HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NATURAL PRODUCTS

I. SEPARATION OF CEPHALOSPORIN C DERIVATIVES AND CEPHALOSPORIN ANTIBIOTICS; ISOLATION OF CEPHALOSPORIN C FROM FERMENTATION BROTH

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Microbonded propylamine silica with a solvent system containing acetic acid, methanol, acetonitrile and water (2: 4: 7.5: 86.5) is suitable for an efficient separation of mixtures containing several closely related cephem derivatives. The same system with preparative columns was used for the isolation of cephalosporin C directly from the filtered broth of *C. acremonium* fermentation.

High performance liquid chromatography (HPLC) has recently been applied to the study of numerous problems associated with cephalosporin antibiotics. These include purity of pharmaceutical preparations,[1] metabolism,[2,3] decomposition[4] of cephalosporin antibiotics and the presence of desacetoxy and desacetyl cephalosporin* C in fermentation broth.[4]

Our interest in the biosynthesis of β-lactam antibiotics[5] prompted us to explore the utility of HPLC in the routine examination of different cephem derivatives. The ultimate goals for the application of this technique were the chromatography of fermentation broths of *Cephalosporium acremonium*, and the isolation of different metabolites contained therein for identification by conventional criteria of identity (spectral data, biological assays, *etc.*).

Earlier HPLC experiments described the use of ion-exchange and reversed phase chromatographies.[1~4] Using microbonded propylamine silica (Micropak NH_2-Varian and Microbondapak NH_2-Waters) with a mixture of appropriate solvents, we were able to achieve greater selectivity, shorter time of analysis and excellent sensitivity and reproducibility.

Materials and Equipment

All chromatograms were obtained using Waters M-6000 pump, U6K septumless injector, 254 nm uv detector (Waters Assoc. Milford, Mass.) with Fischer Omniscribe recorder (Fisher Scientific, Cincinnati, Ohio). Solvents used were glacial acetic acid (J. T. Baker), glass distilled methanol and acetonitrile (Burdick and Jackson, Muskegon, Michigan) and deionized water. The stationary phase was bondedphase propylamine on 10 micron silica (Waters Assoc., 4 mm ID/30 cm, 8 mm ID/60 cm, and/or Varian Aerograph, 2 mm ID $\times$ 25 cm). Broth injections were made using a 38 $\times$ 2 mm ID precolumn, dry and packed with ANH (Cyanoethyl silicone on silica 37~75 μ DuPont, Wilmington, Del.) to act as a disposable filter. In preparative runs appropriate fractions were collected, lyophilized and examined by conventional methods (TLC, NMR, bioassays, *etc.*). In regard to column characteristics, the number of theoretical plates has been calculated for the thiazole derivative (**7**) in Fig. 6. The capacity factor, $k' = t_R - t_0 / t_0 = 5 - 1.1/1.1 = 3.54$. The effective theoretical plates, $N_{eff} = 16(t_R - t_0/t_0)^2$

* Throughout this manuscript the word cephalosporin C has been substituted by ceph C.

=240. The use of the precolumn ANH could be extended as long as the pressure remained constant. This depended upon the effectiveness of filtration of the broth prior to injection. All cephem derivatives were injected as sodium salts in aqueous solutions. Their structures on Figs. 1~6 have been portrayed as free acids since the mobile phase at pH 3.0 would necessarily convert these derivatives towards that ionization state. Injection volumes were in the range of 1~15 μl. Accordingly, the concentration of each compound was adjusted to 0.1~1.0 mg/ml. The limit of detection was dependent upon retention time (t_R) of a given component, since extinction coefficients are reasonably constant at 254 nm. In the case of nuclei (7ACA) and lactones the detection limit was of the order of 0.02 μg. Chromatographies were run at room temperature (26°C).

Results and Discussion

We were particularly interested in the identification of various constituents of the filtered fermentation broth of *C. acremonium*. Ion-exchange methods with a variety of solvents were first tried. Unfortunately, in our hands, the activity of the columns was not consistent, probably due to the great variety of substances present in the broth. Therefore, we proceeded to the use of reversed phase chromatography, such as C_{18}/Corasil II and C_{18}/Porasil B (37~75 μ) B (Waters Assoc.) which is known for its stability. Attempts to separate ceph C, desacetyl ceph C and desacetoxy ceph C using these adsorbents with a variety of solvents as mobile phase were not successful.

