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PROCESS SCALE-UP OF A β-LACTAM ANTIBIOTIC PURIFICATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

In order to assess the feasibility of producing purified cephalosporin antibiotic by preparative high-performance liquid chromatography (HPLC), a series of purifications were carried out on a 60 × 20 cm I.D. HPLC column. The operating parameter most strongly affecting the process throughput and product purity was found to be the column load (g crude sample/g HPLC support). A saturation phenomenon was observed for strongly sorbed impurities, which affected isolated product purity but not chromatographic resolution. Saturation was reversed by routine column washes. Column performance did not decline over the course of the experiment in terms of chromatographic resolution, or column capacity. Loss of packing material by a dissolution process was found to be of no consequence.

INTRODUCTION

The classical isolation and purification process for β-lactam antibiotics has been a series of solvent extractions concluding with the product crystallization[1]. Compounds proving difficult or impossible to crystallize have been purified by techniques such as ion exchange, carbon adsorption, metal salt complexation, and chromatographic processes[2,3].

The excellent resolving power of reversed-phase high-performance liquid chromatography (HPLC) has been used effectively as an analytical method for the quantitation of β-lactams[4,5]. Essentially homogeneous isolates can be obtained after single passes of crude product through a properly selected adsorbent–mobile phase HPLC system. The application of HPLC to the preparation of more than several grams of isolated compound has been limited by the availability of suitably scaled HPLC equipment. Of the more than one hundred papers cited in Chemical Abstracts from 1980 through 1983 dealing with preparative HPLC, only six described the use of large-scale columns capable of purifying kilogram quantities of compound[6–11]. With the appearance of commercially available process-scale HPLC systems, research interest in the area should increase. Recently, Waters Assoc. (Milford, MA, U.S.A.), has made available preparative HPLC equipment capable of separating complex organic solute mixtures at the 0.1–1.0 kg per injection level. The Waters KILOPREP

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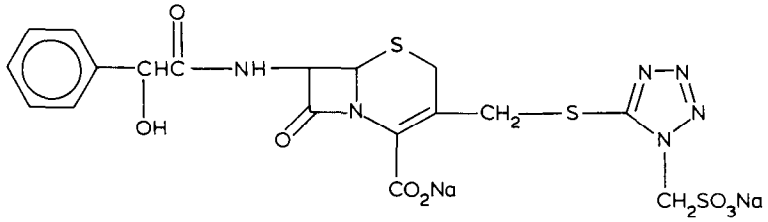
HPLC system was used in this study to evaluate the feasibility of utilizing preparative HPLC for the commercial preparation of cefonicid (Smith Kline Beckman) disodium salt, [7-D-mandelamido-3-(1-sulfomethyltetrazol-5-yl)-thiomethyl-3-cephem-4-carboxylic acid, disodium salt] (I). In this study, a reversed-phase, microparticulate C_{18} silica column and a mobile phase of water were used to separate cefonicid from



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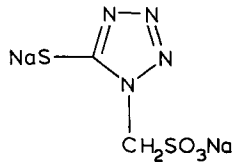

(II)

impurities not readily removed by liquid–liquid extraction. Principal among these is tetrazole sulfonic acid disodium salt (II), although a number of other chemically characterized impurities produced during synthesis or long-term storage have also been identified. The capacity of the process was evaluated by varying load levels and elution rates for a 60 × 20 cm I.D. HPLC column. The effect of initial purity on resolution and the need for column regeneration were also studied. Material balances, capable of detecting the accumulation of strongly sorbed compounds on the column, or the dissolution of the column in the mobile phase on repeated injections were used to estimate column lifetime.

EXPERIMENTAL

Process chromatograph

The chromatograph, Fig. 1, consists of three 60 × 20 cm columns. Constant-flow metering pumps deliver sample, mobile phase, and solvent wash at a flow-rate of 1–5 l/min. A system of three-way valves allows the columns to be operated in series, parallel or singly. For this study the columns were operated as single units. A refractive index detector monitors the eluent stream through a flow splitter. An in-line conductivity detector was also used to monitor the elution profile.

The column packing used was a bonded-phase octadecyl silica, 55–105 μm (Waters Prep-Pak). Approximately 8.8 kg of the material was packed in each column. The column is designed to be radially compressed to improve column stability and packing efficiency. The surface area of the packing material was 325 m^2/g and carbon loading, defined as the weight percent of alkyl phase per unit weight of support, was 12%[12]. A final capping with trimethylchlorosilane was used to minimize available silanol groups.

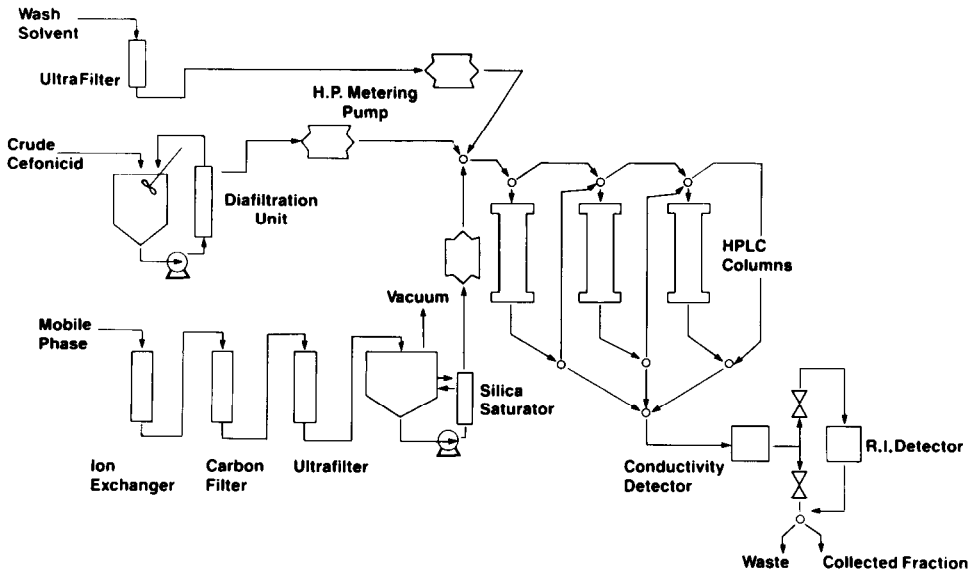

Fig. 1. Process chromatograph flow diagram.

