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A NEW APPROACH TO SEPARATE CEPHALOSPORIN MIXTURES USING ELECTROPHORETIC AFFINITY CHROMATOGRAPHY

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ABSTRACT

An integrated process strategy for the identification and separation of a mixture of five cephalosporins - cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine was developed, based on electrophoretic affinity chromatography (EAC) using a strong cation exchange stationary phase (SCX) under pseudo - affinity conditions enabling high efficiency chromatographic separations. The effect of pH, buffer concentration, and concentration of acetonitrile (ACN) and applied voltage on the resolution of the cephalosporin was examined. An improved result of electrochromatographic separation of cephalosporins was obtained upon introduction of adsorption on SCX, packed under pressurized flow conditions. With the optimized conditions recoveries were determined to be 80.19 - 87.75 % for cefazolin, 86.88 - 88.64 % for ceftaxime, 78.03 - 78.53 % for cefradine, 85.42 - 86.98 % for ceftriaxone, and 81.68 - 85.33 % for ceftazidine. By using the proposed strategy, five cephalosporins were well - separated within only 66.00 min. Preliminary experiments with five cephalosporins dissolved in a fermentation broth indicate that the antibiotic can be selectively extracted from the broth and recovered efficiently without serious loss of potency.

Key words: Cephalosporins, integrated downstream processing, electrophoretic affinity chromatography, mobile phase composition.

[I] INTRODUCTION

The growing interest in various β- lactam antibiotics over the past decade has called upon improvement of their production methods via modification of either the basic process or microbial stain or downstream processing techniques [10, 1]. Most antibiotics are produced by fermentation processes and the extraction of the product is challenging task since the broth contains several metabolites, proteins, amino acids, and salts, etc., along with the desired product which is usually a minor components in the whole broth. In industry, this is usually done in several stages and many of these steps are expensive and generate large quantities of waste water and other chemicals. In view of worldwide importance of antibiotics, it is essential that improvement in their process technologies be made, especially in downstream processing and antibiotic recovery. Recovery of β- lactams are important for both analytical and preparative purposes. Product recovery may involve various methods of extraction and purification and plays an important role in the overall process economics [15]. However, almost all the known processes for commercial scale extraction and purification are based on low-yield operation because of the unfavorable physical properties of the β-lactams, particularly the cephalosporins. While the less hydrophilic penicillin's can be recovered by well known solvent extraction technique, difficulties arise in the case of

cephalosporin's which are of highly hydrophilic possessing zwitterionic properties and have very small differences in polarity.

Methods of analysis of cephalosporin antibiotics include microbiological, titrimetric and chromatographic assays. Literature reveals that cephalosporins were separated by various methods such as liquid chromatography [18,2,5,6,7,16 & 19], and capillary electrophoresis [12,9,3,8,17,21,4], which have been used extensively for the separation and assay of cephalosporins in formulation and biological samples.

In addition to the continuous efforts in improving existing methods to meet the demand of high selectivity and good versatility, study on novel ways to increase separation speed has attracted growing attention. One way is to develop novel chromatography media of superior mass transfer characteristics [22, 14]. An alternative approach to accelerate separation speed is to operate the chromatography in an electric field, as shown by capillary electrochromatography (CEC). However, this method is basically designed for analytical purposes and is not suitable for large scale quantitative separations.

In the present study we attempted the new combination of electrophoresis with affinity chromatography, namely electrophoretic affinity chromatography (EAC). Here we present a study of EAC for lab-preparative separation using commercial preparation of five cephalosporins as a model system in a specially designed glass column and influence of pH, buffer composition and the electric field strength on the adsorption and desorption in order to establish a comprehensive understanding of the process characteristics of the electrophoretic affinity chromatography. In this work we explore the possibility of using EAC for the separation of five cephalosporins dissolved in a fermentation broth. The aim of this work was to investigate if a EAC system can be developed as a method for simple separation of cephalosporins from the fermentation broth.

[II] MATERIAL AND METHODS

The antibiotics, cephalosporins namely cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine were purchased from the shanghai xinya pharmaceutical co, shanghai, China. Double distilled water was used. All other chemicals were of analytical grade.