We considered the use of microbonded propylamine column for two reasons. The stationary phase is much more polar than the octadecyl function; this allows a better retention of metabolites that are not extractable into organic solvents. The amino function acts as a weak anion-exchanger at low pH giving an additional parameter of selectivity. Fig. 1 shows a chromatogram of 7-(5-amino-5-carboxy-valeramido)-3-methyl-thiomethyl-3-cephem-4 carboxylic acid

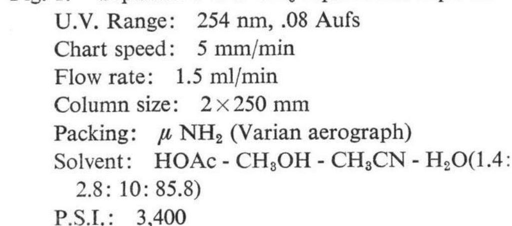


Fig. 1. Separation of S-CH_3-ceph C and ceph C.
U.V. Range: 254 nm, .08 Aufs
Chart speed: 5 mm/min
Flow rate: 1.5 ml/min
Column size: 2×250 mm
Packing: μ NH_2 (Varian aerograph)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O(1.4: 2.8: 10: 85.8)
P.S.I.: 3,400

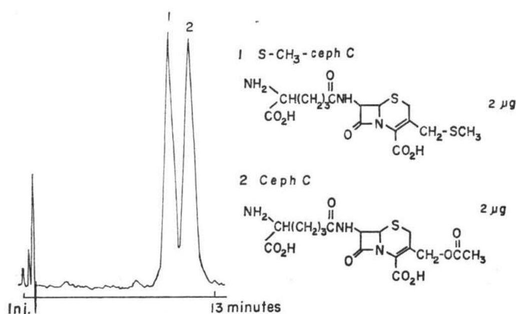


Fig. 2. Separation of desacetoxy, desacetyl, S-CH_3-ceph C and ceph C.
U.V. Range: 254 nm, .08 Aufs
Chart speed: 5 mm/min
Flow rate: 1.5 ml/min
Column size: 2×250 mm
Packing: μ NH_2 (Varian aerograph)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (1.4: 2.8: 10: 85.8)
P.S.I.: 3,400

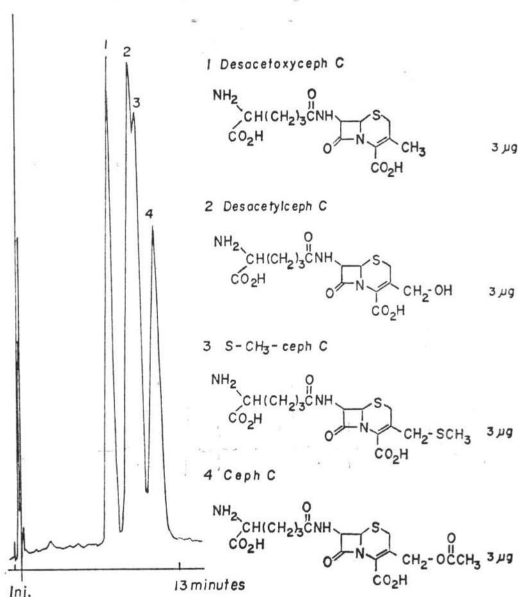


Fig. 3. Separation of desacetoxy, desacetyl, S-CH_3-ceph C and ceph C.
U.V. Range: 254 nm, .08 Aufs
Chart speed: 5 mm/min
Flow rate: 1.5 ml/min
Column size: 2×250 mm
Packing: μ NH_2 (Varian aerograph)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5)
P.S.I.: 3,400

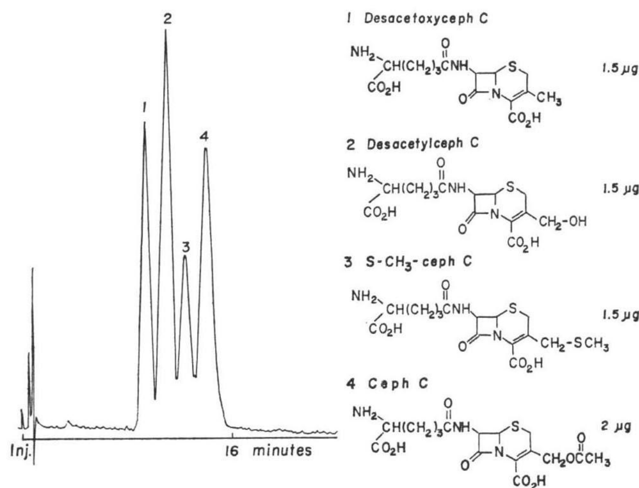
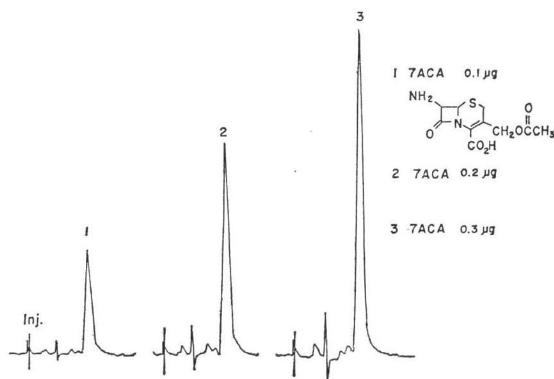


