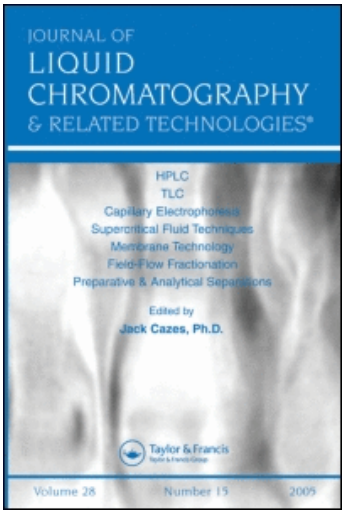

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Lei Tao[a]; Wenjie Zheng[a]; Fengsheng Zhao[a]
[a] School of Pharmacy, Key Laboratory of Microbial Metabolism, Ministry of Education, Shanghai Jiao Tong University, Shanghai, P. R. China

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Preparative Separation of Cephalosporin with Ion-Exchange Planar Electrochromatography

Lei Tao, Wenjie Zheng, and Fengsheng Zhao

School of Pharmacy, Key Laboratory of Microbial Metabolism, Ministry of Education, Shanghai Jiao Tong University, Shanghai, P. R. China

Abstract: A novel device for planar electrochromatography was developed, in which an electric field was applied across a thin layer of strongly acidic ion-exchange resin. Three types of cephalosporin – cefamezin, cefotaxime, and cefradine, at preparative amounts, were separated with different mobile phases and applied voltages. Experimental results showed that planar electrochromatography using 0.05 M alanine-acetate solution containing 7.0 M ethanol and 1% Triton X-100 under 200 or 400 V achieved good separation. Critical micelle concentration of Triton X-100 was measured to identify micelle formation in the buffer.

Keywords: Cephalosporin, Ion-exchange resin, Micelle, Planar electrochromatography, Preparative separation, Triton X-100

INTRODUCTION

Planar electrochromatography (PEC) is an efficient chromatography system, in which an electric field is applied across a layer of chromatography plates to get better separation of charged solutes in analytical or preparative modes. The initial application of planar chromatography in the presence of an electric field could be tracked back to the work of Martin and Synge et al. in the 1940s and 1950s.[1,2] The concept of planar

Correspondence: Prof. Fengsheng Zhao, School of Pharmacy, Shanghai Jiao Tong University, 800 Dong Chuan Road, Shanghai 200240, P. R. China. E-mail: fszhao@sjtu.edu.cn

electrochromatography was first mentioned by Pretorius et al. in 1974.[3] Their work focused on the electrically driven separations of both column chromatography and thin-layer chromatography (TLC). Since then, a great deal of work with a packed column at a high electric field has been made. However, there have been few reports on TLC utilizing an electric field in the past decades.[4] According to Tate and Dorsey,[5] "conventional TLC is still widely used as an analytic technique for its low cost and simple operation. Newer TLC methodologies requiring expensive instrumentation and computer automation may not be cost competitive." The statement explained the slow development of planar electrochromatography.

After an interval of nearly 20 years, the interest in the PEC technique was ignited again. In 1994, Pukl et al. studied the separation behavior of PEC on non-wetted thin-layers.[6] The selectivity of separation of non-ionic and ionic compounds showed evident enhancement. Poole and Wilson brought enlightenment to this technique by their report "Planar electrophoresis and electrochromatography: Time to revisit these techniques."[7] The investigators found the potential of PEC to perform efficient separations of charged compounds, as indicated by its distinct advantages: high solvent migration rate, significantly reduced elution time, and great improvement in band broadening.

