Reversible phase high speed liquid chromatography of antibiotics

II. USE OF HIGH EFFICIENCY SMALL PARTICLE COLUMNS*

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Improved methods for the separation and quantitation of cephalosporins, penicillins, tetracyclines and several miscellaneous antibiotics by reverse phase high speed liquid chromatography are presented. The methods have been improved significantly by the substitution of high efficiency, small particle (~10 μm) reverse phase columns in place of the previously used medium efficiency, pellicular columns. The conditions and procedures described here illustrate that considerable improvements in separation and sensitivity of detection of antibiotics are achieved. Pure compounds, complex mixtures of antibiotics in a variety of dosage forms and fermentation broths are routinely analyzed by the described procedures.

In a previous paper we described conditions for the determination of cephalosporins, penicillins, tetracyclines and other miscellaneous antibiotics by reverse phase high speed liquid chromatography. A second paper dealt with a specific application of this technique to the determination of cephalexin and cephadine in various pharmaceutical formulations and in physiological fluids.

The application of reverse phase methods to the analysis of cephalosporins and penicillins has been reported by other investigators recently. Also, a new method which employs an amino column has been reported for the analysis of cephalosporin C derivatives and cephalosporin antibiotics. Several authors have reported on the use of reverse phase methods for the determination of tetracyclines. For reasons discussed in our previous paper, we prefer to use reverse phase instead of ion exchange chromatography for antibiotics. However, ion exchange high pressure liquid chromatography has also been used successfully for the analysis of cephalosporins, penicillins and tetracyclines.

Most of our previously reported methods as well as much of the work just cited employed large particle (~37~50 μm), medium efficiency pellicular columns. While these columns are adequate for many applications, there are several disadvantages with them such as poor separation, long analysis times, poor sensitivity and low capacity. Recently, high efficiency, totally porous, small particle (~10 μm) reverse phase columns have become available which overcome most of these disadvantages and offer significant improvements in performance. The primary advantages associated with these columns are: (1) better separations; (2) shorter analysis times; (3) sharper peaks; (4) higher sensitivity; (5) greater retentivity and (6) larger capacity.

In this paper improved methods which employ high efficiency, small particle reverse phase columns are presented for the determination of cephalosporins, penicillins and tetracyclines as well as some miscellaneous antibiotics. These methods are applicable to pure chemicals and a wide variety of pharmaceutical formulations.

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Experimental

Apparatus
The following types of liquid chromatographs were used in this study: Dupont Models 820, 830 and 840 (E. I. duPont deNemours and Co., Inc., Instrument Products Division, Wilmington, Delaware); Chromatronics 3100 (Chromatronics Division of Spectra-Physics, Berkeley, California); self-built instruments consisting of pump (Model No. 26980-4, Haskel Engineering and Supply Co., Burbank, California), UV detector (Model No. 153, Altex Scientific Inc., Berkeley, California) and injection port (Chromatronics Division of Spectra-Physics, Berkeley, California). All instruments were equipped with UV detectors (254 nm). In some instances a variable wave length UV detector (Model SF770, Schoeffel Instrument Co., Westwood, New Jersey) was used with the self-built instruments. A refractive index detector (Model R401, Waters Associates, Milford, Massachusetts) was used for some separations. Most samples were syringe-injected with a 10 μl syringe (Catalog No. 160022, C–160 Syringe, Precision Sampling Co., Baton Rouge, Louisiana). However, some instruments were equipped with sample valve injectors as well. All separations were carried out at room temperature.

Reagents
The water in the mobile phases was de-ionized and distilled. Other reagents used in the mobile phases such as various salts and organic solvents were obtained from either Arthur H. Thomas Co., Philadelphia, Pennsylvania or Fisher Scientific Co., Pittsburgh, Pennsylvania.

Materials
Cephalosporins, penicillins and tetracyclines are the three major classes of antibiotics studied. The structures for many of these compounds are given in the first paper.

Column Packings
All of the column packings used in this study were chemically bonded. Octadecylsilyl (ODS) Sil-X–II was obtained from Perkin-Elmer Corporation, Norwalk, Connecticut. C18/Porasil B was obtained from Waters Associates, Milford, Massachusetts.

