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LIQUID CHROMATOGRAPHY OF CEPHALOSPORIN C AND α-AMINO ACID MIXTURES ON POLYFUNCTIONAL POLYSTYRENE RESINS

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SUMMARY

The industrial separation of cephalosporin C from fermentation broths is usually achieved by liquid chromatography using ion-exchange resins, or adsorption on active carbon or neutral macroreticular sorbents. Unfortunately, the efficiency is low because of the low capacity or/and the poor selectivity of the resins used. Some new sorbents were synthesized by coupling three polyfunctional ligands (ε-L-lysine, glycyl-ε-L-lysine and diglycyl-ε-L-lysine) on divinylbenzene-cross-linked polystyrene resins, and tested with an artificial aqueous mixture of cephalosporin C and α-amino acids. These stationary phases exhibit a selective affinity towards the antibiotic compared with the α-amino acids. In order to elucidate the mechanism of this adsorption, the influence of the ionic strength of the eluent on the retention of the different compounds used was investigated. This preliminary study allowed us to define optimal conditions for subsequent application to the preparative separation of cephalosporin C.

INTRODUCTION

Cephalosporin C (Fig. 1), a β-lactam compound prepared by fermentation[1], is the starting material for various antibiotics characterized by a wide bactericidal spectrum, particularly against gram-negative bacteria[2].

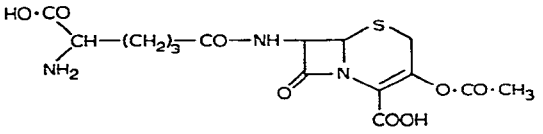

Fig. 1. Cephalosporin C.

After its biosynthesis, cephalosporin C is contained in a broth, mixed with many α-amino acids and other products, and is recovered using ion-exchange resins[3], active carbon[4], neutral macroreticular adsorbents[5–8] or, more frequently, a combination of these. Its isolation consists first in using an adsorption process on a macro-

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porous unfunctionalized divinylbenzene-cross-linked polystyrene resin (Amberlite XAD), followed by an anion-exchange separation[6]. However, for the first operation the pH of the filtered fermentation broth must be adjusted to 3, and even then the adsorption is not very selective and the resins exhibit a low loading capacity (2 g of cephalosporin C per litre of resin[5]).

This paper describes the results of investigations carried out on the retention and separation of aqueous cephalosporin C and α-amino acid mixtures on three polyfunctional polystyrene stationary phases. The first adsorbent was prepared by coupling the ε-amino group of L-lysine on a previously activated divinylbenzene-cross-linked polystyrene; hydrophilic spacer arms consisting of glycyl and glycyl-glycyl residues were inserted into the two other adsorbents. The structures of these resins are shown in Fig. 2. The chromatographic retention of α-amino acids and cephalosporin C on these phases as a function of the ionic strength of the eluent was studied.

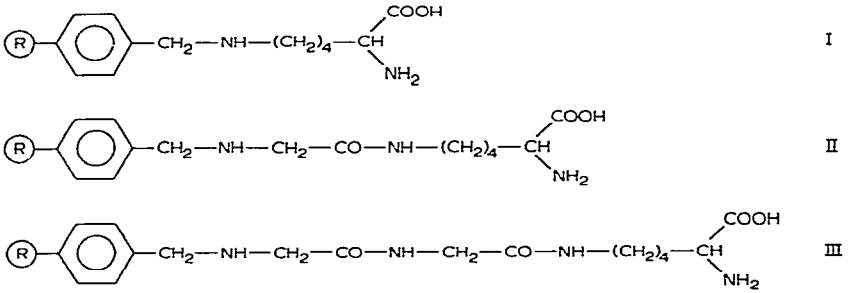

Fig. 2. Structures of the three sorbents; divinylbenzene-cross-linked polystyrene (R) with ε-L-lysine (I), glycyl-ε-L-lysine (II) and diglycyl-ε-L-lysine (III).

This preliminary investigation allowed us to define the optimal conditions for the separation of the antibiotic on the three adsorbents and to establish their potential in the industrial isolation of cephalosporin C.

EXPERIMENTAL

Reagents

α-Amino acids were obtained from Fluka (Buchs, Switzerland). Cephalosporin C was kindly provided by Roussel-Uclaf (Paris, France). The divinylbenzene-cross-linked polystyrene (Bio-Beads SX-1; 2% divinylbenzene; particle size 200–400 mesh) was purchased from Bio-Rad Labs. (Richmond, CA. U.S.A.).