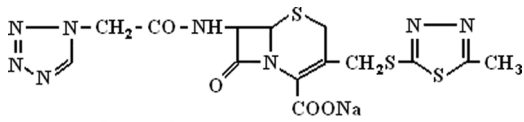
Many investigators made great contributions to the PEC progress by designing and developing new apparatus. Howard et al. carried out experiments in several development chambers especially constructed.[8,9] Nurok et al. designed several novel devices, which contained a vertical TLC plate or a horizontal development chamber, and invented a new pressurized PEC apparatus.[10–13] Dzido et al. applied a commercially available horizontal chamber to the planar electrochromatographic separation of a dye mixture on non-polar stationary phases.[14,15] Polak et al. used pressurized planar electrochromatography with chiral plates to separate the enantiomers of tryptophan and valine.[16] Tate and Dorsey built an instrument composed of three sections: PEC base, cover grid, and voltage divider box.[5,17] Berezkin et al. used a circular technique in the electroosmotic thin-layer chromatography.[18] Great work on the influence of PEC variables has been achieved, such as electric field, salt concentration in buffer, and mobile phase pH. The categories of available stationary phases have been investigated. Besides well known silica, aluminum oxide, cellulose, and polyamide were used to seek for better separation results.[19–21] Hydrophobic compounds were usually analyzed. Proteins and amino acids were also tested to broaden the application of this technique.[22,23]

Cephalosporins are important clinical β-lactam antibiotics. They have a β-lactam ring, a sulfurated six ring, a free carboxyl group, and an amide side chain. The cephalosporins used to investigate the separation effect in this paper were cefamezin, cefotaxime, and cefradine.

Among these cephalosporins, cefamezin only contains an acid group, but cefotaxime and cefradine are zwitterionic compounds. The chemical structure of cefamezin, cefotaxime, and cefradine was shown in Figure 1.

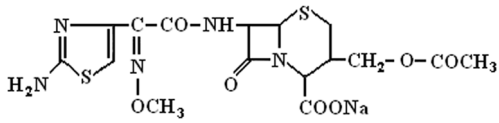
Up till now, the separation of cephalosporins was focused on the application of capillary electrophoresis. Andrási et al. used lyophilization as the pretreatment method and analysed 6 cephalosporins in bronchial secretion by capillary zone electrophoresis.[24] Lin et al. investigated the influences of buffer pH, buffer concentration, and buffer electrolyte on the migration behavior and separation of 12 cephalosporins in capillary zone electrophoresis.[25] Under optimum conditions, 12 cephalosporins could achieve complete separation. Mrestani et al. used capillary zone electrophoresis to determine the dissociation constants of cephalosporins, which relies on measuring ionic mobility of the solute as a function of pH.[26] On the other hand, Bhushan et al. separated cephalosporin antibiotics on thin layer plates impregnated with transition metal ions, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}.[27] New solvent systems were tried for the separation, which was composed of different ratios of propanol-H_2O-butanol or butanol-methanol-H_2O.

This paper designed a novel ion-exchange planar electrochromatography apparatus to obtain online detection results. In earlier reports, adsorption materials such as silica and alumina were mostly used. In this paper, ion-exchange resin was chosen, so that ionic or



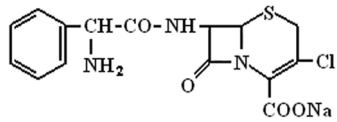
cefamezin

(a)



cefotaxime

(b)



cefradine

(c)

Figure 1. Chemical structure of (a) cefamezin, (b) cefotaxime, and (c) cefradine.

amphoteric compounds could be separated. Most reports focused on trace sample and analytical purpose. In this work, preparative amount of samples could be separated. The new method used aqueous buffer as mobile phase, it greatly reduced organic solvent consumed during the experimental procedure. It also prevented gases into the resin layer and had a better performance of dissipating Joule heat than column electrochromatography.

EXPERIMENTAL

Reagents and Apparatus

DL-Alanine, sodium acetate, ethanol, acetone, and Triton X-100 were of analytical grade from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Water was purified with Milli-Q Ultrapure Water Systems (Billerica, USA). Buffer was prepared as 0.05 M alanine-sodium acetate in purified water, and adjusted to pH 3.0 or 7.0. The buffer was then mixed with ethanol and Triton X-100 in the proper proportion as the mobile phase. Cefamezin, cefotaxime, and cefradine with 95% purity were purchased from Harbin Pharmaceutical Group Co., Ltd. (Harbin, China). DPH (1,6-diphenyl-1,3,5-hexatriene, Fluka, Buchs, Switzerland) was used as a probe molecule to measure critical micelle concentration in buffer. All other chemicals were purchased from local sources (Shanghai, China) at the highest purity available.

The experiments were carried out in a specially designed apparatus composed of four sections (shown in Figure 2): chromatographic planar chamber, electric power supply, UV detector, and fraction collector.

