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The chromatographic behaviour of cephalosporins in gel filtration chromatography, a novel method to separate high molecular weight impurities

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Abstract: The interaction between cephalosporins and the dextran matrix of Sephadex gel in gel filtration chromatography has been thoroughly studied. Twelve cephalosporins with specific structures were examined under different chromatographic conditions, including 12 different mobile phases comprising inorganic or organic compounds of different charge or/and density of electrons on their negative ions, different types of Sephadex gel (Sephadex G-10 and Sephadex G-50) and different flow rates. It was found that the more negative the charge or/and density of electrons on the negative ions of buffer components, the more was the adsorption of cephalosporins on the solid phase; this indicated tht the mobile phase played an important rôle in gel filtration chromatography for cephalosporins. By choice of suitable chromatographic conditions, optimum separation of high molecular weight impurities from cephalosporins could be achieved. The novel method could be used as a routine method for the quality control of cephalosporin preparations.

Keywords: *Gel chromatography; dextran gel; cephalosporins; high molecular weight impurities of cephalosporins.*

Introduction

Cephalosporins, a class of antibiotics with a β-lactam structure are becoming more important in clinical therapy. But the immediate hypersensitivity reaction to β-lactam antibiotics is still a remaining problem. Published work indicates the function of these molecules as haptens [1], and the high molecular weight impurities polymerized by themselves as allergens [2–4]. So it is clear that reducing the impurities from their products should decrease anaphylaxis in patients. There have been some reports on the separation and isolation of the polymers formed by β-lactam antibiotics, such as ampicillin polymers, by anion-exchange chromatography [5], high-performance liquid chromatography (HPLC) [6] or by gel filtration chromatography [7]; similarly, penicillin G polymers have been separated by gel filtration chromatography and reversed-phase HPLC [8]. But all those methods had some problems when used for drug control. Although in the USP XXII the high molecular weight polymers of ceftazidime have been controlled by gel permeation chromatography, there have been few reports on the application of this technique to cephalosporins.

In the present study a novel gel filtration chromatographic system based on the interaction between cephalosporins and a solid-phase Sephadex G-10 has been developed; the influence of reagents in the mobile phase upon the chromatographic behaviour of cephalosporins has been examined. The method could be used routinely for the isolation and assay of the high molecular weight impurities in cephalosporin preparations.

Materials and Methods

Chemicals and reagents

Cefalexin monohydrate and cefadroxil monohydrate were purchased from Dobfar (Italy); cefradine and cefoperazone sodium were obtained from ACS (Italy) cefalothin sodium, cefaloridine hydrate and ceftazidime were from Glaxo (UK); cefotaxime sodium was purchased from Hoechst AG (Germany); ceftriaxone sodium was from Roche (Switzerland); 7-aminocephalosporanic acid (7-ACA) and cefmenoxime hemihydrochloride were from Takeda (Japan); cefamandole sodium was made by Shanghai No. 3 pharmaceutical factory (China). The structural differences of those cephalosporins are shown in Fig. 1.