Water, purified by passage through an ion exchanger and activated carbon was the mobile phase. Treated water was ultra-filtered, saturated with silica, and vacuum-degassed before being stored at 25°C in a temperature-controlled glass vessel.

Crude cefonicid

Crude cefonicid was either a lyophilized product of 87–93% purity (anhydrous basis), or an aqueous solution of 48–65% assay (anhydrous basis). The lyophilized material and the lower-purity aqueous solutions contained the typical impurities derived from the synthesis of the compound or from long-term storage. An analytical chromatogram (Fig. 2) shows well-resolved components. Strongly adsorbed components are eluted by a step gradient of 100% methanol.

Chromatograph operation

Cefonicid was diluted to its injection concentration (50–200 mg/ml) with purified water, prepared as described, and filtered through a polysulfone membrane (Osmonics) with a nominal molecular weight cutoff of 1000. Ultra-filtration experiments showed that 80% of the impurities having a molecular weight (MW) greater than 1000 were removed by this step. The bulk of the >1000 MW impurities remaining in the crude cefonicid solution after ultra-filtration were shown to have MW less than 5000 by size-exclusion chromatography. The pH of the filtered solutions was adjusted to 5.2–5.3. Solutions were assayed immediately before use.

Sample solution was injected at a predetermined flow-rate on a column with a stable baseline conductivity of less than 2 μS. Sample loads were between 7 and 57 mg/g of column material. Compared with analytical column loads of 10^{-1}–10^{-2} mg/g, the column was operated in an overload condition. Under these conditions the retention volume, V_R, of the sample components varies with sample size, and reten-

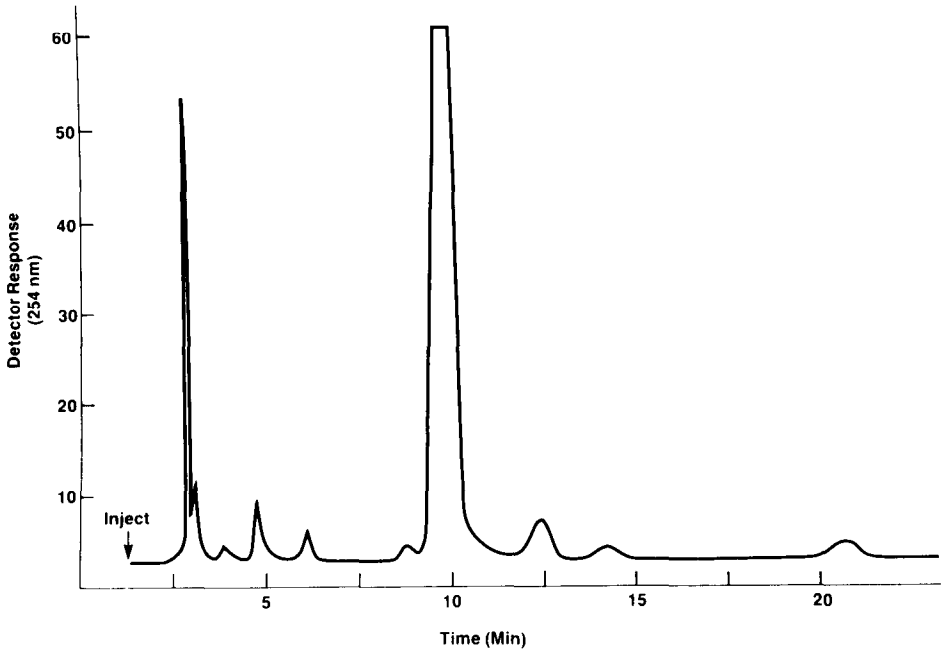

Fig. 2. Analytical chromatogram. Sample: crude cefonicid, 20 mg/ml, 20 μl injection. Column: 30 cm × 3.9 mm I.D., μBondapak C_{18}. Mobile phase: methanol–0.1 *M* ammonium phosphate–water (12.5:10:77.5), pH 5.3.

tion volumes or retention times cannot be used to predict when a component will emerge from the column. Fractions were manually collected by monitoring the recorder output from the refractive index detector for all components other than inorganic salts, which were detected by a conductivity flow cell.

A typical chromatogram (Fig. 3) reveals well-resolved peaks of inorganic salts (peak 1), tetrazole (peak 2), and cefonicid (peak 4). A cluster of other impurities (peak 3) emerge as a shoulder on the leading edge of the cefonicid peak. There is no evidence of late-eluted components. A methanol wash eluted strongly adsorbed impurities (peaks 5–7). The manually fractionated cuts were subsequently analyzed gravimetrically and chemically. A desired purity of greater than 93% cefonicid in the isolated fraction determined where the cut was made between 3 and 4. By taking two or three cuts in the peak 3 region, it was possible to pool only those cuts which would produce a cefonicid fraction having a minimum purity of 93%. Solution conductivity was used to determine the cutoff point for the cefonicid peak. A 300 μS conductivity was selected as a reasonable compromise between product losses in the tailing cefonicid peak, and the dilution of the isolated fraction if collected to baseline conductivity. This cutoff resulted in a *ca.* 3–5% product loss. Maximum recoveries of 95–97% were, therefore, due to the experimental method.