The chromatographic planar chamber was built by two pieces of $60 \times 300 \times 3$ mm glass. The bottom piece was rimmed by two narrow glass bars of $13 \times 300 \times 3$ mm and two thin rubber bars with the same

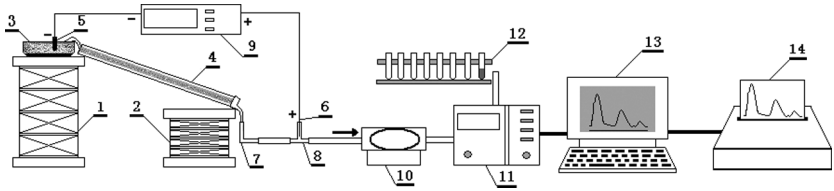


Figure 2. Apparatus for ion-exchange planar electrochromatography. (1, 2) platform, (3) eluent dish, (4) ion-exchange plate chamber, (5, 6) electrode, (7) collecting tube, (8) T-tube, (9) power supply, (10) peristaltic pump, (11) UV detector, (12) fraction collector, (13) computer, (14) printer.

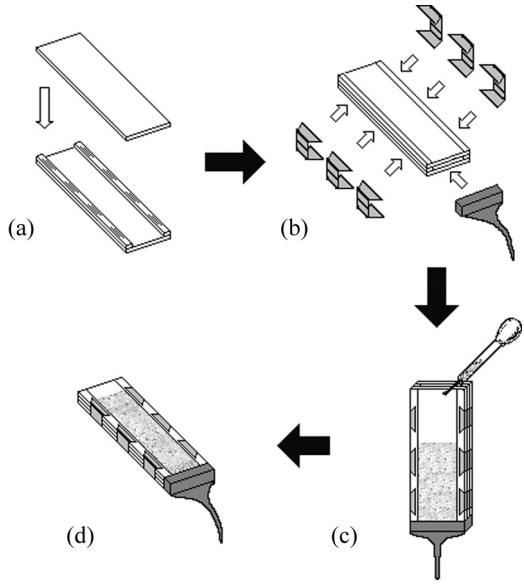


Figure 3. View of fitting process of the ion-exchange plate. (a) Two pieces of glass were held together, (b) clamps and cotton gauze were attached, (c) resin was loaded, (d) sandwiched plate chamber was made up.

length and width as the glass bars. These two pieces of glass were held together by six clamps in order to create a flat space for packing acidic ion-exchange resin. The resin was sandwiched between two pieces of glass (shown in Figure 3), but not filled to the top. A 1–2 cm space was left to contain only mobile phase, which could prevent liquid leaking from the resin to dry. The output end of the chamber was coated with a piece of cotton gauze to prevent the resin from leaking. The gauze was linked with a cotton rope, which introduced the eluent to the collecting tube.

The planar chamber containing acidic ion-exchange resin was shelved on two platforms and the slope could be adjusted by changing their heights: input end was higher and output end was lower. Mobile phase could flow from input end to another end driven by gravity. The flow rate of eluent was controlled by the height difference between two platforms, which may be different according to the flow rate for all separation experiments. The sample was injected by a syringe at the input of the chamber. One electrode cell was placed on the higher platform, which was filled with eluent. Eluent was delivered to the input end of planar chamber through a fibrous wick by capillary action. A T-tube served as the lower electrode cell linking the collecting tube. Platinum wires of Φ 0.2 mm were used as electrodes, which were connected to a DYY-4

electrophoresis power supply (Liuyi Analytical Instrument, Beijing, China). Gas generated by electrolysis near the electrodes was released through open space around the electrodes. The inner diameter of all tubes used was 6 mm. Eluent passed through a UV spectrophotometric detector (280 nm, Huxi Analytical Instrument, Shanghai, China) by a peristaltic pump (Pharmacia Fine Chemicals, Uppsala, Sweden). The peristaltic pump was not used to control the flow rate, but merely to transport the eluent from T-tube to UV detector for the online detection and fractional collection. The flow rate of the peristaltic pump should be adjusted to be the same as the flow rate of eluent in the planar chamber.