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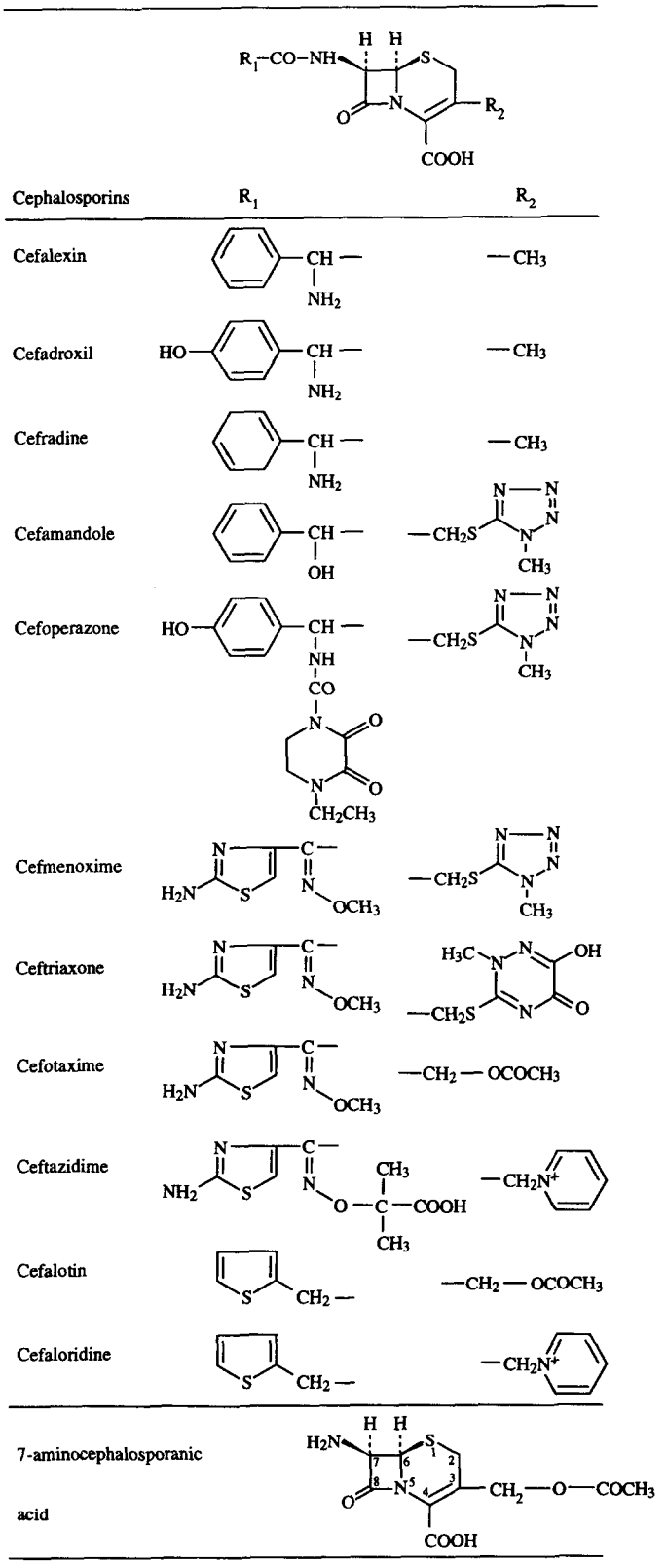

Figure 1
Structure of cephalosporins tested.

Sephadex G-10, Sephadex G-50 and blue dextran 2000 were made by Pharmacia (Sweden). The other reagents made in China were of analytical reagent grade.

Gel filtration chromatography

A LKB 2120 Varioperpex II pump, a ZW-1 UV detector (Tianjin analytical instrument factory, Tianjin, China) and a 4700 chromatogram data-processing micro-computer system (Dongxi Electric Institute, Beijing, China) were used.

An aliquot of sample dissolved in the mobile phase was applied on a column (500 × 13 mm i.d.) filled with Sephadex gels (40–120 μm), previously equilibrated with the mobile phase. Elution was carried out with the mobile phase and the eluates were detected by a UV-detector at 254 nm. The elution volume (V_e) of the cephalosporin was obtained corresponding to the maximum concentration of the compound, at which the eluate had a maximum UV absorbance.

The void volume (V_o) was determined with an aqueous solution of blue dextran 2000, and then the distribution coefficient (K_{av}) of the compound was calculated according to the following equation:

$$K_{av} = (V_e - V_o)/(V_t - V_o), \qquad (1)$$

where V_t is the bed volume of the column calculated from its geometry. The test was performed at room temperature.

Study on the effects of the mobile phase

The cephalosporin was chromatographed on Sephadex G-10 with different mobile phases. The flow rate of eluate was 1 ml min^{-1} and a 0.5-ml sample with a concentration of 0.4 mg ml^{-1} was applied. The K_{av} values were calculated.

To find the influence of ionic strength of the mobile phase upon the K_{av} value, 12 cephalosporins with different structures were tested. The ionic strength (μ) of the mobile phase was given by the expression:

$$\mu = 1/2 \sum M_i z_i^2, \qquad (2)$$

where M is the molarity and the z is the charge of the ion. Four mobile phases were tested separately: mobile phase A (sodium chloride 0.22 M, μ 0.22 M); mobile phase B (phosphate buffer 0.1 M, pH 7.0, μ 0.022 M); mobile phase C (sodium chloride 0.5 M in mobile phase B, pH 7.0, μ 0.72 M); and mobile phase D (phosphate buffer 0.01 M, pH 7.0, μ 0.022 M).

To study the effects of different ionic types in the mobile phase, ceftazidime, ceftriaxone and cephalothin were selected. Twelve different mobile phases consisting of inorganic or organic compounds (see Results) had the same concentration (0.1 M) and pH (7.0) which was adjusted by sodium hydroxide solution or by an acidic solution having the negative ions of the mobile phase in common.