Synthesis of the polyfunctional sorbents

The sorbents were synthesized by the procedure of Sacco and Dellacherie[9], by coupling the ε-amino group of L-lysine either directly on the previously activated polystyrene matrix or after the introduction of a hydrophilic spacer arm (glycyl or glycylglycyl). According to elemental analysis and potentiometric titration, the chemical capacities were 0.6–0.8 mmol (sorbent I) and 0.4–0.6 mmol (sorbents II and III) of fixed lysine per gram of dry resin (Table I). Table I gives also the swelling capacities in pure water.

TABLE I

PROPERTIES OF THE POLYSTYRENE SORBENTS

*Property**	*Sorbent*		
	I	*II*	*III*
Capacity in lysine sites (mequiv./g)	0.6–0.8	0.4–0.6	0.4–0.6
Swelling capacity in water	0.23	0.29	0.33

* The capacity in lysine sites is given in mequiv. per gram of dry resin; the swelling capacity is determined by the ratio of the weight of solvent to the weight of dry resin.

Procedures

Stainless-steel tubing (0.95 cm I.D.) and fritted end fittings were purchased from Waters Assoc. (Milford, MA, U.S.A.). Columns were 30 cm long and were packed with the sorbents in the conventional manner by preparing a slurry in water and forcing it into the column at 2000 p.s.i. The column was then connected to the chromatographic apparatus and equilibrated with the elution solvent. A Waters Model ALC 200 liquid chromatograph, equipped with an M-6000 A pump, an M-440 UV detector with a 12.5-μl flow cell, an R.401 differential refractometer and an U 6K sample injector fitted with a 2-ml sample loop, was used. Cephalosporin C was detected at 254 nm at sensitivities of 1–2 absorbance units full-scale. Cephalosporin C standards and sample solutions were injected in a volume of 20–150 μl with a Hamilton syringe.

Capacity factors were calculated using the equation

$$k' = \frac{V_R - V_0}{V_0}$$

where V_R is the elution volume for a chromatographic peak and V_0 the column void volume.

RESULTS AND DISCUSSION

It is known[10] that Amberlite XAD polystyrene sorbents are able to separate α-amino acids ($\overset{+}{N}H_3$–$\underset{R}{\underset{|}{C}}$H–$COO^-$) in water and that the hydrophobic nature of the R portion is the prime cause of the selectivity. Accordingly, the dicarboxylic α-amino acids aspartic acid and glutamic acid are retained less in water than, for example, glycine or alanine. By combining some weak ionic interactions with this hydrophobic adsorption process, the selectivity of the separation should be materially improved. Thus, by using a microbonded propylamine silica column, Miller and Neuss[11] succeeded in fractionating cephalosporin C from a fermentation broth, but by means of mixed eluents consisting of organic solvents and water.

The adsorbents that we have synthesized (Fig. 2) have both hydrophobic and

ionic properties, but all the chromatographic experiments described below were performed merely with aqueous eluents of different ionic strengths. Under these conditions, α-amino acids and cephalosporin C are fully ionized. For neutral α-amino acids these conditions correspond to the isoelectric point pH, whereas trifunctional compounds such as aspartic acid, glutamic acid, lysine and cephalosporin C are not electrically neutral in water and will be able to interact with ionic sites of the stationary phases. All of the ionogenic functions of the resins are also dissociated under the conditions used.

Physico-chemical properties of the columns

The chromatographic features of the columns packed with sorbents I, II and III were determined by means of standard polyethylene glycols (PEGs) with molecular weights between 200 and 2000 (Table II). From these data, it can be shown that above a molecular weight of 1500 the elution volume of the PEG sample is constant whatever the sorbent, and this value (6.3 ml) was considered to be the void volume of the columns.

TABLE II

CALIBRATION OF THE DIFFERENT COLUMNS

Length = 30 cm; I.D. = 0.95 cm; injection volume = 20–40 μl; concentration = $6 \cdot 10^{-2}$ *M*. Elution with water at a flow-rate of 1 ml/min.

Sorbent	$V_{e(PEG)}$ *(ml)*					
	200	*300*	*400*	*1000*	*1500*	*2000*
I	9.5	8.8	8.2	6.4	6.3	6.3
II	9.4	8.1	6.7	6.3	6.3	6.3
III	9.3	8	6.6	6.3	6.3	6.3

Isocratic elution in water and aqueous saline solutions

Several α-amino acids were injected separately onto stationary phases I, II and III and eluted under an isocratic flow with water and sodium chloride solutions of various concentrations.