Evaluation of chromatograph performance

In order to evaluate the effect of the operating parameters on chromatographic performance, the capacity factor k', the theoretical plate number N, and the sepa-

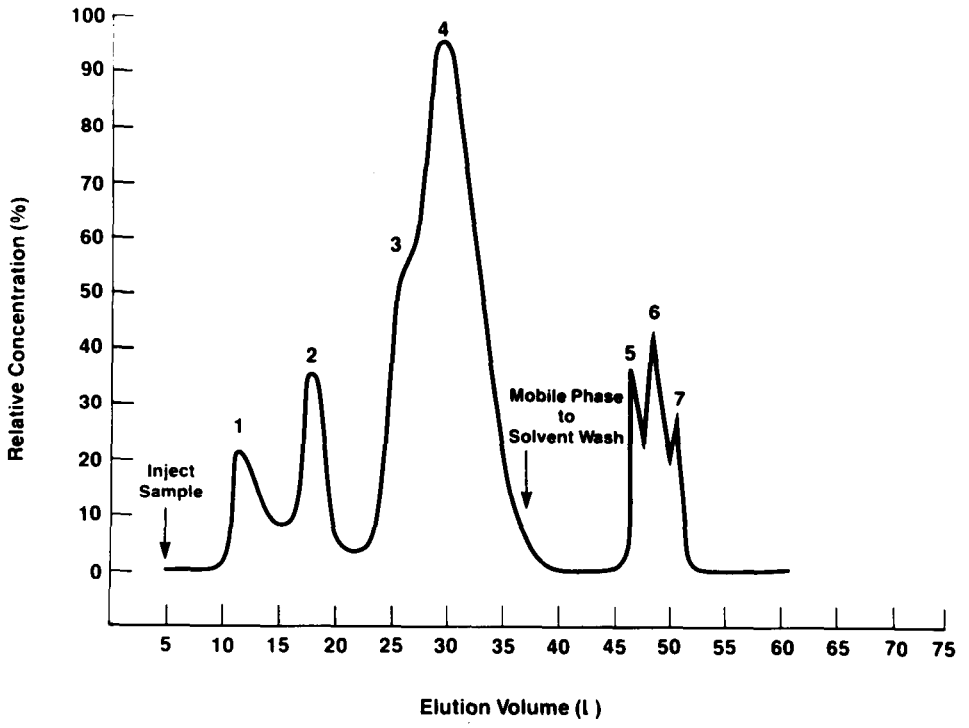

Fig. 3. Process scale chromatogram. Sample: crude cefonicid, 10% solids, 81% purity, 270 g injected. Column: 60 × 20 cm I.D., 8.8 kg C_{18} silica, 55–105 μm. Mobile phase: water. Detection was by conductivity (peaks 1–4) and refractive index (peaks 5–7).

ration factor α, were calculated from the measurements of the chromatograms according to the methods described by Snyder and Kirkland[13]. Chromatographic resolution, R_s, was calculated from the resolution equation.

The operating parameters known to influence R_s were varied through a series of experiments in order to determine the extent of control each operating parameter exerted on resolution. The operating parameters varied were: sample mass, sample purity, sample volume, mobile phase flow-rate, and column age. The mobile phase consisted only of water, except for two experiments which were performed with the analytical system mobile phase.

By controlling purity to a level greater than 93% for all experiments, the values for R_s could be correlated with other performance criteria for preparative separations. Recovery of cefonicid and volume of the isolated fraction were determined by physical measurement and chemical analysis. Correlations between these measurements and the values of R_s were then possible. The additional performance criterion of chromatographic throughput (mass of compound purified per unit weight of column support per unit time) could also be evaluated under a number of operating conditions in order to select the optimum for routine purification of cefonicid.

Chromatographic reproducibility, an important performance criterion for a process operation, required a stable system without gradual performance decline after

repeated cycles. The stability of the stationary phase was investigated by attempting to determine the rate of loss of bonded phase and silica due to erosion or chemical change. The possibility that compounds in the injected sample are irreversible bound to the column with a resulting gradual decline in column performance was also investigated by performing material balances of total weight and weight of individual components. Isolated fractions were lyophilized to determine total solids. A portion of the solids was chemically analyzed to determine composition, and results were reported on an anhydrous solids basis.

RESULTS AND DISCUSSION

Effect of load on resolution

A solute moving through a column of bonded-phase silica is partitioned between the mobile phase and the stationary phase according to its equilibrium distribution constant K, when the amount of solute relative to the amount of bonded phase is low. This condition is required in analytical HPLC for constant retention volumes with variable sample loads. Typically, loads of 10^{-5}–10^{-4} g solute/g bonded phase are employed in analytical HPLC. As solute loads increase, the concentration of the solute in the mobile phase begins to exceed the sorption capacity of the bonded phase. This condition is known as column overload. Although column overload is avoided in analytical HPLC, it is a necessary condition in preparative HPLC. Without column overload, the process throughput would be unacceptably low. For example, using the 8.8-kg column of our experiments, less than 1 g of cefonicid could be injected at the 10^{-4} load level of an analytical system. However, as the overload becomes excessive, resolution between peaks declines to the point where either unacceptable losses of product result if high purification is the objective, or if high recovery is desired, lower purity must be accepted.

An objective of this investigation was to determine how the operating parameters of sample load, sample purity, sample volume, mobile phase flow-rate, and column age influence resolution. By inspection, values of k', α, and N for all eluted components of interest can be calculated from the recorder-generated chromatograms. The familiar equations for these factors, expressed in volume terms, are:

Capacity factor: $k' = (V_R - V_0)/V_0$ where V_0 = retention volume of an unretained component, *e.g.* sodium chloride and V_R = retention volume of component of interest, *e.g.* tetrazole or cefonicid.

Separation factor: $\alpha = k'_2/k'_1$ where k'_1 = capacity factor for the first peak of interest, *e.g.* tetrazole and k'_2 = capacity factor for the second peak of interest, *e.g.* cefonicid.

Plate count: $N = 16(V_R/V_w)^2$ where V_w = elution volume of component of interest, *e.g.* tetrazole or cefonicid and V_R = retention volume of component of interest, *e.g.* tetrazole or cefonicid.

Resolution: $R_s = (V_{R2} - V_{R1})/0.5\,(V_{w1} + V_{w2})$, can be expressed in terms of the three factors α, k', and N as: $R_s = (\sqrt{N}/4)\,[k'/(k' + 1)]\,(\alpha - 1)$.

Having calculated values for α, k', N, and R_s under a variety of operating conditions plots of R_s *vs.* the parameters exhibiting an influence on R_s were generated.