Using citrate buffers of 0.1 M (pH 7.0, 5.6 and 4.0), the K_{av} values of cephalosporins (ceftazidime, ceftriaxone and cephalothin) were compared.

Study of the interaction between cephalosporins and Sephadex

Sephadex G-10 beads (5 g) were put separately in each of 50-ml graduated flasks and immersed in distilled water or 0.3 or 0.2 or 0.01 M phosphate buffer (pH 7.0) or 0.2 M NaH_2PO_4 (pH 5.4) for 20 h; 1 ml of ceftriaxone solution (0.05 mg ml^{-1}) was placed in each flask and the solution was diluted to 50 ml. The flasks were shaken for 30 min at room temperature and the UV absorption spectra of the supernatants were recorded with a Shimadzu UV-265 spectrophotometer.

The K_{av} values of all cephalosporins were determined with Sephadex G-10 and with Sephadex G-50 for comparison of the effects of the dextran concentration of Sephadex gels. The elution was carried out with phosphate buffer (0.1 M, pH 7.0) and the flow rate of elution was 1 ml min^{-1}. The relative K_{av} ($K_{av'}$) values were calculated as the ratios of K_{av} (7-ACA) to K_{av} (cephalosporin).

The influence of flow rate of elution upon the K_{av} values of all cephalosporins with the above Sephadex G-10 chromatographic system was examined; the flow rates tested were 1 and 4.5 ml min^{-1}.

The free energy change (ΔG) relevant to the transformation of the adsorption of a cephalosporin with the dextran matrix of Sephadex gel in different mobile phases (mobile phase B and mobile phase D) can be obtained from the formula:

$$-\Delta G = RT \ln K_{av_2}/Kav_1, \qquad (3)$$

where R is the universal gas constant (1.987 cal

mol^{-1} K^{-1}), T is the temperature (Kelvin) and K_{av_2} and the K_{av_1} are the K_{av} values obtained with mobile phase D and mobile phase B, respectively.

Optimization of the chromatographic condition

In order to find an optimal chromatographic system for the isolation and assay of the high molecular weight impurities in the products, the chromatographic parameters, tailing factor (T_f) and column efficiency were used. The T_f was evaluated using blue dextran 2000 according to the method described in ref. 9. The column efficiency is defined in terms of the number of theoretical plates per meter (n) by the expression [10]:

$$n = 5.54\ V_e^2/LW_h^2,$$

where L is the length of the column in metres and W_h is the width at half peak-height.

Results and Discussion

Effects of the mobile phase on the chromatographic behaviour of cephalosporins

The K_{av} values of cephalosporins in four different mobile phases were obtained (Table 1). Use of a different mobile phase affected the K_{av} value of a cephalosporin so that the chromatographic behaviour was different. High concentration of electrolyte (e.g. 3 M NaCl) could enhance the strength of the adsorptive interaction for substances in aqueous solutions on the chromatographic system of Sephadex gels [11]. If the type of electrolyte was not changed, increasing the concentration of electrolyte in the mobile phase could increase the K_{av} values of compounds. Therefore the K_{av} values of cephalosporins obtained from mobile phase B were higher than those from mobile phase D, as the concentration of phosphate in mobile phase B was 10 times higher than that in mobile phase D. But if the electrolyte was changed, it was thought that it was not ionic strength but the type of mobile phase that played a direct rôle in influencing the chromatographic behaviour since mobile phase A (sodium chloride solution) and mobile phase B (phosphate buffer) had the equivalent ionic strength (μ 0.22 M), which did not result in an equivalent K_{av} value; for the cephalosporin similarly, mobile phase C (sodium chloride in mobile phase B) had a stronger ionic strength than mobile phase B, which also did not result in a higher K_{av} value.

Since the four types of mobile phases were composed of sodium phosphates and/or sodium chloride, it was thought that the change in chromatographic behaviour of cephalosporins in different mobile phases was caused by the negative ions. This result was confirmed (Tables 2 and 3).

Table 2 shows the influence of the mobile phase composed of different negative ions on the chromatographic behaviour. The density of electrons on negative ions in the mobile phase was the main influence on the chromatographic behaviour of cephalosporins. In sequence, the density of electrons on some ions used at pH 7.0, were citrate, sulphate, phosphate, acetate, bicarbonate and nitrate.