L-Lysine, which has a positive net charge in water, was strongly excluded by the resins (repulsive electrostatic interaction) and was always eluted in the void volume ($k' = 0$).

As shown in Fig. 3, the effect of the sodium chloride concentration on the capacity factors of the solutes tested depends both on the nature of the R group and the structure of the adsorbent. The retention of neutral α-amino acids (alanine, phenylalanine, methionine) is not influenced by the ionic strength of the eluent, which suggests that hydrophobic or π-binding interactions are the main factor in the adsorption process. On the other hand, the k' values of these compounds are higher in all instances for sorbent I than for II and III. It is likely that in the two last supports, the hydrophilic spacer arms shield the aromatic and lipophilic backbone of the adsorbents to some extent, resulting in a decrease in the hydrophobic or π-binding interactions. On the other hand, the dicarboxylic α-amino acids (aspartic and glutam-

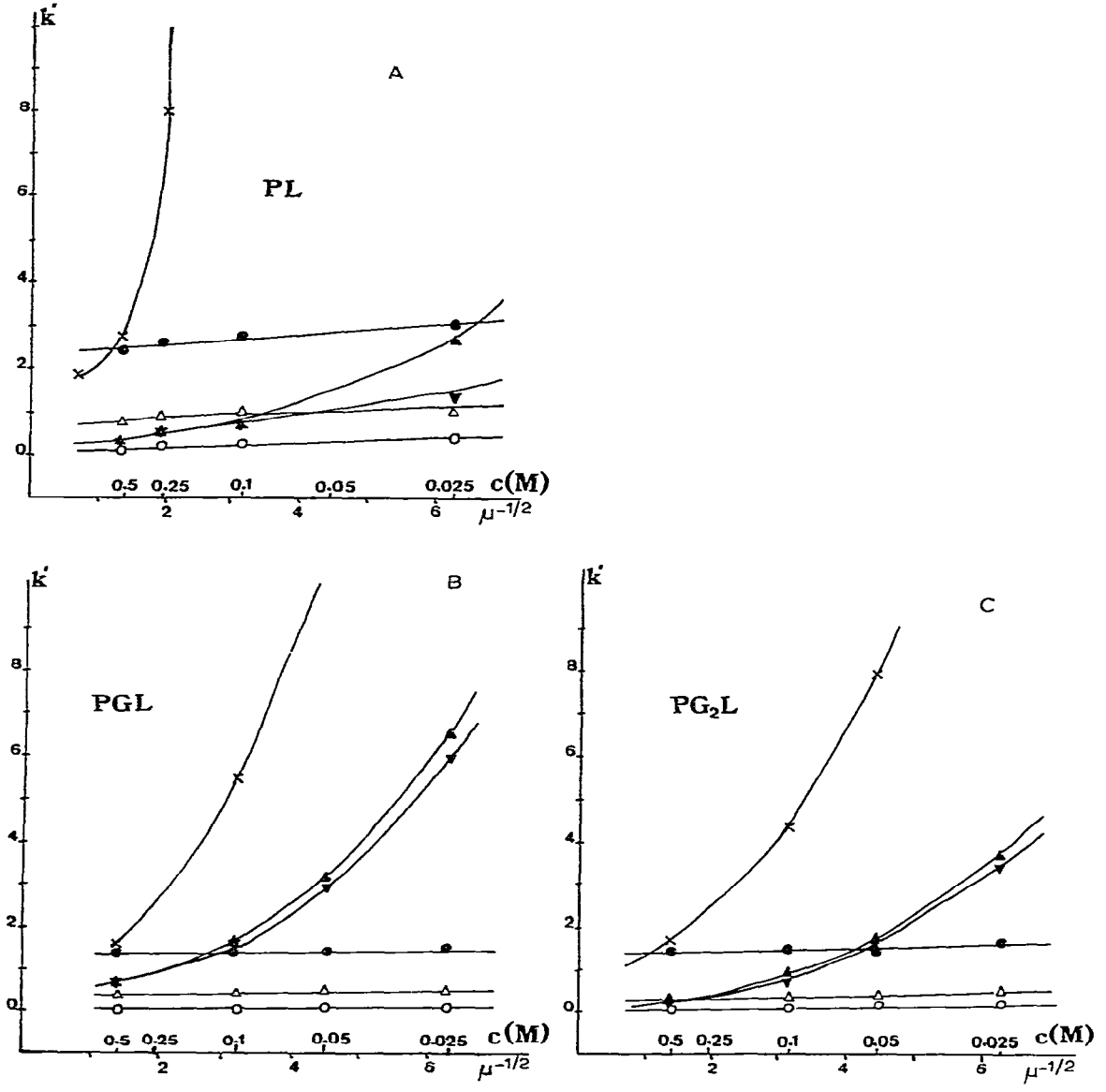

Fig. 3. Influence of the ionic strength (μ) of the eluents on the capacity factors of cephalosporin C and α-amino acids. A, sorbent I; B, sorbent II; C, sorbent III. Successive injections of 20–50 μl; concentration of the solutions, $6 \cdot 10^{-2}$ M; flow-rate, 1 ml/min; room temperature; $c(M)$ = molarity of NaCl solution. ○, L-Alanine; △, L-methionine; ●, L-phenylalanine; ▲, L-glutamic acid; ▲, L-aspartic acid; ×, cephalosporin C.

ic acids) are strongly adsorbed in water ($k' = \infty$), whatever the stationary phase, probably because of an ionic interaction involving the side-chain carboxyl function of the solutes and the secondary amine attached to the different resins. This effect is also observed at low sodium chloride concentrations and especially with sorbent II (Fig. 3B).

Cephalosporin C is retained much more strongly on all three sorbents than any other α-amino acids provided that the sodium chloride concentration remains below 0.5 *M*. Its adsorption increases as the ionic strength decreases and the fixation becomes quasi-irreversible when the salt concentration reaches 0.025 *M*.