The result indicated that of all the operating parameters sample load had the

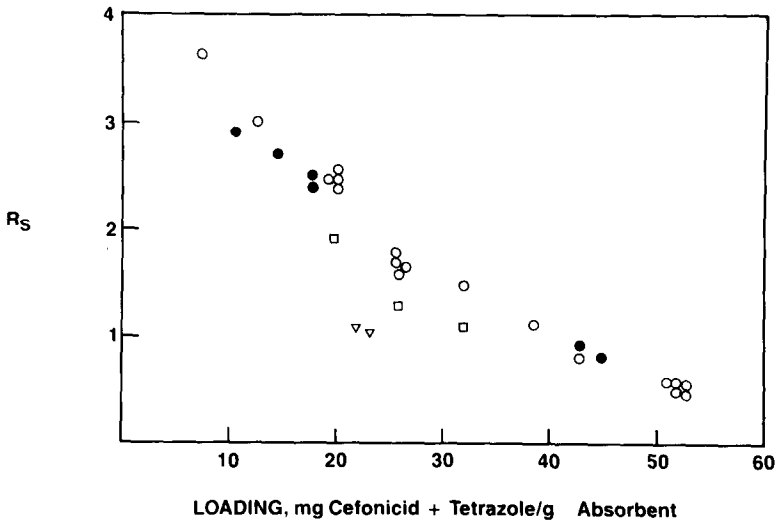

Fig. 4. Resolution *vs.* column load for crude cefonicid solutions. Column 60 × 20 cm I.D., 8.8 kg C_{18} silica. ○, Sample purity 85–90%; mobile phase, water; flow-rate, 2.7 l/min. ●, Sample purity 40–65%, mobile phase, water; flow-rate, 2.7 l/min. ▽, Sample purity 88%; mobile phase, methanol–0.1 *M* ammonium phosphate–water (12.5:10:77.5); flow-rate, 2.7 l/min. □, Sample purity 85–90%; mobile phase, water; flow-rate, 5 l/min.

most pronounced influence on R_s. Fig. 4 illustrates this. When initially plotted against total solids in the sample, the correlation between R_s and load exhibited variations when the purity of the injected sample was changed. It was found that samples having a high percentage of impurities other than tetrazole appeared to give R_s values higher than that predicted by Fig. 4. When the R_s values for these low-purity samples were plotted *vs.* the load of cefonicid and tetrazole only, their R_s values approximated the R_s values for the purer injected samples of the same cefonicid plus tetrazole load. Obviously, R_s as a measure of the separation of tetrazole from cefonicid is not significantly influenced by impurities that are eluted quite early, *e.g.* inorganic salts, or quite late, *e.g.* non-polar impurities. It appears reasonable to state that for this system resolution was governed by the amount of the injected individual solutes incorporated in the resolution equation rather than by the total weight of the sample mixture.

Figs. 5–7 illustrate the influence of sample load on the factors k', α, and N. Plate count, N, was most sensitive to load changes and, therefore, had the largest effect on resolution. Plate count is most effectively increased by decreasing the size of the stationary phase and by increasing column length at the expense of higher operating pressure. Since operating pressure for this series of experiments was in the 70–150 p.s.i. range, it would appear that longer columns and smaller diameter support would be appropriate, since the mobile phase delivery system and column hardware can handle pressures up to 500 p.s.i.

Effect of operating parameters other than load on resolution

Although Fig. 4 shows that column load was the principal determinant of resolution in this series of experiments, other operating parameters did have a mea-

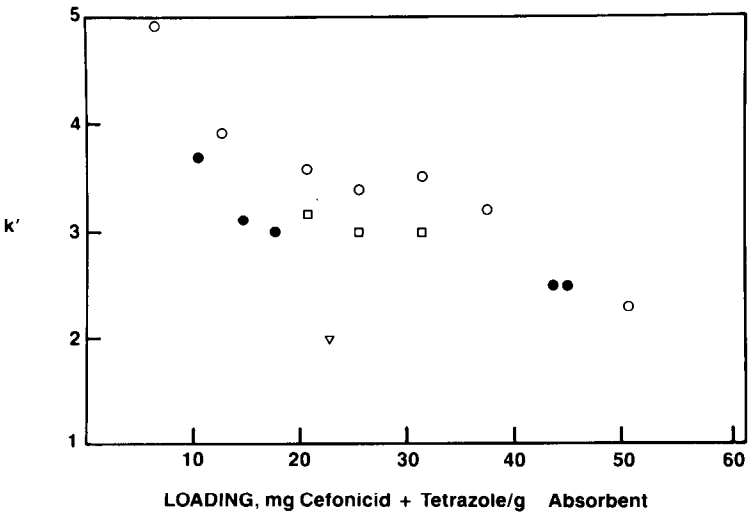

Fig. 5. Capacity factor *vs.* column load for crude cefonicid solutions. Column: 60 × 20 cm I.D.; 8.8 kg C_{18} silica. ○, Sample purity 85–90%; mobile phase, water; flow-rate, 2.7 l/min. ●, Sample purity 40–65%; mobile phase, water; flow-rate, 2.7 l/min. ▽, Sample purity 88%; mobile phase, methanol–0.1 *M* ammonium phosphate–water (12.5:10:77.5), pH 5.3; flow-rate, 2.7 l/min. □, Sample purity 85–90%; mobile phase, water; flow-rate, 5 l/min.

surable effect on resolution. Several experiments, in which all parameters were held constant except the flow-rate, indicated that resolution declined with increasing flow-rate. For example, at a load of 20 mg/g, a doubling of flow-rate (from 2.7 l/min to 5.0 l/min) resulted in a decline of R_s from 2.5 to 1.9. Results further below demon-