The data was obtained by the connection of the UV detector and computer. On the resin plate, the resin particles formed a dark background and the sample band could not be observed. So the samples should be eluted and detected one by one online.

Preparation of Strongly Acidic Ion-Exchange Resin

The strongly acidic ion-exchange resin was provided by Prof. Zhixiang Wang in Shanghai Institute of Pharmaceutical Industry. The resin was prepared by synthesis of styrene and divinyl benzene, which reacted in water (containing 0.5% polyvinyl alcohol). Divinyl benzene content was 2% of the total amount of styrene and divinyl benzene, in which 0.6% benzoperoxide was used as initiator. Reaction temperature changed as follows: 55°C (1 hr), 70°C (2 hr), 80–85°C (2 hr), 95–100°C (6 hr). Obtained particles were filtered and washed, then reacted with sulfuric acid in the presence of dichloromethane (as swelling agent). Temperature changed as: 80°C (2 hr), 95–100°C (2 hr), 120–125°C (2 hr). The acidic ion-exchange resin was washed and filtered. The ion exchange capacity was 0.685 mmol/mL bed volume.

Ion-Exchange Planar Electrochromatographic Separation of Cephalosporin

During the experiment, the apparatus was set up and the resin was equilibrated with acidic mobile phase (pH 3.0). The cephalosporin sample was prepared by mixing 100 μL of 10.0 mg/mL cefamezin, 100 μL of 10.0 mg/mL cefotaxime sodium, and 200 μL of 10.0 mg/mL cefradine. After injecting 400 μL of above sample to the upper end of the chamber, the power supply was turned on. The sample was then eluted with neutral mobile phase (pH 7.0) at a flow rate range of 0.6 – 1.0 mL/min. All experiments were performed with 200 V voltage and 10 mA current, unless otherwise stated. The current was kept constant by adding 1.0 M

NaCl frequently into the mobile phase. According to the data and image obtained online, the elution fractions were collected.

Cephalosporin Assay

Cephalosporin in eluted fractions was determined by an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, Delaware). The HPLC analysis was carried out with reversed phase ZORBAX SB C18 column (4.6 × 250 mm, 5 μm, Agilent). Mobile phase consisted of 0.01 M NaH_2PO_4-methanol (80:20, v/v), with flow rate 1.0 mL/min and UV detection at 280 nm. The column temperature was kept at 35°C.

In the electrochromatographic separation, the recovery of individual analyte was determined by the ratio of analyte amount obtained from its peak to amount of injected analyte. The purity of the individual analyte was determined from the ratio of analyte amount in its peak to total amount of all cephalosporins in the same peak.

Critical Micelle Concentration Measurement

The critical micelle concentration (CMC) of Triton X-100 in 0.05 M alanine-acetate buffer containing 7.0 M ethanol was measured by the DPH solubilization technique.[28] The above buffer was prepared as several samples, which contained different Triton X-100 concentrations from 0.1 mM to 2.0 mM. Each sample was taken for 5 mL, in which 0.5 μL of DPH methanol solution (0.4 mM) was added. Then the samples were stored in the dark for 5 hours. The absorbance of the samples was measured with a spectrophotometer (Lengguang Analytical Instrument, Shanghai, China) at 356 nm wavelength. A curve was drawn according to Triton X-100 concentration on the x-axis and absorbance on the y-axis. CMC was determined by the turning point of the curve.

RESULTS AND DISCUSSION

Principle of Zwitterionic Compounds Separation by Planar Electrochromatography

Zwitterionic compounds can act either as proton donors (acids) or proton acceptors (bases). Ion exchange is a common practice to separate zwitterionic compounds.

As shown in Figure 4, acidic ion-exchange resin was used as the separation media. The buffer system provided a gradient range of pH

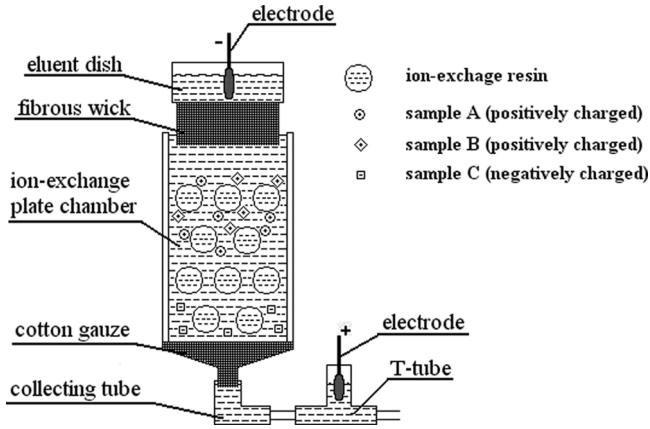


Figure 4. Principle of acidic ion-exchange planar electrochromatographic separation of amphoteric compounds.