2.1 Instrumentation

The apparatus for EAC used in this experiment was composed of a peristaltic pump (P-1, Pharmacia fine chemicals, Sweden), a manually designed glass chromatography column-fabricated by shanghai based analytical company (as seen in figure: 1), a UV - detector (HD-2, Shanghai analytical company), a high voltage power supply (DYY- 4, Beijing, China) and a laboratory built reservoir equipped with a platinum electrode. The reservoirs were set at the inlet

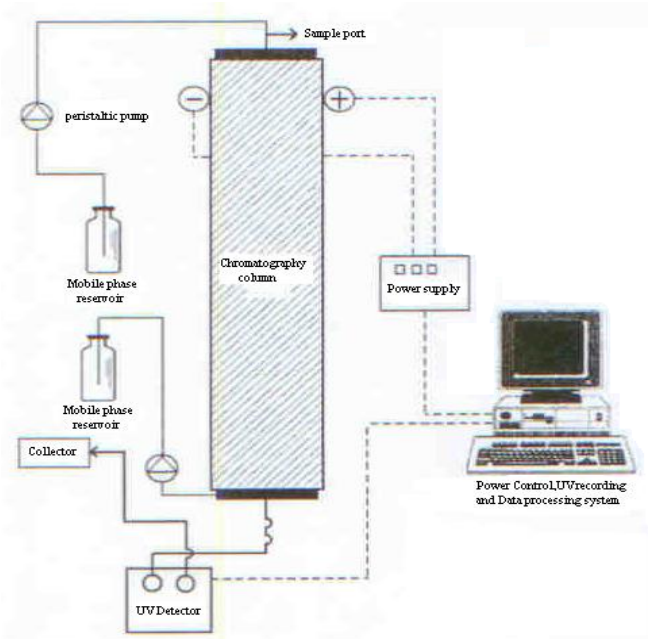

Fig: 1 Experimental system of electrophoretic affinity chromatography. (Insert) photograph of a specially designed and fabricated glass chromatography column with a length of (250 x 50mm I.D) has been used.

and outlet end of the column. In this apparatus, sample port was at the inlet end of the column. Therefore, high voltage was applied at both the reservoirs of the column. The specially designed chromatography column of (250 x 50 mm I.D) was packed with 50 – 70 µm strong cation exchange (SCX) stationary phase (East China University of Science and Technology, shanghai, china). Packing of column was carried by applying 1000 psi. The temperature of the column was maintained at 35^{o}C. The applied voltage was 400 volts and the current level was at 5.5 Amps. Detection of samples was performed at a wavelength of 254 nm. All analytical experiments were carried out using HPLC (Agilent Technologies; column C8, 4.6x150 mm Agilent Zorbax, Palo, CA, USA; mobile phase consisting of 0.05M, pH= 6.0, NaH_2PO_4- 30% methanol, flow rate1ml/min).

2.2 Procedure

The buffers containing ammonium acetate/acetic acid were adjusted to the desired pH with either 0.1M NaOH or 30% HCl. After the pH was adjusted, the buffer and acetonitrile were mixed. All the buffer solutions were filtered through 0.45 µm filter and degassed by ultrasonication for approximately 10 min before they were transferred to the inlet/outlet vials. Five standard cephalosporins, cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine, were dissolved in methanol at concentrations of each 1mg/ml, respectively, degassed in an ultrasonic bath and filtered through a 0.45 µm membrane filter. Equilibration of a packed SCX column is required for successful operation. The column was washed with buffer containing ammonium acetate/acetic acid (20 mM each, pH 5.0) with 50% ACN under an electric field and waited until the current and detector baselines were stable. The column was conditioned with every new run buffer of chromatography for atleast 30 min before any samples were injected. Likewise the mixture of cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine at 1.0 mg/ml each, were applied to the column for adsorption for one hour in the absence of electric field and during which flow rate of inlet/outlet buffer tanks were blocked in total, whole experimental setup was kept undisturbed for one hour. Therefore, elution buffer containing 20 mM ammonium acetate/ 20mM acetic acid/0.2 M NaCl, pH 6.0 and 50 % ACN was introduced at a flow rate of 2ml/min in the presence of electric field and products from the column outlet were collected after their detection by N3000 chemstation software.

2.3 Preparation of fermentation broth

Saccharomyces cerevisiae was grown in a culture medium consisting of yeast extract (2%), peptone (1.5 %), and glucose (5 %). After 24 h of growth in a shake flask at 200 rpm and 30 °C, the cells were centrifuged at 15000 rpm. The supernatant was used as the broth for further studies.

