Thus, an analyte must possess an appropriate chemical functional group so that it may exist in at least two distinct states, with different velocities, in the capillary. By far, focusing using a dynamic pH junction has been successfully applied to zwitterions, epinephrine and catecholamines, nucleosides, and nucleotides [18–21]. However, the discontinuous sample introduction mode confined

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the sample throughput and precision. Furthermore, the capillary used in above methods was relatively long which made the analysis time relatively long. Hitherto, no applications of microfluidic CE–FI systems for the on-line focusing based on dynamic pH junction have been reported.

On the basis of knowledge of chemical properties of trimethoprim (TMP) and sulfamethoxazole (SMZ), a novel technique based on dynamic pH junction for continuous on-line preconcentration of TMP and SMZ by microfluidic CE–FI system is developed. The combined microfluidic CE–FI system has the favorable potentials in achieving efficient continuous sample introduction for CE [22–27], including enhanced sampling frequencies, improved reproducibility, as compared to conventional sample introduction. Additionally, the microchip with an H-channel design in this work was produced from plastic slides, Tygon pump tubing, polyvinyl chloride (PVC) pump tubing and short 7.5 cm long separation capillaries, and was readily coupled to a flow injection sample introduction system. It presented a low-cost alternative for more basic studies on a microfluidic system and can be inexpensively produced without resorting to microfabricated techniques [28–32]. Furthermore, this focusing technique is simple to perform, using conventional instrumentation without complicated procedures. The inherent simplicity and effectiveness of this focusing procedure may be a facile way to enhance sensitivity in CE.

2. Experimental

2.1. Reagents and materials

TMP and SMZ were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. All the reagents used were of analytical grade and distilled water was used throughout the study.

Stock standard solutions (300.0 μg/ml) of TMP and SMZ were prepared in (1) ethanol–water (1:1, v/v) and (2) 0.1 M HC1. Working standard solutions were obtained by diluting the corresponding stock standard solutions (1) with ethanol–water (1:1, v/v) and stock standard solutions (2) with water or water + HCl to the desired concentration.

Buffer solutions were prepared from 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 stock solutions. The pH of the buffer was adjusted by mixing NaH_2PO_4 with the same concentration NaH_2PO_4 to give the required pH. All buffer solutions were filtered through a 0.45-μm syringe filter before use.

TMP–SMZ tablets (nominally containing 80.0 mg of TMP and 400.0 mg of SMZ per tablet) were produced by Xi'an Pharmaceutical Factory (Xi'an, Shanxi, China).

2.2. Apparatus

A model HPE-100 CE system with 12 kV maximum voltage (Bio-Rad, Hercules, CA) was used for the separations, which was connected to a 486 personal computer with a chroma chromatography collection system (Bio-Rad) for integration and data treatment. Uncoated silica separation capillaries of 75 μm i.d., 375 μm o.d., and 75 mm length (45 mm effective length) (Yongnian Optical Fiber Factory, Hebei, China) were used throughout the study. UV detection was performed at 214 nm.

A FIAstar 5020 analyzer (Tecator, Sweden) equipped with two 4-channel peristaltic pumps was used for automated FI operations. 0.5 mm i.d. polytetrafluoroethylene (PTFE) tubing was used for connecting all components of the FI system, including 16-cm length transport line from the valve to the split-flow interface. A sample loop of 40 μl was made from PTFE, and Tygon pump tubes were used for delivering all solutions. The time period for the injecting sample was defined through thumbwheel settings.

The detailed description of the H-channel microchip has been given elsewhere [32].

2.3. Procedures

The manifold of the microfluidic CE–FI system was shown schematically in Fig. 1. For the FI operations, with the valve in the 'load' position (Fig. 1a), sample solution (S) was pumped by pump 2 (P2) to fill the sample loop (SL) of the injector valve (V), simultaneously, the carrier solution (C), which also functioned as the running buffer, was pumped by pump 1 (P1) through the split-interface (anodic