Table 1
Influence of ionic strength of the mobile phase on the K_{av} value of cephalosporins

Cephalosporins	K_{av} values with mobile phase			
	A(NaCl, μ = 0.22 M)	B(PB*, μ = 0.22 M)	C(PB* + NaCl, μ = 0.72 M)	D(PB*, μ = 0.022 M)
7-ACA†	0.236	0.379	0.312	0.227
Ceftazidime	0.227	0.254	0.254	0.124
Cefalexin	0.394	0.545	0.409	0.397
Cefradine	0.364	0.424	0.370	0.339
Cefadroxil	0.603	0.697	0.667	0.561
Cefaloridine	0.667	0.721	0.776	0.645
Cefalothin	0.812	1.424	1.218	0.621
Ceftriaxone	0.497	1.364	0.939	0.230
Cefotaxime	0.721	1.364	1.664	0.455
Cefmenoxime	1.394	2.455	2.030	0.818
Cefamandole	1.115	2.394	2.030	0.818
Cefoperazone	1.182	1.939	1.758	0.742

*PB = Phosphate buffer.
†7-ACA = 7-aminocephalosporanic acid.

Table 2
Influence of mobile phases of different negative ions on the K_{av} value of cephalosporins

Compounds in mobile phase (0.1 M, pH 7.0)	K_{av} values		
	Ceftazidime	Ceftriaxone	Cefalothin
Sodium citrate	0.45	2.52	2.06
Ammonium sulphate	0.36	1.76	1.55
Sodium phosphate	0.31	1.36	1.42
Sodium acetate	0.27	0.79	1.00
Sodium bicarbonate	0.21	0.55	0.88
Sodium nitrate	0.15	0.21	0.58
Potassium chlorate	0.18	0.36	0.70
Tris–HCl	0.18	0.38	0.70
Glucose	0.06	0.11	0.09
Aminoacetic acid	0.08	0.08	0.12
Diethylamine	0.14	0.23	0.61
Water	0.05	0.06	0.09

Table 3
Influence of pH of the mobile phase on the K_{av} value of cephalosporins

Cephalosporins	K_{av} value with citrate buffer*		
	pH 7.0	pH 5.6	pH 4.0
Ceftazidime	0.45	0.39	0.30
Ceftriaxone	2.52	1.91	1.76
Cefalothin	2.06	1.64	1.21

*pK_a values of citric acid are 3.13, 4.76 and 6.40.

Because a salt formed by a polyprotic acid can be dissociated in more than one step in water, the ionization extent of a polyprotic acid in solution is affected by the pH value. According to the pK_a values, the charges of a citric acid ion in solution of pH 7.0, 5.6 and 4.0 are mainly −3, −2 and −1; under each condition the density of electrons on the ions was special, the most dense is the triacid and the least dense is the monoacid. Thus the cephalosporins had the highest K_{av} values with the mobile phase at pH 7.0 and the least at pH 4.0 (Table 3).

Interaction between cephalosporins and the dextran matrix

In gel permeation chromatography, if there is no adsorption between the solute and the solid phase, the K_{av} value of a compound should be smaller than 1. But some K_{av} values of cephalosporins under the chromatographic conditions were greater than 1 (Tables 1 and 2), which means that there was adsorption on the matrix of Sephadex gel formed by dextran.

The interaction between cephalosporins and the dextran matrix was increased by adding negative ions in the mobile phase. In Fig. 2, it was found that less ceftriaxone was detected in the supernatant which meant that more was

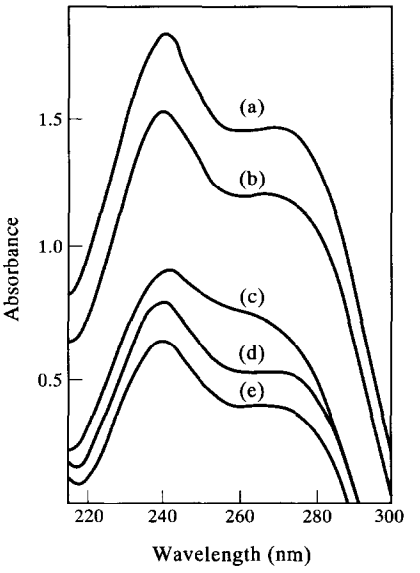

Figure 2
Adsorption phenomena of cefriaxone on Sephadex G-10. UV absorption spectra of supernatants of Sephadex G-10–Ceftriaxone mixture in (a) distilled water; (b) 0.01 M, pH 7.0 phosphate buffer; (c) 0.2 M, pH 5.4 NaH_2PO_4; (d) 0.2 M, pH 7.0 phosphate buffer; (e) 0.3 M, pH 7.0 phosphate buffer.