Assay of cephalosporin

The antibiotic assay was performed by standard bacteriological diffusion discs for cephalosporin using *Bacillus anthracis* as per the instructions given in [20]. Measurement of the inhibition zones indicated the potency of the antibiotic.

[III] RESULTS AND DISCUSSION

3.1 Choice of electrolyte solutions

a) Effect of pH

In order to find the suitable conditions for the separation of five cephalosporins, a series of experiments was conducted at different pH and buffer compositions. By comparison, ammonium acetate/acetic acid buffer was proven to be better, however it was necessary to optimize the ammonium acetate/acetic acid buffer concentration and pH.

The ammonium acetate/ acetic acid buffer systems with different pH values were tested. It was found at higher (> 7.0) or at lower pH (< 4.5), the peaks could not be well separated.

Therefore we have selected the buffer pH value of 6.0.

b) Effect of buffer concentrations

To optimize the method, the concentrations of ammonium acetate/acetic acid were changed from 10 mM to 50 mM. The result showed that at a lower concentration of 10 mM, the peaks were partially overlapped, while at higher concentration of 50 mM, the system became unstable. A reasonable good separation of all five cephalosporins was obtained using 20 mM ammonium acetate/ 20 mM acetic acid at a shorter runtime [Fig: 2] due to optimal ionic strength, and thereby high electrophoretic mobility and lower current, which decreased from 10.0 Amp (50 mM ammonium acetate/ acetic acid) to 2.0 Amp (10 mM ammonium acetate/acetic acid)

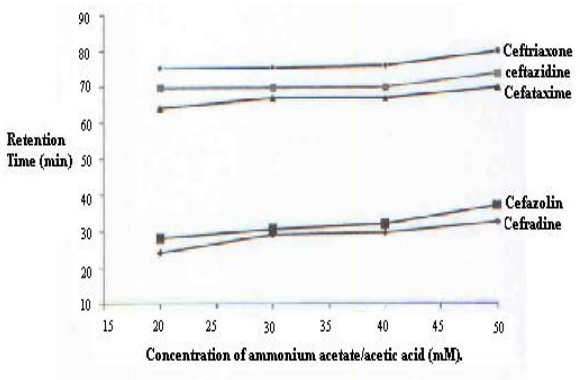

Fig: 2 Effect of ammonium acetate/ acetic acid concentration in the buffer. Conditions: mobile phase, ammonium acetate/acetic acid (pH 6.0) with 50 % ACN.

c) Effect of ACN concentrations

Addition of organic modifiers can modify the electro osmotic flow (EOF) greatly and influence the resolution. Several reports [13] indicate that ACN is often used as an organic modifier in electro chromatography. On the other hand, the addition of ACN to the electrolyte can enhance the solubility of the analytes studied. After a series of experiments with different concentrations of ACN from 20 to 60 % were tested. It was indicated that at lower concentration of ACN (< 20 %) the peaks tailed, whereas at higher concentration of ACN (> 60 %) the peaks became sharp, therefore the concentration of ACN (50 %) was demonstrated to be optimum. Higher concentration of ACN shortened the analysis time, enhanced the resolution and sharpened peaks. An increase in the ACN concentration reduces the retention of the analytes, because the EOF inversely depends on ionic strength and viscosity. The retention times of five cephalosporins decreases from 28.297, 33.230, 75.207, 83.747 and 89.860 min to 26.233, 30.292, 66.824, 66.249 and 69.111 min, respectively, when ACN concentration was increased from 30 % to 50 % in electrolyte (20 mM ammonium acetate/ acetic acid pH 6.0) at 300 V. In addition, increasing the amount of ACN in the electrolyte also made the solvent stronger, and thus the reversed - phase mechanism would dominate the separation. The peak sequence of the cephalosporins in electro chromatography is interpreted as that obtained by HPLC. However, a very highly concentrated addition of ACN (> 60 %) cannot be selected. The volatization of ACN can make the electrolyte non reproducible in this experiment, especially for the electrolyte with much higher ACN concentration at 35 ºC Column temperature. Therefore the concentration of ACN of 50 % was adopted. The ACN volatization may even cause the formation of bubbles, which can result in the current breakdown during the experimental work.

d) Effect of applied voltage

The influence of the applied voltage on the resolution of the analytes was studied. The EOF velocity in a designed column is most likely smaller compared with that in an open chromatography tube because of the tortuosity and porosity of the packed bed [11] thus we can increase the applied voltage to increase the flow rate. Our studies showed that an increase in the

voltage resulted in reductions in elution times while the resolutions hardly changed [Fig: 3]. After a series of comparative experiments with voltages from 100 – 400 V, 400 V at 35 °C was proved to be the best. Voltage was therefore not increased continually for the designed apparatus limitation and electro chromatography column maintenance requirements.