Fig. 4. Relationship of the peak area and the quantity of injected 7ACA.
U.V. Range: 254 nm, .08 Aufs
Chart speed: 5 mm/min
Flow rate: 1.5 ml/min
Column size: 2×250 mm
Packing: μ NH_2 (Varian aerograph)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (1.4: 2.8: 10: 85.8)
P.S.I.: 3,400



(3-thiomethyl-ceph C-analog) and ceph C. In Fig. 2, 3-thiomethyl-ceph C-analog (S-CH_3-ceph C) and desacetyl ceph C are only partially resolved. This separation could be improved and in Fig. 3 is shown the separation of these compounds and two other cephem derivatives by the use of less acetonitrile (greater retention) and more acetic acid and more methanol (greater efficiency).

Our studies indicated that acetic acid and methanol were most effective in a 1:2 ratio. The increases of the total content of these two solvents in relation to acetonitrile and water sharpened the peaks and improved the resolution. Identification of peaks in a mixture was achieved by spiking with a known standard in a similar procedure and is not shown.

The linear relationship of the peak area to the quantity injected is indicated in Fig. 4.

The absolute retention time varied with column age. However, it was observed that equilibrium was reached after several hours of use and did not change significantly with time.

After completion of the above-mentioned separations on analytical columns (Varian Aerograph), we subjected a mixture of cephem derivatives to chromatography on Waters columns (300×4 mm) in anticipation of preparative runs with filtered fermentation broth. The degree of separation of a mixture of several cephalosporin antibiotics is shown in Fig. 5. In runs involving mixtures of compounds each component was introduced separately into the loop of the U6K injector (Waters) prior to injection of the entire content of the loop into

Fig. 5. Separation of the cephalosporin antibiotics.

Column: Bonded phase propylamine on 10 μ silica (Waters) 300×4 mm
Solvents: HOAc - MeOH - CH_3CN - H_2O (2: 4: 50: 44)
Speed: 6 ml/min U.V.: 254 mm (0.08 Aufs) $\Delta P \sim 4,000$ psi

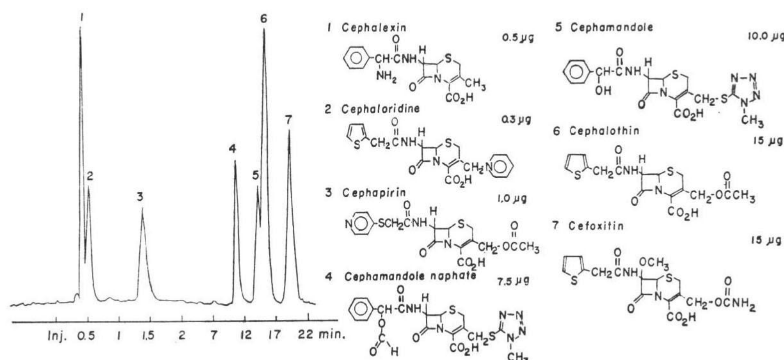
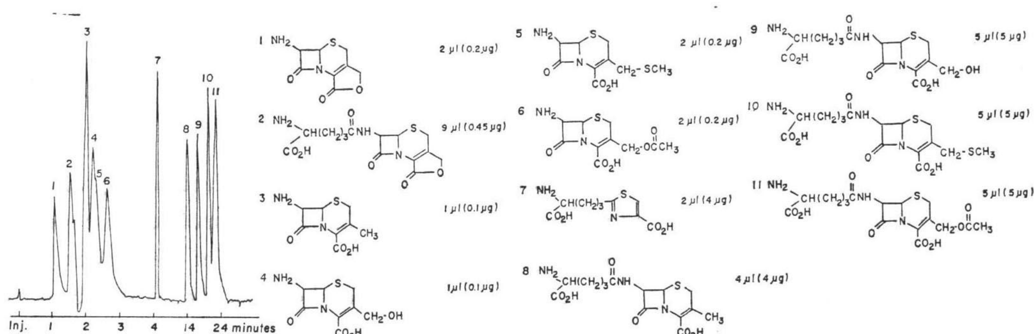