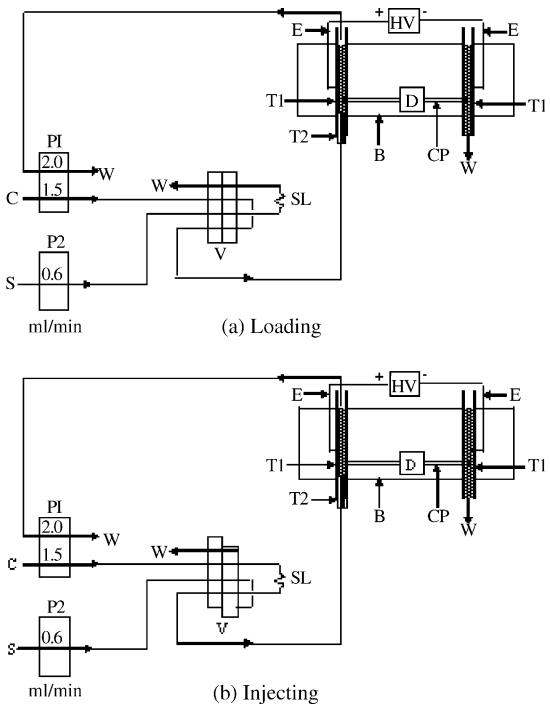

Fig. 1. The manifold of the combined microfluidic CE–FI system (not to scale): (a) loading and (b) injecting. C, carrier solution; S, sample; P1 and P2, peristaltic pumps; SL, sampling loop; V, injector valve; B, planar plastic base; T1, Tygon tubing; T2, PVC tubing; CP, separation capillary column; E, platinum electrode; W, waste; HV, high voltage; and D, detector.

reservoir). When the SL was full of sample solution, P2 was stopped, P1 resumed, and the valve was switched to the 'inject' position (Fig. 1b), the sample solution in the SL was transported by the carrier solution (pumped by P1) through the connecting conduit into the flow-through reservoir (anodic reservoir), where the flow was split and a fraction of the sample zone was introduced into the separation capillary by electrokinetic means. After the injection sequence, the valve returned to the 'load' position (Fig. 1a), and the next cycle began. A series of samples was injected continuously without interrupting the voltage (1.5 kV). For CE operations, the capillary was flushed sequentially with distilled water (5 min), 0.1 M NaOH (5 min) and distilled water (5 min) followed by running buffer (5 min) from the capillary outlet reservoir using a syringe. The mechanism of dynamic pH junction will be introduced in Section 3.1.

3. Results and discussion

3.1. Analytes chemical structure and focusing rational

For SMZ, two dissociation equilibria exist [33]. One is the equilibrium between the positively charged, protonated amino group of SMZ and its electrically neutral conjugate base, the other is the equilibrium involving the loss of the SMZ proton to yield its negative charged conjugate. TMP can be dissolved not only in acidic media but also in basic media [34,35], therefore, depending on the pH of the buffer employed, TMP and SMZ can exist either as negatively charged, deprotonated species, neutral special or as positively charged, protonated species. A cartoon is given in Fig. 2 to show dynamic pH junction in the system. At low-pH sample zone (Fig. 2a), the two analytes are positive and migrate toward the cathode end. When migrating from low-pH sample zone to high-pH electrolytes, the analyte molecules at the front of the injected sample acquire a negative charge, as the hydroxide ions in the higher pH BGE invade the sample zone, and migrate backward (Fig. 2b). The molecules at the back of the sample zone are faster than the molecules at the front until the hydroxide ions reach the end of the sample zone. The pH of the whole capillary now becomes the same, and the negatively charged or neutral analytes are brought to the detector by electro-osmotic flow (EOF), as in a normal CE separation (Fig. 2c). The focusing and the separation are achieved in the same run without any extra step. The pH junction in this type exists only for a short period of time.

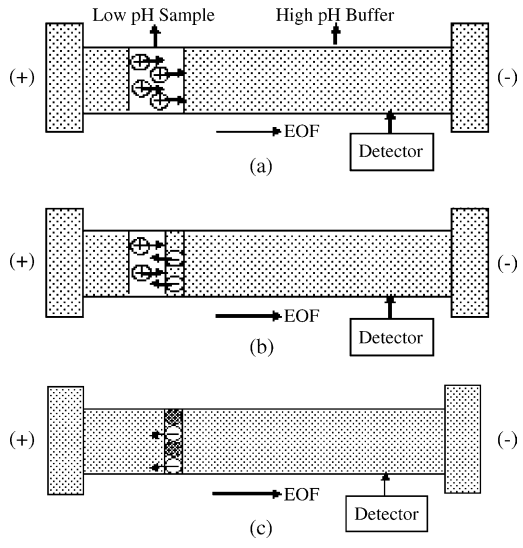

Fig. 2. Mechanism of dynamic pH junction: (a) injection of sample, (b) stacking of sample, and (c) separation of sample. (⊜) Neutral, (⊖) negative, (⊕) positive.