The enhanced adsorption observed upon passing from simple α-amino acids to cephalosporin C must be attributable to the combination of several interactions. Obviously, the ionic interactions play a major role in the retention of the antibiotic but hydrophobic and π-binding interactions must be also involved in the retardation phenomenon. This is demonstrated by the fact that, on sorbent I, which has the most hydrophobic and aromatic character, the capacity factor of cephalosporin C is as high as 2.75 in 0.5 *M* sodium chloride solution, whereas glutamic and aspartic acids are almost excluded from the column ($k' = 0.25$).

Separation of cephalosporin C from α-amino acid mixtures

The practical application of the above observations was demonstrated in fractionation experiments in which artificial mixtures consisting of several α-amino acids and cephalosporin C were applied to a column filled with sorbent I. A chromatogram obtained on using isocratic elution with 0.25 *M* sodium chloride solution is shown in Fig. 4. The antibiotic was well separated from all of the α-amino acids tested but the elution peak was wide, and consequently the concentration of cephalosporin C in the corresponding fractions was low. In another experiment (Fig. 5), the α-amino acids were first eluted using an eluent of low ionic strength (0.025 *M*, sodium chloride solution), then cephalosporin C was rapidly desorbed by increasing the sodium chloride concentration to 1 *M* and collected in well separated fractions.

Similar experiments were performed using sorbents II and III. Because of the hydrophilic spacer arms attached to the resins, the fixation of the antibiotic was less strong than with sorbent I. Thus, as shown in Fig. 6, cephalosporin C can be salted out by elution with a low salt concentration (0.1 *M* for sorbent III) or desorbed in a reduced volume using a higher sodium chloride concentration (for example, 0.25 *M*).

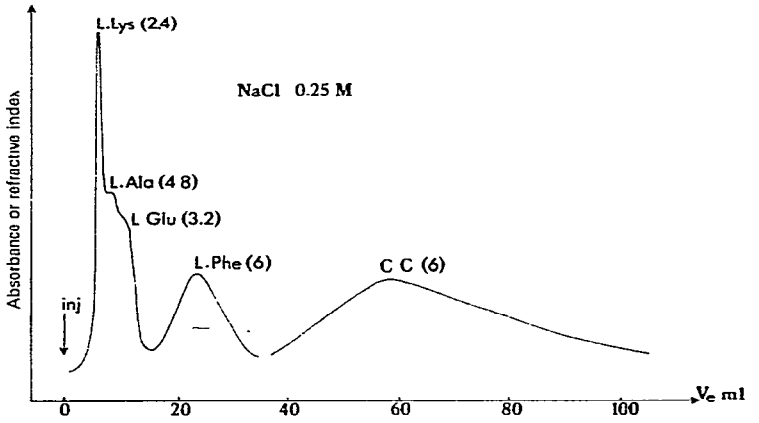

Fig. 4. Chromatogram of a mixture of α-amino acids and cephalosporin C on sorbent I under isocratic conditions (0.25 *M* NaCl). UV detection at 254 nm for cephalosporin C and refractometric detection for the other products. Flow-rate, 1 ml/min; room temperature. The numbers in parentheses correspond to the amounts injected (μmol); total volume of the injection, 100 μl.