3.0 to 7.0. The negative electrode was set in the inlet of plate chamber, and the positive one in the outlet. The resin was initially balanced with pH 3.0 buffer. The buffer pH value was near the pK_{a1} values of acid groups of zwitterionic cephalosporin samples, therefore, the sample molecules charged positively were adsorbed on the acidic resin or moved to the negative electrode direction. The buffer of pH 7.0 was gradually induced to the resin layer. When the pH value of mobile phase rose towards the pK_{a2} value of base group on one kind of compound, the charged state of this compound changed to be negative. The molecules of the compound would be eluted from the resin and moved to the outlet end. With the increase of the pH value, different compounds were eluted and collected according to the sequence of transferring to the state charged negatively. If there was no electric field, the eluted compound would be driven only by the flow of mobile phase. When the electric field was added, different samples were acted by the electric field force with different strengths, so the migration of the compounds became faster and the separation was more efficient.

In this paper, three cephalosporins were used as samples. It was reported that many cephalosporins possess a carboxylic acid group with pK_{a1} values in the range 1.5–3.1 and an amino group with pK_{a2} values in the range 6.8–7.4.[25] The pK_{a1} value of cefamezin is 2.5.[29] The pK_{a1} value of cefotaxime is 2.09.[26] The pK_{a1} and pK_{a2} values of cefradine are 2.63 and 7.30, respectively.[30] Because cefamezin has no basic group, it could not be adsorbed by acidic resin and was eluted at first. Cefotaxime and cefradine are zwitterionic compounds, which were eluted during the increasing buffer pH.

Effect of Ethanol Concentration and Triton X-100 Concentration in Buffer

Some conditions were investigated for their effects to the separation of cephalosporin mixture: ethanol concentration and Triton X-100 concentration in mobile buffer and applied voltage.

In the mobile phase, certain amounts of ethanol and Triton X-100 were necessary for effective separation of cephalosporin. Generally, ethanol exerts its effect mainly in these ways: changing polarity of buffer and swelling state of resin, and influencing interaction between ion-exchange resin and cephalosporin. The eluting power of eluent increases with ethanol concentration. However, as a result of resin swelling caused by ethanol, flow rate decreased while increasing ethanol concentration. Triton X-100 could form micelles in water, which could solubilize cephalosporin selectively, so the separation of cephalosporin samples improved.

When there was no ethanol in the buffer, no separated peak of cefotaxime could be observed, as shown in Figure 5a. With no Triton X-100 in the buffer, the cephalosporin mixture was partially separated in a very