3.2. Separation of TMP and SMZ

To achieve satisfactory separation using the microfluidic CE technique, the buffer pH, concentration, and voltage were investigated.

In CZE, the pH of the buffer played an important role in the separation. In this study, the effect of buffer pH on peak heights and resolution in the pH range 7.0–10.0 with 30 mM phosphate and 1.5 kV applied voltage was investigated. The best resolution was achieved at pH 7.5, however, the sensitivity was best at pH 8.0 and 8.5 for TMP and SMZ, respectively. With concurrent consideration in resolution and sensitivity of the analytes, pH 8.5 was preferred for further studies.

Buffer concentration also had obvious influence on the separation because it could influence the EOF and the viscosity of the electrolyte. The effect of the concentration of phosphate at pH 8.5 on the separation and peak heights was studied in the range from 10 to 50 mM and 1.5 kV applied voltage. As expected, the resolution increased almost linearity with the increase of buffer concentration. But the best sensitivity was achieved at 30 mM. So, 30 mM phosphate was chosen.

The effect of varying the separation voltage from 1.2 to 1.8 kV was investigated under the conditions selected above. The resolution decreased significantly with increasing separation voltage from 1.2 to 1.8 kV, however, the best sensitivity was achieved at 1.5 and 1.6 kV for TMP and SMZ, respectively. So, the separation voltage of 1.5 kV was considered to be a good comprise between sensitivity and resolution.

3.3. Stacking of TMP and SMZ

In many literatures [33,36] for the separation of sulfonamides, TMP and SMZ were dissolved in ethanol–water (1:1, v/v). However, TMP and SMZ can also be dissolved in acidic media and basic media. At acidic media, they exist as positively charged. Based on the mechanism of dynamic pH junction, in this study, TMP and SMZ were dissolved in HCl.

It was observed that the presence of HCl in the sample could improve the sample stacking performance. Fig. 3 shows the electropherograms of TMP and SMZ in different concentration of HCl solutions. The peak heights of TMP and SMZ

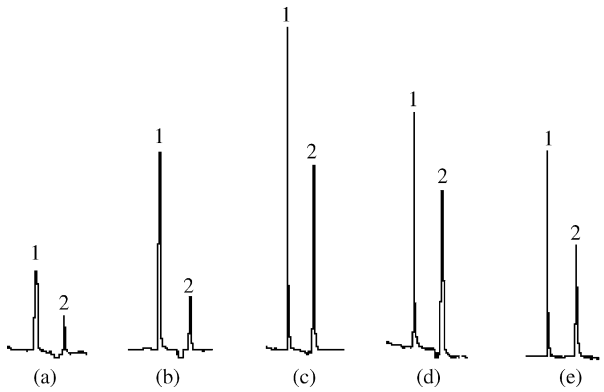

Fig. 3. Electropherograms of different concentration HCl on stacking. Sample, 60.0 μg/ml TMP and SMZ in: (a) ethanol–water (1:1, v/v, 0 mM HCl); (b) 20 mM HCl; (c) 50 mM HCl; (d) 75 mM HCl; (e) 100 mM HCl. Experimental conditions were: uncoated separation capillary, 75 μm i.d. × 375 μm o.d. × 75 mm length (45 mm effective length); detection wavelength, 214 nm; sample volume, 40 μl; carrier flow-rate, 1.5 ml min^{-1}. Separation conditions: buffer, 30 mM phosphate; pH 8.5, voltage; 1.5 kV.

Table 1
The comparison of the sensitivity of dynamic pH junction with no stacking

Analyte	No stacking (N_1)	Dynamic pH junction (N_2)	N_2/N_1
TMP			
Peak height (μV)	1.4×10^2	6.3×10^2	4.5
Peak area (μV s)	7.3×10^3	2.1×10^4	2.9
SMZ			
Peak height (μV)	6.4×10^1	3.0×10^2	4.7
Peak area (μV s)	4.9×10^3	1.5×10^4	3.1

increased with the concentration of HCl from 0 to 75 mM and 50 mM, respectively. Further increasing HCl concentration to 100 mM, the peak heights decreased. The sensitivity in terms of peak height could be improved by ∼4.6-fold in 50 mM HCl compared to the case when the sample was dissolved in ethanol–water (1:1, v/v) (Table 1).