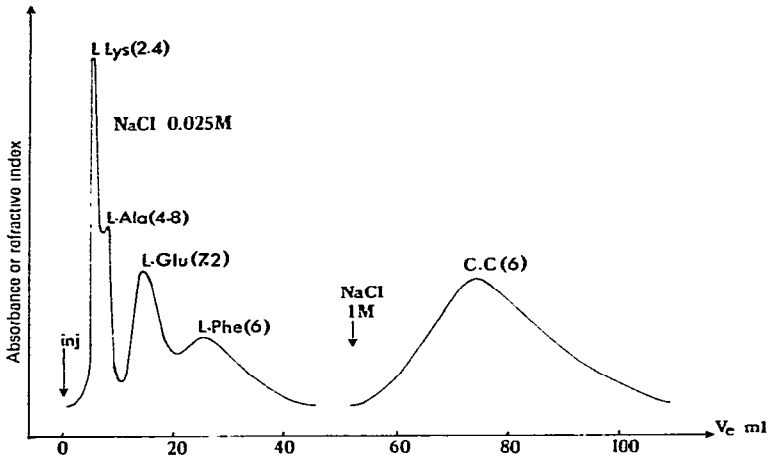

Fig. 5. Chromatogram of a mixture of α-amino acids and cephalosporin C on sorbent I. Eluent: 0.025 *M* NaCl, then 1 *M* NaCl. Other conditions as in Fig. 4.

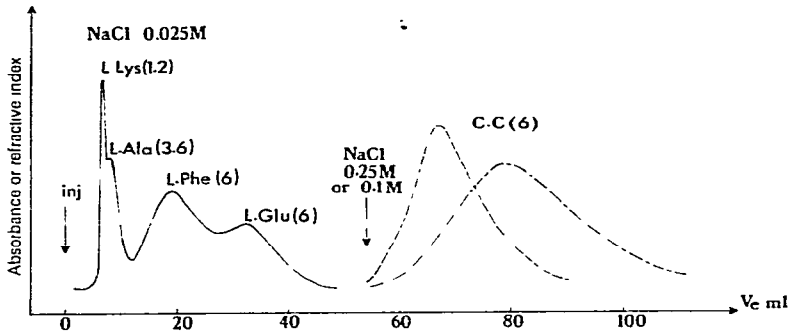

Fig. 6. Chromatogram of a mixture of α-amino acids and cephalosporin C on sorbent III. Eluent: 0.025 *M* NaCl, then 0.1 *M* NaCl (— - — - —) or 0.25 *M* NaCl (— — —). Other conditions as in Fig. 4.

CONCLUSION

The sorbents used appear to be suitable for the efficient separation of cephalosporin C from an artificial mixture of α-amino acids. A good selectivity for the retention of the antibiotic was achieved owing to the combination of three types of interaction: hydrophobic, π-binding and ionic. As in affinity chromatography[1,2], these different interactions probably must be synergistic for the antibiotic, which explains the tight binding of this compound with stationary phases in water and dilute salt solutions. By optimizing the elution conditions and the nature of the adsorbent, it is possible to elute first the α-amino acids and then cephalosporin C in well separated fractions, the concentration depending both on the ionic strength of the eluent and the stationary phase. The loading capacity of sorbent I for cephalosporin C was tested in preparative runs by gradually increasing the concentration and the volume of the injections. This capacity was found to be higher than 40 mg of cephalosporin C per gram of dry resin, *i.e.*, 9 g of cephalosporin C per litre of swollen packing.

The application of this new type of sorbent to the industrial isolation of cephalosporin C from fermentation broths requires the preparation of selective polystyrene resins from large-diameter particles, for example Amberlite XAD-4 (particle size 20–50 mesh), and this work is now being carried out.

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