Columns
All analytical columns used in this study were made from 6.3 mm O. D. 316 stainless steel of various lengths and internal diameters.

Results and Discussion
In the previous paper it was shown that reverse phase chromatography was applicable to a wide variety of antibiotics. This was demonstrated by the separation of cephalosporins, penicillins, tetracyclines and miscellaneous antibiotics that were carried out on the state of the art large particle (~37~50 μm), medium efficiency columns. However, now that high efficiency, small particle (~10 μm) columns are available, significant improvements in the separations have been made. These improvements are best presented by a visual comparison of the separations on a medium efficiency column versus a high efficiency column. For this reason, two figures will be presented for each application—(a) medium efficiency; (b) high efficiency.
6-APA and 7-ACA

In order to separate 6-APA and 7-ACA, the nuclei for penicillins and cephalosporins, respectively, it is necessary, because of their highly polar nature, to employ a heavily loaded column such as C18/Porasil B. Fig. 1a shows the separation obtained with 0.1% ammonium carbonate solution as the mobile phase. The ammonium carbonate was necessary to obtain sufficient retention of the two compounds. As can be seen, the separation is incomplete, the sensitivity for 6-APA is poor at 254 nm and the analysis takes a rather long twenty minutes. Fig. 1b shows a much improved separation on a high efficiency 10 µm C18/Lichrosorb column. Now there is baseline resolution between the two compounds and the analysis is completed in about one third the time. Detection with a variable wave length detector at 225 nm gives much better sensitivity for 6-APA. The improvement in separation is mainly due to the much higher efficiency of the C18/Lichrosorb column -10,000 plates per meter versus 700 plates per meter for the C18/Porasil B column. Note that sodium dihydrogen phosphate rather than ammonium carbonate was used for the separation in Fig. 1b. There are two reasons for this change in mobile phase.

![Fig. 1a. Separation of 6-APA and 7-ACA.](image1)

![Fig. 1b. Separation of 6-APA and 7-ACA.](image2)

![Fig. 2a. Separation of some cephalosporin starting materials.](image3)

![Fig. 2b. Separation of some cephalosporin starting materials.](image4)
First of all, it was found that sodium dihydrogen phosphate gave sharper peaks and a better separation. Secondly, because of the basic nature of ammonium carbonate (~pH 9), it tends to dissolve the small silica particles and thus damage the column. Deterioration by ammonium carbonate was not noticed with the large particle columns.

**Cephalosporin Starting Materials**

It is important to be able to quantitate starting materials, since they are potential trace impurities in final cephalosporin products. The separation of 7-ADCA, 7-ACA, cephalosporin C and 7-tetrazolyl-amino-cephalosporanic acid (7-TACA) on a large particle C18/Porasil B column is shown in Fig. 2a. Notice that there is incomplete separation of the first three compounds.

The same mixture plus an additional compound, desacetylcephalosporin C, are separated on a 10 μm C18/Lichrosorb column as shown in Fig. 2b. Each component is now separated with practically baseline resolution and the analysis time is shortened significantly. This system is in continuous use for quantitating cephalosporin C content in fermentation broths. With the aid of an automatic sample injector as many as 100 samples per day are run completely unattended.

**Separation of Cephalosporin Mixtures**

There are many cephalosporin antibiotics which are commercially available that can be separated by reverse phase liquid chromatography. Shown in Fig. 3a is the separation on a large particle column of cefazolin, cephradine, cephaloglycin and cephalothin, which are four of the most well-known and effective compounds available. Note that this separation is incomplete. A fifth compound, cephalexin, is not shown because it cannot be separated from cefazolin.

The separation of a mixture of all five compounds carried out on a 10 μm C18/Lichrosorb column is shown in Fig. 3b. On this column the resolution of all compounds is practically complete and cephale-
xin and cefazolin are adequately separated from one another. Naturally, not all of these compounds would be present in a real mixture. However, as an example, the determination of cephalexin impurity in cephradine would be quite straight forward because of the large amount of separation between the two compounds. An important point in connection with this separation is the fact that, depending on the compound being determined, one of the other cephalosporins is a suitable internal standard.