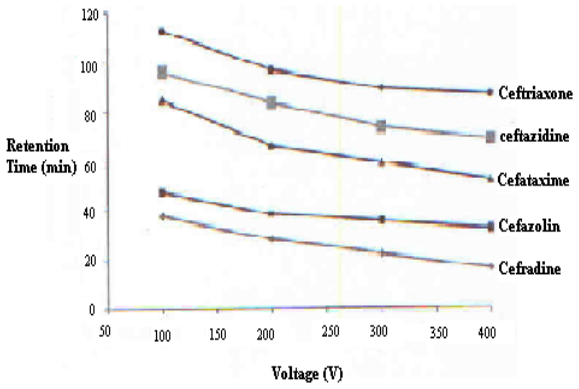

Fig: 3 Effect of voltage. Conditions: mobile phase, 20mM ammonium acetate/acetic acid, pH 6.0 with 50 % ACN.

3.2 Final optimization

Detection was performed at a wavelength of maximum UV absorption of the five cephalosporins at 254 nm. Detection at 254 nm showed best signal-to-noise ratio and lower interface. Finally a electrolyte consisting of 20 mM ammonium acetate/ acetic acid, pH 6.0 with 50 % ACN under conditions of 400 V applied voltage, 35 °C column temperature, 254 nm UV detection was found to produce the best resolution. [Fig: 4] is the electropherogram showing the separation of the mixture of cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine with the migration time of 24.733 min for cefradine, 26.527 min for cefazolin, 60.694 min for cefataxime, 62.136 min for ceftazidine, and 65.428 min for ceftriaxone, respectively. The five cephalosporins could be separated with in 66.00 min with acceptable resolution, when compared to the experiment that was carried with unpressurized column packing and without adsorption of solutes for one hour on SCX. The total run time for resolution was 8 hours and each peak lasted for more than one hour as seen in [Fig: 4 (Insert-B)]. Thus the result showed that the developed

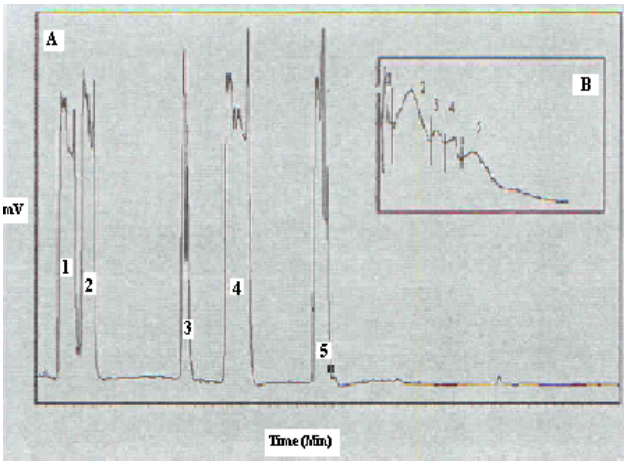

Fig:4 Electophoretic pattern of five cephalosporin fractions obtained with the electrophoretic affinity chromatography (EAC) which was performed on the chemstation software N3000; (a) with pressurized column packing and adsorption on SCX for one hour, (Insert –B) un pressurized column packing and without adsorption on SCX.

Peaks: 1, cefradine; 2, cefazolin; 3, cefataxime; 4, ceftazidine; 5, ceftriaxone.

method is a simple and rapid method for the separation and determination of cephalosporins.

3.3 Quantitative analysis

a) Calibration curves for cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine:

Calibration curves (peak area ratio y, vs. concentration x, $mg.ml^{-1}$) were constructed in the range of 0.1 mg/ml - 1.5 mg/ml for all five cephalosporins.