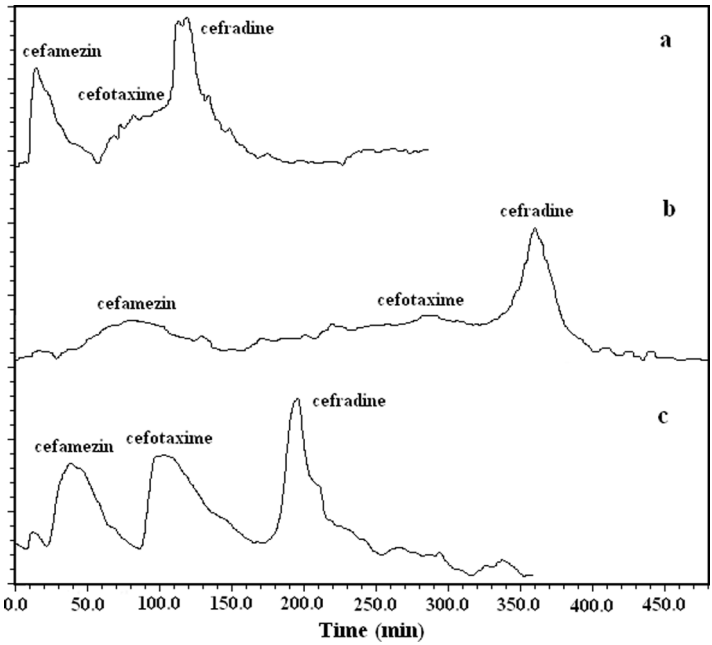


Figure 5. Planar electochromatographic separation of cefamezin, cefotaxime, and cefradine with or without ethanol and Triton X-100 in buffer. (a) 0 M ethanol, 1.0% Triton X-100; (b) 7.0 M ethanol, 0% Triton X-100; (c) 7.0 M ethanol, 1.0% Triton X-100.

long procedure and no evident cefotaxime peak was observed (Figure 5b). Raising ethanol concentration to 7.0 M and Triton X-100 concentration to 1.0%, the cephalosporin mixture achieved baseline separation and formed three definite peaks (Figure 5c).

However, the increased ethanol would prolong the retention time of peaks. With higher ethanol concentration (10.5 mol/L) in buffer, the peaks were broadened and flattened except for the peak of cefotaxime (data were not shown). It could be seen that at low Triton X-100 concentration the flow rate increased when Triton X-100 concentration increased. This suggested that moderate Triton X-100 concentration would accelerate the separation. While higher Triton X-100 concentration (2%) was used, it would delay the elution, because the viscosity of solution increased.

The recovery yield and peak purity of cephalosporin are listed in Table 1. It was indicated that suitable concentration of ethanol and Triton X-100 led to higher recovery yield and peak purity.

Effect of Applied Voltage

The experimental results shown in Figure 5 were obtained in the buffer containing 0.05 M alanine-acetate, 1% Triton X-100, and 7.0 M ethanol. It could be seen that ion-exchange chromatography without applied electric field was unable to separate cefamezin, cefotaxime, and cefradine (Figure 6a). An applied voltage of 400 V gave a much better separation (Figure 6b). However, under 600 V voltage the samples were eluted so fast that the peaks were confused (Figure 6c). The peak height increased

Table 1. Recovery yield and peak purity of cephalosporin with or without ethanol and Triton X-100 in buffer

Ethanol concentration (M)	Triton X-100 concentration (%, V/V)	Cephalosporin	Recovery yield (%)	Peak purity (%)
		Cefamezin	45.0	92.6
0	1.0	Cefotaxime	44.2	52.0
		Cefradine	73.9	65.6
		Cefamezin	6.0	64.4
7.0	0	Cefotaxime	20.0	39.2
		Cefradine	79.3	70.1
		Cefamezin	49.5	99.9
7.0	1.0	Cefotaxime	63.4	84.1
		Cefradine	78.0	81.4

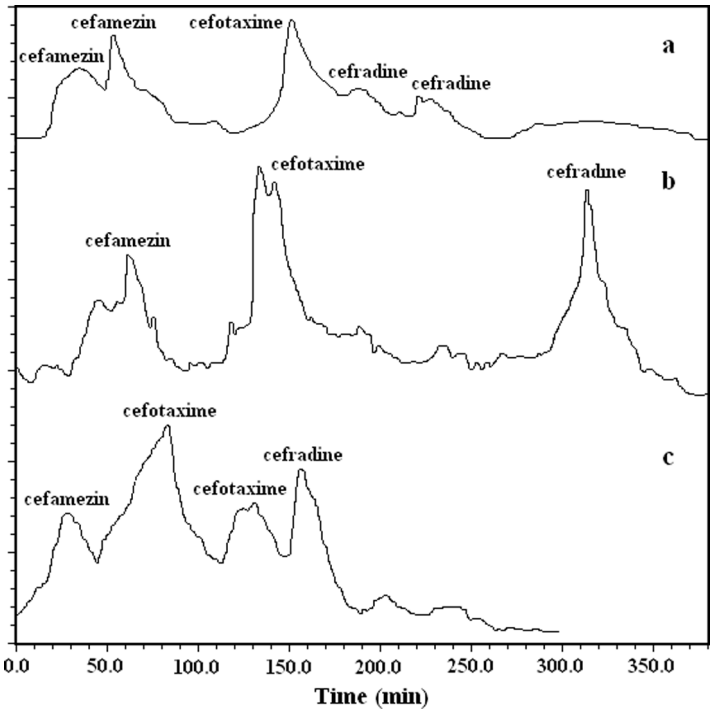


Figure 6. Planar electochromatographic separation of cefamezin, cefotaxime, and cefradine under different voltages. (a) 0 V, (b) 400 V, (c) 600 V.