adsorbed on the Sephadex beads when the concentration of the phosphate ions of the solution was increased from 0.01 to 0.3 M, or when the extent of ionization of the ions was enhanced by changing the pH of the solution from pH 5.4 to pH 7.0; at this pH range the ionization extent of ceftriaxone changed very little. This explained the reason for the change in K_{av} of a cephalosporin in different mobile phases using the chromatographic system.

Sephadex matrix contains many hydroxyl groups which could be present as proton donors combining with proton acceptors by hydrogen bonding. Yano *et al.* [12] have dis-

cussed the interaction between Sephadex matrix and water in solvent. Water molecules were firmly bound or co-operatively hydrated in the highly condensed gel matrix to form small pores. Accordingly, the water film formed should prevent a solute interacting from the matrix and keep the solute in the central region of the pore. Janson [3] attributes enhancement of gel–solute interaction by electrolyte to a smaller layer of hydration surrounding the solute molecule [13]. The negative ions as a proton acceptor could not only interfere with the water film in the pores of the gels but could substitute for the water molecules bound to the matrix and the solutes. Those made contact between the solute and the matrix easy so that secondary interactions between the solute and the matrix such as ionic attraction, hydrogen bonding and hydrophobic interaction come into play. The higher the density of electrons of the negative ions, the more efficient was the electrolyte binding to the solutes or the interference with the water film. That may be the reason that negative ions help cephalosporins to be adsorbed to the Sephadex gels.

The free energy changes of a cephalosporin from the mobile phase of 0.01 M to that of 0.1 M phosphate buffer on the Sephadex G-10 system are listed in Table 4. Hyslop and Milligan have studied the chromatography of penicillins on dextran gels [10]. It was found that the anomalous behaviour of penicillins could be best explained by their side-chain adsorptive effects; polar side-chain substituents such as the amino group of ampicillin and the carboxyl group of carbenicillin appeared to interfere with side-chain-directed adsorption.

Table 4
The free energy change of cephalosporins

Cephalosporins	$-\Delta G = RT\ln K_{av_2}/K_{av_1}$*
7-ACA	303.72
Ceftazidime	546.73
Cefalexin	187.74
Cefradine	132.57
Cefadroxil	128.62
Cefaloridine	66.00
Cefalothin	490.48
Ceftriaxone	1054.75
Cefotaxime	650.52
Cefmenoxime	651.20
Cefamandole	625.52
Cefoperazone	569.17

* K_{av_2} and K_{av_1} are the K_{av} values obtained with 0.01 and 0.1 M phosphate buffer, respectively.

A similar situation has been found in cephalosporins. Furthermore, in comparison of Fig. 1 with Table 4, it can be seen that not only had both R1 and R2 substituents a close relation to the adsorption, but usually compounds with rich π-electron substituents, an extending system of double bonds, or a planar configuration such as cefotaxime, ceftriaxone, cefmenoxime, cefamandole, cefoperazone and cefalothin, had a larger $-\Delta G$ value; this result suggested that the ions of the mobile phase strongly interfered with the adsorption of those compounds. The interference of the ions was influenced by the presence of ionizable substituents of a molecule such as the pyridyl group in cefaloridine, the amino group in cefalexin, cefradine or cefadroxil; this made the binding of cephalosporins to the Sephadex matrix difficult and resulted in a small $-\Delta G$ value. The phenomenon indicated that the hydrophobic interaction played an important part in the interaction between cephalosporins and the Sephadex matrix.