Fig. 6. Separation of different cephem derivatives.
$N=16(5/1)^2=400$

Column: Bonded phase propylamine on 10 μ silica (Waters)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5)
U.V.: 254 nm (0.08 Aufs) $\Delta P \sim 2,000$ psi



the system. In Fig. 6, a mixture of ten cephem derivatives and a thiazole derivative[6] was subjected to separation and an excellent resolution obtained.

These results prompted us to use the same system for the preparative isolation of ceph C from fermentation broth using slightly larger columns. We first established the peak position of the antibiotic by spiking with an authentic sample of ceph C. A typical analytical run is shown in Fig. 7. Next we switched to the preparative column (8×600 mm, Waters). In the preparative run, repeated many times, amounts of 0.5~1.5 ml of filtered fermentation broth were injected and the appropriate fraction collected and lyophilized. In Fig. 8 is shown a preparative run of 1.3 ml of filtered broth. The yield of antibiotic varied between 55~70% based on the biological assay of ceph C in the starting material. The identity and purity of ceph C obtained was established by comparison of TLC and NMR with those of an authentic specimen.

On the basis of our experience with the isolation of ceph C from filtered fermentation broths and mycelial extracts of *C. acremonium*, it is apparent that the microbonded propylamine silica lends itself very well to the isolation of small amounts of metabolites. In this study we found that the lifetime of

Fig. 7. Analytical chromatography of *C. acremonium* fermentation broth.
U.V. Range: 254 nm, .08 Aufs
Chart speed: Var. mm/min
Flow rate: 3 ml/min
Column size: 4×300 mm
Packing: μ NH_2 Bonded phase propylamine on 10 μ silica (Waters)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5)
P.S.I.: 3,400

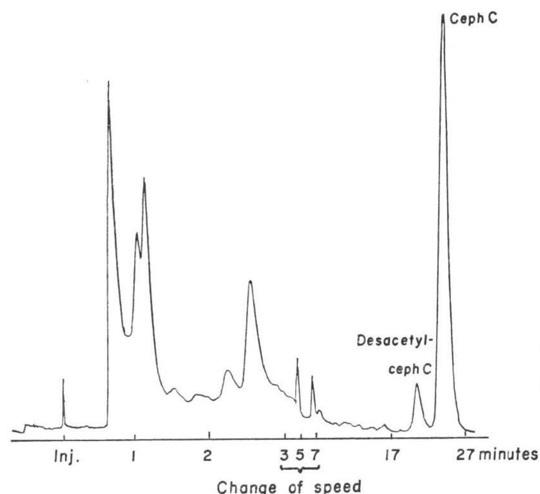
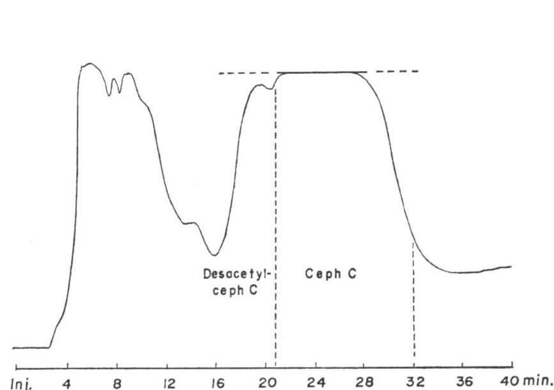


Fig. 8. Preparative chromatography of *C. acremonium* fermentation broth.
U.V. Range: 254 nm
Chart speed: 6.35 mm/min
Flow rate: 4 ml/min
Column size: 8×600 mm
Packing: μ NH_2 Bonded phase propylamine on 10 μ silica (Waters)
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5)
P.S.I.: 2,000



the column was limited to approximately six months of daily use with intermittent purging with methanol after 6~8 hours of operation. It should be pointed out that the pH of the mobile phase used in our experiments approaches the extreme value suggested for the operating range of the packing. Under such circumstances, the performance of the column over that period of time is most satisfactory considering the variety of substances present in fermentation broths.

From analytical runs depicted in Figs. 1~6, involving cephalosporin antibiotics and various cephem derivatives, it is apparent that the separation system used is efficient and versatile. Our microbonded propylamine silica columns are still performing well after one year of use.

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