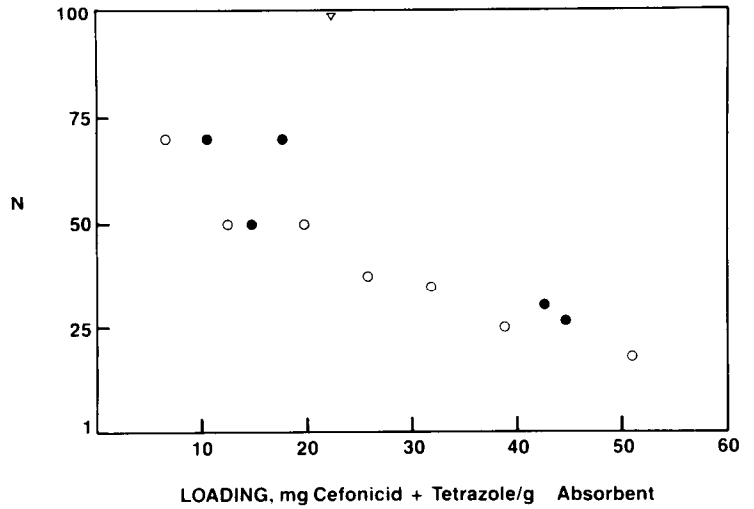

Fig. 6. Plate count *vs.* column loading for crude cefonicid solutions. Column: 60 × 20 cm I.D.; 8.8 kg C_{18} silica. ○, Sample purity 85–90%; mobile phase, water; flow-rate, 2.7 l/min. ●, Sample purity 40–65%; mobile phase, water; flow-rate, 2.7 l/min. ▽, Sample purity 88%; mobile phase, methanol–0.1 *M* ammonium phosphate–water (12.5:10:77.5), pH 5.3; flow-rate, 2.7 l/min.

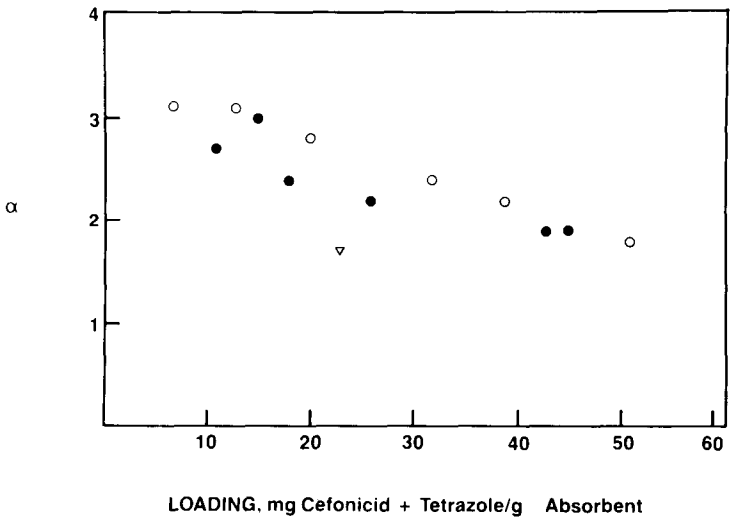

Fig. 7. Separation factor α *vs.* column loading for crude cefonicid solutions. Column: 60 × 20 cm I.D.; 8.8 kg C_{18} silica. ○, Sample purity 85–90%; mobile phase, water; flow-rate, 2.7 l/min. ●, Sample purity 40–65%; mobile phase, water; flow-rate, 2.7 l/min. ▽, Sample purity 88%; mobile phase, methanol–0.1 *M* ammonium phosphate–water (12.5:10:77.5), pH 5.3; flow-rate, 2.7 l/min.

strate that favorable gains in process throughput can be obtained at the expense of modest declines in resolution when the flow-rate is increased.

Sample volumes ranging from 1–6 l did not appear to affect resolution. Since the largest injection volume is approximately 25% of the elution volume of the cefonicid fraction (20–30 l), it is reasonable to assume that injection volumes at this level do not influence resolution significantly.

As already mentioned, the presence of impurities which have elution volumes much smaller or much larger than tetrazole and cefonicid did not influence resolution significantly. However, it was noted that when sample purity was high, due to prior purification by, *e.g.*, adsorption chromatography, the resolution declined. This result is illustrated in Fig. 8. The primary contributor to this decline was the significant increase in k' of tetrazole and a corresponding drop in R_s. Apparently, when the concentration of tetrazole in the injected sample approaches 1%, the column is no longer overloaded with tetrazole. For example, a 200-g sample containing 1% tetrazole would give a tetrazole load of $2.2 \cdot 10^{-4}$ g/g for the 8.8-kg column. The k' of tetrazole would approach the higher value of a non-overloaded column, while the k' of cefonicid would remain relatively constant. Since the change in k' in moving from a non-overload condition to an overload condition is non-linear[14], it is apparent that the k' of tetrazole will increase rapidly as purity is increased, approaching the k' of cefonicid. In certain situations this behavior would place an upper limit on the purity of the isolated product and, indeed, in this chromatographic system it would appear that cefonicid would not be efficiently isolated in greater than 97% purity.

The effect of column age or the number of injections a column can tolerate before performance declines and the column must be regenerated by washing with a stronger eluting solvent is an important consideration since it will determine the

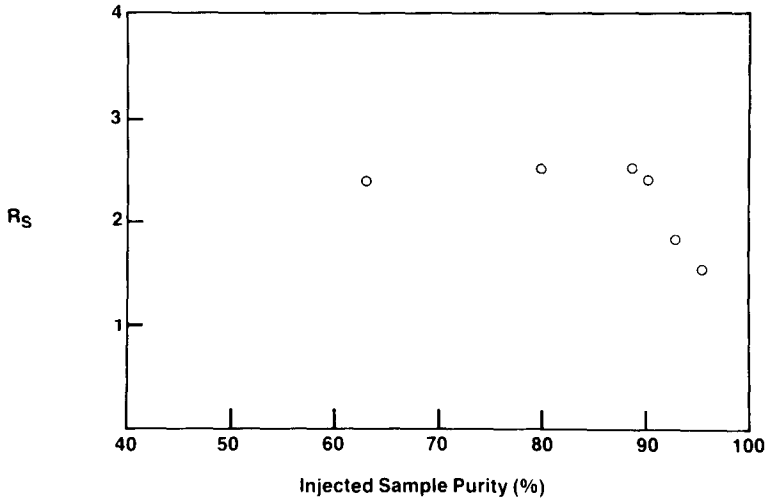

Fig. 8. Chromatographic resolution *vs.* injected sample purity for cefonicid solutions. Column: 60 × 20 cm I.D.; 8.8 kg C_{18} silica. Mobile phase, water; sample loading, 18–24 mg crude cefonicid/g absorbent; flow-rate, 2.2–2.8 l/min.

time the column must be taken out of service. Column performance, as measured by separation of tetrazole from cefonicid, did not decline with repeated injections of the load levels used in our experiments. Table I illustrates the results of repeated injections at three load levels.