The regression equations of these curves and the correlation coefficients were calculated as follows:

Cefazolin, y= 21516x - 166.990 (r = 0.9998, n = 7) ;
Cefataxime, y = 24821x - 56.149 (r = 0.9995, n = 7) ;
Cefradine, y= 14017x - 105.500 (r = 0.9994, n = 7) ;
Ceftriaxone, y = 32741x - 70.149 (r = 0.9994, n = 7) ;
Ceftazidine, y = 29348x - 109.080 (r = 0.9997, n = 7) ;

b) System suitability test

The reproducibility (relative standard deviation) of this proposed model, on the basis of peak area ratios for 3 replicate experiments for, cefataxime, cefazolin, cefradine, ceftriaxone, and ceftazidine were shown in [Table: 1]. The ranges of recovery were 80.19 - 87.75 % for cefazolin, 86.88 - 88.64 % for cefataxime, 78.03 - 78.53 % for cefradine, 85.42 - 86.98 % for ceftriaxone, and 81.68 – 85.33 % for ceftazidine.

Compoud	Added (mg)	Found (mg)	Recovery (%)	RSD (%)
	1.0176	0.8930	87.95%	1.95
Cefazolin	0.6878	0.5516	80.19%	3.42
	1.1114	0.9253	83.25%	3.96
	0.2925	0.2581	88.25%	0.98
Cefataxime	1.1430	0.9930	86.88%	3.29
	0.6565	0.5820	88.64%	3.46
	1.0839	0.8600	79.34%	1.76
Cefradine	0.7106	0.5581	78.53%	1.94
	0.4176	0.3259	78.03%	3.44
	0.5593	0.4829	86.34%	2.94
Ceftriaxone	1.0488	0.9123	86.98%	1.01
	0.7632	0.6520	85.42%	1.94
	0.6753	0.5516	81.68%	3.44
Ceftazidine	0.1661	0.1418	85.33%	1.91
	0.6673	0.5523	82.70%	3.28

Table: 1 Recoveries of cefataxime, cefazolin, cefradine, ceftriaxone & ceftazidine (n = 5)

3.4 Extraction and purification of cephalosporins from fermentation broth:

On the basis of the above results, the principle of chromatographic separation by EAC was successfully applied to the extraction and purification of cephalosporins from a fermentation broth of baker's yeast (*Saccharomyces cerevisiae)* to which commercially available cephalosporin mixture of cefataxime, cefazolin, ceftriaxone, and ceftazidine was added at 1mg/ml. This broth was chosen as a case study since it satisfies the criteria of possessing all the standard ingredients of a fermentation broth of an antibiotic producing organism (although the exact composition would differ from case to case). After adjusting the pH to 6.0, the broth extracted with acetate buffer ([ammonium acetate/ acetic acid]), 10 - 50 mM; [ACN], 20-60 %; [NaCl], 0.05-1M). HPLC analysis at 220 and 254 nm showed that unwanted impurities were also extracted at low salt and high acetate concentrations ([NaCl], 0.2M; [ammonium acetate/ acetic acid], 20mM, purity of cephalosporins 70-75 %). At high salt concentrations (≥ 0.5 M) and low acetate concentrations (≤ 15 mM), the level of impurities decreased to < 5 %. Best results were obtained with 0.5 M NaCl and 15 mM acetate (extraction efficiency ≥ 80 %, product purity > 95 %). The biological activity of recovered cephalosporins was found to be 70 – 75 % of the starting material [20].

[IV] CONCLUSIONS

Electrophoretic affinity chromatography is a new combination of electrophoresis with affinity chromatography which is designed for small scale separations. In the present study, the effect of adsorption onto the SCX and influence of electric field strength on resolution of five cephalosporins was experimentally evaluated, in which the introduction of adsorption and an electric field led to the changes, including enhanced selectivity in the separation mechanism compared to that in the absence of electric field.

The work presented demonstrates that the five cephalosporins can be separated in a relatively short time (< 70.00 min) by EAC, the method developed shows satisfactory and retention time repeatability. It is also indeed possible to use EAC for selective extraction and purification of antibiotics from fermentation broths. The selectivity and efficiency of extraction can be maximized by simple operation such as changing the pH, electrolyte concentration, and the buffer concentration. We propose that the analysis time be reduced by shortening the column and applying programmed voltage if EAC systems can provide higher voltage. With this strategy it is possible to separate and purify the closely related

cephalosporins by greatly reducing the number of steps in the downstream processing. This process can also be easily implemented on a large scale upon further optimization and this methodology can be a promising alternative industrial technique in near future.

[V] ACKNOWLEDGEMENT

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