with applied voltage. Recovery yield and peak purity are listed in Table 2. The recovery yield and peak purity was the highest under 400 V. Thereby, use of moderate electric field could maintain a good separation.

Critical Micelle Concentration Measurement

Of various methods used to measure critical micelle concentration (CMC) of Triton X-100, DPH solubilization technique appeared to offer greater precision in the presence of 0.05 M alanine-acetate and 7.0 M ethanol in buffer. At Triton X-100 concentration below CMC, DPH exists predominantly in an aqueous environment and exhibits low optical absorbance. When Triton X-100 concentration increases to CMC, formation of Triton X-100 micelles results in preferential partitioning of DPH into hydrophobic regions of micelles and sharp increase of absorbance.

As can be seen in Figure 7, the increase of absorbance with the concentration of Triton X-100 resulted in a sharp turn at 1.25 mM

Table 2. Recovery yield and peak purity of cephalosporin with variation of applied voltage

Applied voltage (V)	Cephalosporin	Recovery yield (%)	Peak purity (%)
	Cefamezin	29.0	79.3
0	Cefotaxime	48.8	76.1
	Cefradine	66.1	72.8
	Cefamezin	29.9	99.9
400	Cefotaxime	53.1	98.5
	Cefradine	83.4	97.2
	Cefamezin	35.5	99.9
600	Cefotaxime	44.8	82.5
	Cefradine	45.1	86.1

concentration, which indicated CMC. In the experiments of this paper, the concentration of Triton X-100 added in buffer was far beyond this value. These results confirmed that Triton X-100 might form micelles in buffer. It was suggested that various cephalosporin samples could distribute differently in micelles and get efficient separation. In aqueous solution, CMC of Triton X-100 is 0.24 mM, but in 7.0 M ethanol solution it becomes 1.25 mM, which means that ethanol interferes with the formation of micelles and raises the CMC of Triton X-100.

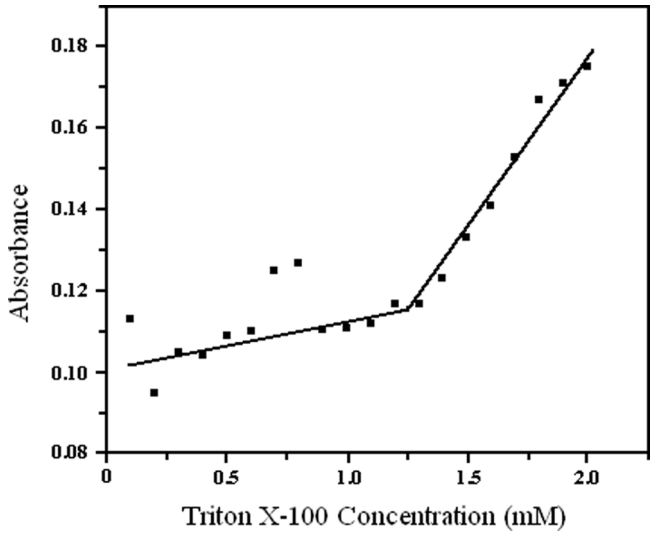


Figure 7. Variation of the absorbance of DPH at 356 nm as a function of Triton X-100 concentration in buffer of 0.05 M alanine-acetate and 7.0 M ethanol.

CONCLUSION

The specially designed apparatus stated in this paper could be applied to separate ionic compounds. It has some advantages such as the preparative amount of sample and effective heat elimination. The separation effect of planar electrochromatography was better than that of chromatography without the electric field; otherwise, the apparatus offered online detection and collection of samples. More work is currently underway to further develop the novel apparatus and to investigate the mechanism of electrochromatography with ion-exchange resin.

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