In each case the results showed only random scatter in the values for R_s. The isolated cefonicid fractions were also chemically assayed for cefonicid purity. In one case, the cefonicid purity declined with repeated sample injection. Interestingly, this

TABLE I

THE EFFECT OF REPEATED INJECTIONS ON COLUMN PERFORMANCE AND FINAL PRODUCT PURITY

Sample: crude antibiotic solution 20% solids in water. Column: 60 × 20 cm I.D., 8.8 kg C_{18} silica, 55–105 μm. Mobile phase: water. All injections performed without intervening column wash.

Injection No.	*Sample weight (g)*	*Flow-rate (l/min)*	R_s	*Injected sample purity (%)*	*Isolated sample purity (%)*
1	500	5	0.6	89	93.9
2	500	5	0.5	89	93.9
3	500	5	0.6	89	92.5
4	500	5	0.6	89	93.3
5	500	5	0.5	89	93.2
1	200	1.5	2.5	88.8	97.7
2	200	1.5	2.5	88.8	98.4
3	200	1.5	2.5	88.8	97.2
1	547	2	1.1	48	96.8
2	547	2	0.9	48	96.8
3	567	2	0.9	48	93.7

was cefonicid known to have a high concentration of impurities that are strongly adsorbed on the HPLC column. Apparently, in this case, the column became saturated with these non-polar impurities. The issue of column regeneration is discussed in further detail in the results section dealing with material balances.

Effect of mobile phase on resolution

The obvious advantages of water as a mobile phase in terms of cost and ease of removal from the isolated cefonicid have limited the investigation of this parameter to two experiments in which the mobile phase developed for analytical HPLC was used. The results (Table II) indicate that while the analytical mobile phase significantly reduced peak tailing for cefonicid, thereby increasing plate count, it also reduced the capacity factor for the cefonicid peak, causing it to be eluted much closer to the tetrazole peak. The overall result was a significant decrease in resolution. Since with water as the mobile phase no impurities are eluted after the cefonicid peak, there is no advantage in a mobile phase which reduces peak tailing other than the reduction in cycle times and cost of product concentration. Actually, the advantages of reduced cycle time were negated because it was found that an impurity, which remains on the column with water as the mobile phase is eluted with the analytical mobile phase after the cefonicid peak. This results in a longer cycle time for the analytical mobile phase than for the water mobile phase.

TABLE II

COMPARISON OF MOBILE PHASE EFFECT ON RESOLUTION

Sample: crude antibiotic solution, 20% solids, 88% purity. Column: 60 × 20 cm I.D., 8.8 kg C_{18} silica, 55–105 μm. Analytical mobile phase: methanol–0.1 *M* ammonium phosphate–water (12.5:10: 77.5), pH 5.3.

Mobile phase	*Sample loading (mg/g)*	*Elution volume (l)*	*Cycle volume (l)*	k'_{CEF}	N_{CEF}	R_s
Water*	20	21	50	3.7	38	2.5
Water*	26	23	52	3.5	34	1.7
Analytical**	23	11	90	2.0	100	1.1

* Average of four runs.
** Average of two runs.

The additional cost of the mobile phase and the complexity of removing organic solvent and inorganic salts from the isolated product clearly outweigh the advantages of a more concentrated isolate. This result illustrates the difficulty of adopting results from non-overloaded analytical columns for overloaded process systems. Mobile phase selection for analytical systems should be performed at the load intended for the preparative systems in order to be useful for the scale-up.

Effect of chromatographic resolution on product yield

Product yield is defined as the percentage of the cefonicid isolated from the injected sample as a purified fraction. In our experiments the isolated fraction was the portion of the total cefonicid peak giving purity of at least 93%. The portion of

the total cefonicid peak with purity in excess of 93% increased with higher chromatographic resolution, and a plot of yield *vs.* R_s (Fig. 9) shows the dependence of yield on the chromatographic separation. Below R_s values of 1.0 product yield from starting material of 89% purity declined sharply as larger volumes of the cefonicid peak were contaminated with the tetrazole impurity. Above R_s values of 1.0 product yield was essentially constant, since the tetrazole and cefonicid peaks were fully resolved.

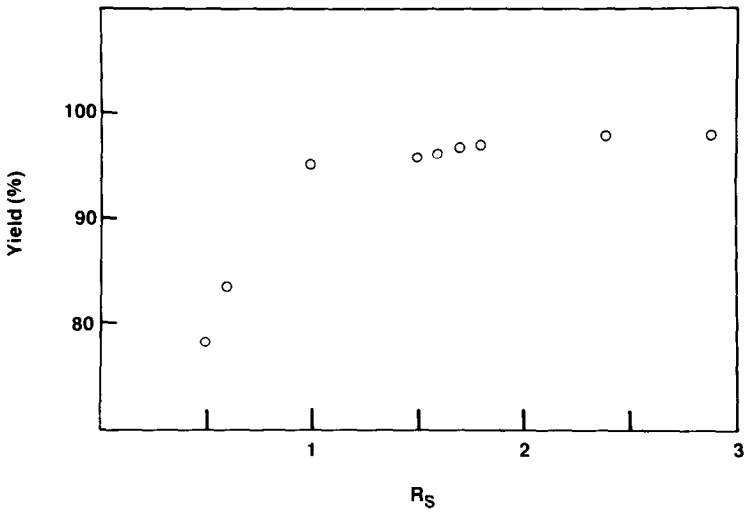

Fig. 9. Isolated product yield *vs.* chromatographic resolution. Column: 60 × 20 cm I.D.; 8.8 kg C_{18} silica. Mobile phase, water; sample loading, 7–57 mg crude cefonicid/g absorbent; product purity, 93% minimum; sample purity, 89%; flow-rate, 2–5 l/min.