The effect of dextran concentration (C_f) of swollen Sephadex gels on chromatographic behaviour was investigated. The values of C_f (g/100 ml) for Sephadex gels were 12.9 for G-50 and 57.4 for G-10 [12]. In Table 5, when 7-ACA was chosen as a reference compound since the other cephalosporins were its derivatives and the relative K_{av} values of cephalosporins to 7-ACA were calculated, it was found that the relative K_{av} values obtained on the Sephadex G-10 system were smaller than those obtained on the Sephadex G-50 system and the variance of the former was larger than that of

Table 5
Influence of dextran concentration of Sephadex gels on the K_{av} value of cephalosporins

Cephalosporins	Sephadex G-50		Sephadex G-10	
	K_{av}	$K_{av'}$*	K_{av}	$K_{av'}$*
7-ACA	0.891	1	0.379	1
Ceftazidime	0.894	0.997	0.312	1.215
Cefalexin	0.924	0.964	0.545	0.695
Cefradine	0.924	0.964	0.424	0.894
Cefadroxil	0.970	0.919	0.697	0.544
Cefaloridine	0.970	0.919	0.721	0.526
Cefalothin	0.970	0.919	1.424	0.266
Ceftriaxone	1.133	0.786	1.364	0.278
Cefotaxime	0.970	0.919	1.364	0.278
Cefmenoxime	1.073	0.830	2.455	0.154
Cefamandole	1.045	0.853	2.394	0.158
Cefoperazone	1.067	0.835	1.939	0.196

* $K_{av'}$ is the relative K_{av} value K_{av} (7-ACA)/K_{av} (cephalosporin).

Table 6
Influence of flow rates of elution on the K_{av} value of cephalosporins

Cephalosporins	K_{av} values at different flow rates		ΔK_{av}
	1 ml min^{-1}	4.5 ml min^{-1}	
7-ACA	0.379	0.348	0.031
Ceftazidime	0.312	0.106	0.206
Cefalexin	0.545	0.485	0.060
Cefradine	0.424	0.384	0.076
Cefadroxil	0.697	0.606	0.091
Cefaloridine	0.721	0.636	0.085
Cefalothin	1.424	1.364	0.060
Ceftriaxone	1.364	1.061	0.303
Cefotaxime	1.364	1.212	0.152
Cefmenoxime	2.455	2.273	0.182
Cefamandole	2.394	2.152	0.242
Cefoperazone	1.939	1.948	0.091

the latter; this indicates not only that the C_f value affected the binding of cephalosporins to the matrix but also that other factors could affect the interaction.

Comparison of the effects of flow rate in the chromatographic system on the K_{av} values (Table 6) showed that rapid movement of a solute could reduce its adsorption to the matrix. It is well known that there are many pores in Sephadex gel beads and the possibility of movement of a solute within the pores is higher than that of movement outside the pores; slowing down the flow rate of the mobile phase during the process increases the possibility of contact between the solute and the matrix. Thus adsorption of a cephalosporin to the matrix inside the pores is stronger than that outside the pores and this may be one of the reasons for enhancement of the K_{av} values of cephalosporins in the tests.

For explanation of Tables 5 and 6, it is thought that the strong adsorption inside the pores of Sephadex gel was caused by two main factors: the inner surface of the gel beads is larger than the outer surface; and the narrow channels inside the beads enables a compound to make contact more easily with the inner surface.

Optimization of the chromatographic conditions

As could be observed, the chromatographic conditions could control the behaviour of cephalosporins. But in the Sephadex G-10 system, the peak of high molecular weight impurities of cephalosporins was always found at the position of $K_{av} = 0$ under all the experimental conditions which indicated that the behaviour of the high molecular weight impurities was little affected. Thus a chosen special chromatographic condition could lead to an optimum chromatographic result in isolation of the impurities from their products for different purposes; for example, the impurities could be isolated to one or two or even three fractions according to their molecular weight

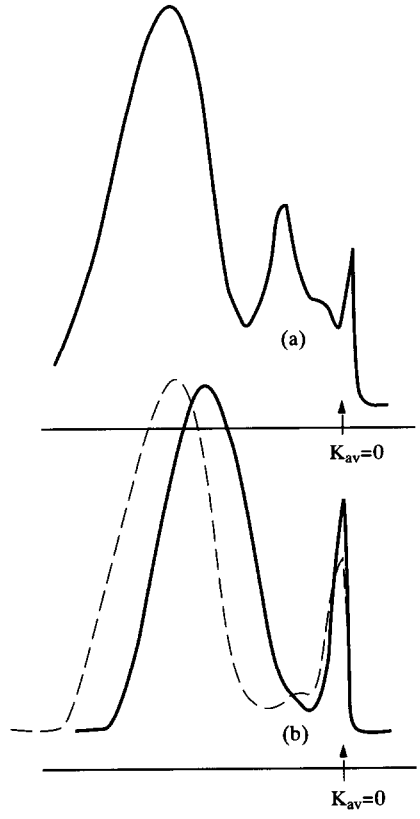

Figure 3
Effect of chromatographic conditions on the chromatography. (a) Three fractions of high molecular weight impurities of 48-h-old cefalothin solution of pH 9.6. (b) Different chromatograms of cefotaxime at different flow rates. Flow rate: ——, 2.5 ml min^{-1}; -----, 1.0 ml min^{-1}. The sample was stored at 60% RH for more than 1 month.