3.4. Performance of the combined microfluidic CE–FI system

Calibration graphs were obtained by injecting standard solutions (2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 μg/ml). Each point on the calibration graph corresponded to the mean value obtained from four independent peak area measurements. The corresponding regression equations, as well as other characteristic parameter for the determination of TMP and SMZ were shown in Table 2. The limits of detection (LODs) were estimated from the calibration curve of peak height versus standard concentration and based on the concentration necessary to yield a net height equal to three times the standard deviation of the baseline noise, i.e. LOD = 3 s/k (s does the standard deviation of the baseline noise, k does the slope of the regression equation of peak height versus sample concentration). The baseline noise was evaluated by

Table 2
Analytical performance of the microfluidic CE–FI in pharmaceutical preparation testing system ($n = 4$)

	TMP	SMZ
LOD (μg/ml)	0.31	0.70
Peak height RSD (%)	3.1	5.8
Peak area RSD (%)	2.7	5.2
Linear range (μg/ml)	2.0–100.0	2.0–100.0
Regression equation[a]	$y = 508.29 + 330.07x$	$y = 340.42 + 253.41x$
Correction coefficient	0.9946	0.9990

[a] y, Peak area; x, standard concentration (μg/ml).

recording the detector response every 3 s over a period about 2 min.

The reproducibility of the microfluidic CE–FI system was evaluated under investigated conditions by using a standard solution containing 60.0 μg/ml TMP and SMZ. The analyte was repeatedly injected into the CE system every 1.5 min. The separation could be achieved within 2 min and sample throughput rate can reach up to 38 h^{-1}. The calculated repeatability values were 2.7%, 5.2% with peak area evaluation and 3.1%, 5.8% with peak height evaluation for TMP and SMZ, respectively.

3.5. Application

The practical applicability of the system was demonstrated through the separation and determination of the TMP–SMZ tablets in pharmaceutical preparations.

Four tablets of each commercial were weighed, powdered and the contents were mixed thoroughly. Then a quantity of the powder equivalent to one tenth of one tablet was extracted with 0.1 M HCl for 1.0 h in an ultrasonic bath, and the supernatant was filtered through a 0.45-μm syringe filter. The solution was diluted by certain fold, and then was directly injected into the CE equipment by the FI system. The typical electropherograms for TMP–SMZ extracts were shown in Fig. 4. The peaks were identified using the standard addition methods. The results for the determination of TMP and SMZ in pharmaceutical preparations, presented in Table 3, showed agreement between the claimed and found values. Recoveries (Table 4) of the spiked analytes for these samples were satisfactory.

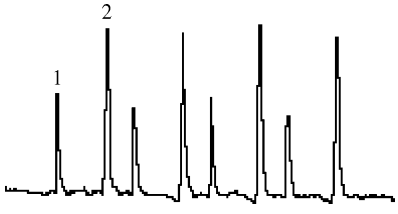

Fig. 4. Capillary electropherogram of TMP–SMZ tablets for four consecutive injections. Peaks: 1, TMP; 2, SMZ. Sample: extracts of TMP–SMZ tablets in 50 mM HCl. The other experimental conditions were as Fig. 3.

Table 3
Assay results for the TMP–SMZ tablets ($n = 4$)

Sample	Ingredient	Amount found (mg/tablet)	Label claim (mg/tablet)	Relative error (%)
TMP–SMZ	TMP	83.3	80.0	4.1
Tablets	SMZ	396.1	400.0	−1.0

Table 4
Recovery of the two analytes ($n = 4$)

Ingredient	Concentration spiked (μg/ml)	Concentration found (μg/ml)	Recovery (%)	Average (%)	RSD (%)
TMP	50.0	52.8	105.6		
	25.0	27.5	110.0	108.1	2.1
	12.5	13.6	108.8		
SMZ	50.0	45.9	91.8		
	25.0	23.9	95.6	92.3	3.3
	12.5	11.2	89.6		

4. Concluding remarks

The continuous on-line preconcentration approach based on dynamic pH junction using microfluidic CE–FI system was useful for the stacking of TMP and SMZ that displayed significant velocity differences in the sample and buffer matrixes selected. This technique further exploited the potential of on-line focusing methods in CE. The focusing technique is simple to perform, using conventional instrumentation without complicated procedures. The inherent simplicity and effectiveness of this focusing procedure makes it a facile way to enhance sensitivity in CE.

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