Maximizing chromatographic process throughput

Chromatographic throughput, defined as the weight of purified product isolated per unit time per unit weight of adsorbent, can be expressed in terms of measurable parameters:

$$\text{Throughput} = \frac{(\text{sample load})\,(\text{product yield})\,(\text{flow-rate})}{(\text{total elution volume})}$$

The results shown in Fig. 9 demonstrate that above an R_s value of 1.0, product yield is 95–98%. Therefore, at acceptable R_s values, the influence of product yield on throughput is insignificant. Similarly, it was found that total elution volume, the volume of eluent used from injection until baseline is re-established and a second injection can be made, was relatively insensitive to the operating parameters of sample load, sample purity, sample volume, and mobile phase flow-rate. The total elution volume was lowest for small loads and low flow-rate, and highest for large loads and high flow-rate. The range was 42–65 l, with an average of 52 l. For the operating conditions providing acceptable product purity and recovery, the process throughput can be approximated as:

(Throughput, mg/min g) = ($0.02\,l^{-1}$) (flow-rate, l/min) (sample loading, mg/g)

A plot of the results for two sample load levels is shown in Fig. 10. The de-

pendence of throughput on flow-rate, assuming constant elution volume and product yield, is shown by the straight lines for two load levels. In the small load series (20 mg/g) the deviation from linearity in the actual experiments is primarily due to increased elution volume. In the large load series it is apparent that the deviation from linearity is more pronounced. This is not only due to the increasing elution volume, but also to the decrease in resolution at higher flow-rates and the corresponding loss in product yield.

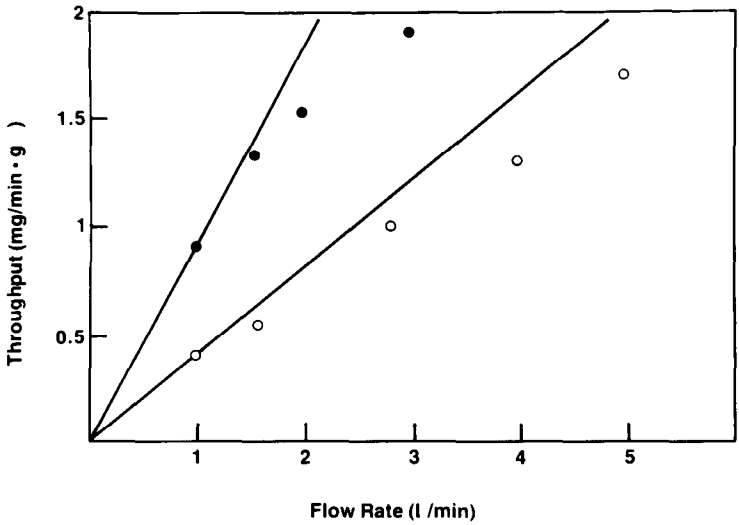

Fig. 10. Process throughput *vs.* elution flow-rate. Column: 60 × 20 cm I.D.; 8.8 kg C_{18} silica. Mobile phase, water. Sample loading: ●, 50 mg crude cefonicid/g absorbent; ○, 20 mg crude cefonicid/g absorbent; —— assuming constant elution volume and product yield. Mobile phase, water. Product purity, 93% minimum. Sample purity, 89%.

Our results indicate that increasing the flow-rate rather than sample load is the most favorable means of increasing process throughput. A general procedure for maximizing throughput would consist of increasing the mobile phase flow-rate to the highest level compatible with the chromatographic system. Pressure limits or pump capacity would determine this value. At this flow-rate, sample load would be increased until the chromatographic resolution between the product peak and the nearest significant impurity peak approaches 1.0. This would be the region of maximum process throughput.

Analysis of material balance results

The ability to account in the eluate for all the material injected into the column gives assurance that there is no net loss of material due to irreversible binding to the stationary phase. Similarly, if it can be shown that no stationary phase appears in the eluate during routine operation, it can be assumed that column erosion or solubilization is not occurring to any significant degree. If either of these conditions were to occur, column performance upon repeated use would decline. Short column-lifetime would adversely affect the economics of process chromatography, since suitable column packings are presently in the 500–1000 US$/kg range.

Careful material balances were performed where the weight of non-volatile anhydrous solid introduced into the column was compared with the weight of non-volatile anhydrous solid recovered in the total eluate. This showed that the recovery of injected samples was 100 ± 3%. Table III gives the results of individual injections as well as multiple injections. It is notable that a significant amount of the injected sample is retained on the column until it is eluted with methanol. A relatively small quantity of methanol (4 l) is effective in removing more than 90% of the strongly adsorbed material. Successive methanol washes show practically no additional solids and the material balance has reached 100% recovery. It was noted that low-purity starting material had a higher percentage of "methanol-eluted" compounds. Interestingly, when repeated injections of the low-purity starting material were chromatographed without intervening methanol washes, the percentage of "methanol-eluted" solids declined. Chemical analysis showed that the purity of the cefonicid fraction declines with successive injections, despite the fact that the R_s values are constant and the fraction volumes and cutoff points are identical. This is evidence for a saturation phenomena in which the capacity of the column for the strongly adsorbed impurities is exceeded. This results in a lack of resolution of these impurities and they are eluted throughout the entire elution process.

TABLE III

COLUMN MATERIAL BALANCES FOLLOWING: (1) REPEATED INJECTIONS WITHOUT INTERVENING COLUMN WASH; (2) SINGLE INJECTIONS FOLLOWED BY COLUMN WASH

Sample: crude antibiotic solution, 10% solids, 81% purity in (1), 42% purity in (2). Column: 60 × 20 cm I.D., 8.8 kg C_{18} silica, 55–105 μm. Mobile phase: water.