(Fig. 3) by changing the concentration of negative ions and/or flow rate of the mobile phase. From experience, if the K_{av} value of a cephalosporin was greater than 0.5 in the Sephadex G-10 system, the impurities (K_{av} = 0) could be well separated from the antibiotic. The chromatographic conditions with phosphate buffer (pH = 7.0) on the Sephadex G-10 system are listed in Table 7, in which the K_{av} values of cephalosporins were about 0.5.

The type of negative ions in mobile phase could affect the results of separation of high molecular weight impurities from cephalosporins. Unsuitable ions such as citric acid ions increased the tailing factor of the peak of high molecular weight impurities and decreased the column efficiency (Table 8). Usually, phosphate ions were used in the mobile phase.

Another important point in the chromatography was sometimes using a mobile phase with a high ionic concentration to prevent cephalosporins from non-covalent polymerization. This meant that cephalosporins could be bound together by non-covalent bonds such as ionic bonds, hydrogen bonds or hydrophobic interactions under some conditions so that the non-covalent polymer had a larger molecular weight; however, the polymer could be dissociated to separate cephalosporins by destroying the interactions among the molecules under other conditions. The effect of non-covalent polymerization of ceftazidime can be observed in Fig. 4. At the lowest ionic strength, ceftazidime could not move in the pores of Sephadex gel beads so that the K_{av} value of the solute was 0; this should have influenced determination of the content of the high molecular weight impurities in products but non-covalent polymerization had been prevented by increasing the concentration of the phosphate buffer. As ceftazidime molecules have both positive and negative groups, it is thought that non-covalent polymerization was caused by some ionic attraction.

Table 7
Chromatographic conditions for assaying high molecular weight impurities of cephalosporins

Cephalosporins	Concentration of PB* (M)	Flow rate (ml min^{-1})
7-ACA	0.1	0.5
Ceftazidime	0.1	0.5
Cefradine	0.1	0.75
Cefalothin	0.01	1.0
Cefaloridine	0.01	1.0
Cefmenoxime	0.01	1.0
Cefoperazone	0.01	1.0
Cefadroxil	0.01	1.0
Cefalexin	0.1	1.0
Cefamendole	0.01	1.4
Ceftriaxone	0.1	2.0
Cefotaxime	0.1	2.5

*PB = Phosphate buffer.

Conclusions

A novel gel filtration chromatographic

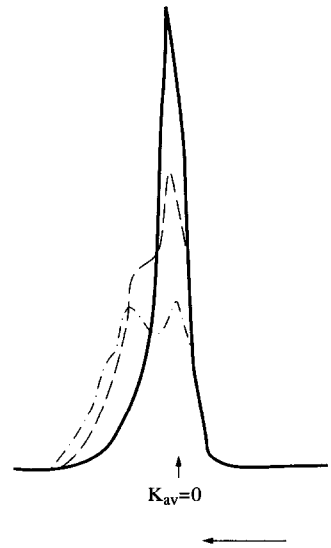

Figure 4
Detection of non-covalent polymerization of ceftazidime by gel chromatography. Mobile phase and distilled water, flow rate 1 ml min^{-1}. ———, Sample dissolved in distilled water; ·-·-·-·, in 0.016 M, pH 7.0 phosphate buffer; -----, in 0.046 M, pH 7.0 phosphate buffer.

Table 8
Influence of mobile phases on the chromatography

Column number	Mobile phase	Column efficiency* (n)	Tailing factor
1	Phosphate buffer	3191	1.43
	Citrate buffer	2380	2.45
2	Phosphate buffer	4121	1.43
	Citrate buffer	3120	2.40
	Ammonium sulphate	3770	1.45

*Calculated from the component of high molecular weight impurities of ceftazidime.

system based on the interaction between cephalosporins and the matrix of the solid phase and on the influence of the negative ions of the mobile phase has been developed. Choice of optimum conditions of chromatography including the nature of the mobile phase (ionic type, pH value and molarity) and the flow rate for elution can result in a useful chromatographic method for the isolation and determination of high molecular weight impurities in cephalosporins.

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