Penicillins

The separation of three common penicillins—ampicillin, penicillin G and penicillin V—on a large particle C18/Porasil B column is shown in Fig. 4a. The separation is incomplete and sensitivity is poor.

Fig. 4a. Separation of penicillins.
Column: 1 m x 2.1 mm i. d., C18/Porasil B; mobile phase, 30% methanol, 70% 0.05 M ammonium carbonate; pressure, 500 psi; flow, 0.5 ml/min; detector, UV (254 nm); sensitivity, 0.04 AUFS; sample solvent, 0.05 M ammonium carbonate; sample size, 5 µl.

This separation can be improved significantly on a 10 µm C18/Lichrosorb column. This is shown in Fig. 4b in which these compounds as well as methicillin and oxacillin are adequately resolved. Because of the higher efficiency of the column and with the use of a variable wave length UV detector at 225 nm the sensitivities are about a factor of ten higher.

Tetracyclines

Tetracyclines are nicely separated by reverse phase chromatography. This is demonstrated in Fig. 5a in which four typical compounds—oxytetracycline, doxycycline, demeclocycline and tetracycline itself—are separated in 30 minutes. With tetracyclines it is necessary to include a small amount of ethylenediaminetetraacetic acid (EDTA) in the mobile phase to prevent their complexing with the metal tubing. If EDTA is not included badly tailing peaks are obtained which are useless for quantitation.

The separation is vastly improved on a 10 µm C8/Lichrosorb column as shown in Fig. 5b. Two additional compounds, methacycline and chlortetracycline, are also resolved on this column. The C8 column is the only small particle column which accomplished this separation. Other columns tried but which gave unsatisfactory results are C18/Lichrosorb, Spherisorb-ODS and µ Bondapak C18. All of
these columns give badly tailing peaks, even with the presence of EDTA in the mobile phase. The superior performance of the C8 column is presumably due to a very even and complete coverage of the silica surface with the C8 monolayer.

Miscellaneous Antibiotics

Chloramphenicol and intermediates have been separated on a Micropak-NH₂ column in the normal phase mode. However, chloramphenicol can also be separated by the reverse phase technique. Its separation on a large particle, pellicular column is shown in Fig. 6a. However, a rather poorly shaped peak is obtained, sensitivity is poor and the analysis time is a long 25 minutes.

This separation can also be accomplished on a small particle C18 column as shown in Fig. 6b. However, now the peak shape is excellent, the sensitivity is about 10 times higher and the analysis time has been decreased to about 4 minutes.

Fig. 7 shows a recently developed separation of erythromycin, a common macrolide antibiotic,
as carried out on a 10 pm C18/Lichrosorb column. OMURA\textsuperscript{22} et al. have also reported on the separation of erythromycin and other macrolide antibiotics by reverse phase methods. A small amount of ammonium hydroxide is used in the mobile phase to suppress the ionization of the basic group. Since this compound absorbs weakly in the UV a refractive index detector was used. Because of the high efficiency of the column and the sharp peak that is obtained, the sensitivity is fairly good for this type of detector.

Preparative Applications

One of the most important uses of liquid chromatography is in preparative applications. Because of limited capacity, it is not possible to carry out preparative separations on large particle, pellicular columns. However, several mgs of sample can be injected onto a small particle, totally porous, 10 pm C18 column without overload. This is illustrated in Fig. 8 where the separation of 5 mg of cephradine is shown. The impurities were separated away from the major component and nearly 5 mg of pure cephradine was obtained simply and easily. Since only water was used as the mobile phase, the sample was recovered easily by vacuum distillation of the water on a "roto-vap."

Conclusions

The results given in this paper illustrate that high efficiency, small particle reverse phase columns are very useful for the analysis of antibiotics. The methods given here are currently being used routinely for the quantitation and isolation of both naturally occurring and synthetically produced antibiotics. Work is in progress to improve the methods even further by the use of 5 pm reverse phase columns.
References


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