Injection No.	*Solids injected (g)*	*Solids in product fraction (g)*	*Solids in all mobile phase fractions (g)*	*Solids in wash solvent fractions (g)*	*Recovery (%)*
Repeated injection					
1	502	421	470	–	93.7
2	502	416	495	–	98.6
3	502	442	491	–	98.0
4	502	459	495	–	98.6
5	502	447	492	62	110.4
Run total	2510	2185	2443	62	99.8
Single injection					
1	567	216	513	69	102.6
1	180	110	159	16	97.2
1	131	77	117	16	101.5

Evidence thus far indicates that these strongly adsorbed impurities (See Fig. 3, peaks 5–7), are non-polar, and have MWs of 2000–5000. The results shown in Table I indicate that product purity declines once the column has become saturated with the strongly adsorbed impurities. This saturation phenomenon occurs after *ca.* 170 g of the strongly adsorbed impurities have accumulated in the column by repeated injection without an intervening methanol wash. Interestingly, the presence of strongly adsorbed impurities on the stationary phase does not influence the resolving power

of the column for cefonicid and the principal impurity, tetrazole, as evidenced by the constancy of R_s values upon repeated injections (Table I). It should be possible to introduce a pre-column for the removal of strongly adsorbed impurities. Such a pre-column should have a high capacity for the impurities, possibly by having a high bonded-phase content of the silica. The pre-column would not function as a chromatographic column since it would be highly overloaded with injected crude cefonicid. However, by absorbing the strongly adsorbed impurities it would allow longer operation of the chromatographic column between methanol washes, and if regenerated separately, it could totally eliminate the need for main column regeneration, thereby significantly increasing the process throughput.

Column dissolution study

The results of column material balances (Table III) show that there is no decline in column performance (as measured by repeated R_s values under the same operating conditions) due to irreversible binding of strongly adsorbed impurities on C_{18} silica. Similarly, a material balance test to determine whether there is slow dissolution of the bonded phase or silica support demonstrated negligible solid phase losses under the column operating conditions used in this investigation.

Purified water was pumped through a clean C_{18} silica column at a flow-rate of 2 l/min. A 100-l fraction of eluent was collected and evaporated to 2 l. The concentrate, taken to dryness by lyophilization, yielded 78 mg of anhydrous solids. The elemental analysis of the solids was Si, 18.1%; O, 25.7%; C, 46.8%; H, 8.1%. These are elements expected from dissolution of the silica support and the octadecyl bonded phase. In the course of the investigation, *ca.* $4 \cdot 10^3$ l of water was pumped through the chromatographic column. Assuming a constant dissolution rate, total solid phase loss can be estimated to be 3.1 g over the course of this investigation.

A second method for evaluating column dissolution relied on the ability to measure the column radial compression pressure at the beginning and end of the investigation. As the column volume declines due to column dissolution, the pressure of the hydraulic fluid which fills the annular space between the PTFE column liner and the steel column shell declines. Over the course of the investigation the radial compression pressure declined from 155 to 90 p.s.i. Using a bulk modulus of $9 \cdot 10^{-10}$ m^2/N for the hydraulic fluid and an initial column volume of 12 l, the column volume change can be calculated from the bulk modulus equation: $M_s = P_2 - P_1/(V_1 - V_2/V_1)$, where P_1, P_2; V_1, V_2 are the initial and final pressure and volume, respectively. The column volume change was calculated as 10.2 cm^3. Using a bulk density for the column of 0.4 g/cm^3, a column dissolution of 4.1 g can be estimated. Thus the material balance and radial pressure drop estimates are in good agreement.

Since methanol was used for column regeneration and the column was stored in methanol when not in use, an experiment was conducted to estimate column dissolution in methanol. After the column was washed twice with 4.0 l of methanol, the column was saturated with methanol and stored 48 h. The methanol-equilibrated column was then eluted with water and the methanol fraction was collected and evaporated to dryness. Elemental analysis and analytical size exclusion chromatography indicated that the residue consisted of three distinct components. The principal component (50.6%) was octadecyl alcohol, derived from the bonded phase. The second component (41.2%) was identified as an oligomeric decomposition product

of cefonicid. This was the same material as that identified as the principal component in the first two methanol washes of a used column. The balance (7.8%) was sodium silicate, derived from the silica support. The total solids in the 4-l methanol volume was 11 mg of octadecyl bonded phase, and 1.7 mg of silica support, or *ca.* 1.5 ppm of the total stationary phase. These results indicate that the dissolution of the column under the conditions used in this investigation resulted in trivial losses of column material and no loss in column performance.

CONCLUSIONS

Crude antibiotic preparations of cefonicid have been purified with 12-l octadecyl silica columns to 93% purity (minimum anhydrous) in 95% yield. The purity and yield were shown to be a sensitive function of sample load. Other process variables, such as mobile phase flow-rate, sample volume, sample purity, and column conditions influenced the purity and yield, but to a much lesser extent than sample load. The chromatographic resolution was shown to be a valid predictor of chromatographic performance. After demonstrating that plate count was the major contributor to improved column performance, a strategy for maximizing process throughput was designed. This consists of utilizing the smallest particle size of silica packing material compatible with the pressure limitations of the HPLC system and operating the system at the maximum flow-rate achievable. Under these conditions, sample loads are increased until R_s drops to a value of 1.0. These conditions provide maximum process throughput and favorable purity and yield of isolated product. The concern that column lifetime would be a significant determinant of overall process economics was shown to be unfounded. Repeated cycles of the process showed no irreversible loss in column performance. Material balances demonstrated that all components applied to the column could be recovered by a suitable wash cycle. Column dissolution with repeated use was shown to be trivial.

Upper limits on the purification capability of the HPLC were shown to be dependent upon the elution order of the product and the associated impurities. Early-eluted impurities typically merge with a later-eluted product fraction as the concentration of the impurity decreases. However, late-eluted impurities are better separated from an earlier-eluted product as the impurity concentration decreases.

Selection of a suitable chromatographic system for conducting a given separation has a significant impact on the process capacity and the process economics. Since preparative chromatography is performed at sample–column ratios much higher than the ratios used in analytical HPLC systems, it is important to select a chromatographic system under load conditions approximating those which will be used for the process scale rather than those used in the analytical system. Under similar load conditions, the results from small-scale experiments can be confidently applied to the larger process systems.

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