REVERSE PHASE HIGH SPEED LIQUID CHROMATOGRAPHY OF ANTIBIOTICS

III. USE OF ULTRA HIGH PERFORMANCE COLUMNS AND ION-PAIRING TECHNIQUES

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Improved methods for the separation and quantitation of cephalosporin, penicillin, aminoglycoside and anthracycline antibiotics are presented. The use of ultra high performance 5 μm reverse phase columns combined with the added dimension of ion-pairing greatly increases the ease of separation and speed of analysis of complex antibiotic mixtures. Antibiotics in a variety of dosage forms and in fermentation broths have been examined in order to provide the maximum data on impurities to meet regulatory requirements for drug safety, purity and efficacy. Mixtures of antibiotics have been analyzed to demonstrate the improved separations, increased efficiency and shortened analysis times possible with ultra high performance columns. Under these improved conditions, the danger of multiple components in a single peak are markedly reduced.

In the first paper[1] of this series we described conditions for the determination of cephalosporins, penicillins, tetracyclines and other miscellaneous antibiotics by reverse phase high speed liquid chromatography. In the second paper[2] we presented improved methods which use high efficiency, small particle (10 μm) reverse phase columns for the determination of these compounds.

The application of reverse phase methods to the analysis of cephalosporins and penicillins has also been reported by other investigators[3~8]. A review article which discusses penicillins and cephalosporins as well as other types of antibiotics and drugs has also been published[9]. Cephalexin and cephradine have been determined in plasma and urine by reverse phase methods[10,11]. Penicillin and cephalosporin diastereoisomers have been separated by reverse phase HPLC[12,13]. Also, a normal phase method[14,15] which employs an amino column has been reported for the analysis of cephalosporin C derivatives and cephalosporin antibiotics.

For reasons such as discussed in our first paper[1], we prefer to use reverse phase instead of ion exchange chromatography for antibiotics. However, ion exchange high pressure liquid chromatography has been used successfully by other investigators for the analysis of cephalosporins[16,17,18], and penicillins[19,20]. Most of the methods reported in our first paper[1] as well as much of the work reported by other investigators used large particle ($\sim 37 \sim 50$ μm), medium efficiency pellicular columns. These columns are adequate for many applications, but there are many disadvantages such as poor separation, long analysis times, poor sensitivity and low sample capacity. The use of high efficiency, totally porous, small particle (~ 10 μm) reverse phase columns as reported in our second paper[2] overcame most of these disadvantages and gave significant improvements in performance. The primary advantages associated with these columns are: (1) somewhat better separations; (2) shorter analysis times; (3) sharper peaks; (4) higher sensitivity; (5) greater retentivity and (6) larger sample capacity.

In this paper we present methods that are improved even further for the determination of cephalosporins, penicillins, aminoglycosides and anthracycline antibiotics. These methods employ ultra high efficiency, small particle (~ 5 μm) reverse phase columns and, when necessary, are combined with ion-pairing techniques. The methods are applicable to pure chemical and a wide variety of pharmaceutical formulations.

Experimental

Apparatus

The instrument used in this work was self-built and consisted of a pump (Model 26980-4, Haskel Engineering and Supply Co., Burbank, California), UV detector (Model No. 153, Altex Scientific, Inc., Berkeley, California) and sample valve (Model No. 70–10, Rheodyne, Berkeley, California). In some instances, a variable wavelength UV detector (Model SF770, Schoeffel Instrument Co., Westwood, New Jersey) was used. A refractive index detector (Model R401, Waters Associates, Milford, Massachusetts) was used for some separations. All separations were carried out at ambient 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 and penicillins are two of the major classes of antibiotics studied. The structures for many of these compounds are given in the first paper.[1]

Cephacetrile was obtained from CIBA Pharmaceutical Company. Adriamycin was obtained from the National Cancer Institute. Carminomycin was obtained from Bristol Laboratories. Daunomycin was obtained from Calbiochem, La Jolla, California. Other antibiotics used in this study were obtained from commercial sources and from the Investigational Products Laboratory of Smith Kline and French Laboratories, Philadelphia, Pennsylvania.

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.

The Spherisorb-ODS column was obtained from Spectra-Physics, Berkeley, California.

The 10 μm C8/Lichrosorb column was obtained from Altex Scientific Inc., Berkeley, California.

The 10 μm C18/Lichrosorb, 5 μm C8/Lichrosorb and 5 μm C18/Lichrosorb columns were prepared in our laboratories by a slurry packing procedure[21] using Lichrosorb S1 100 which was subsequently chemically bonded with octyltrichlorosilane or octadecyltrichlorosilane by an in-situ technique[22].

Results and Discussion

In the first paper[1] of this series 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 then state of the art large particle ($\sim 37 \sim 50$ μm), medium efficiency columns. In the second paper[2] it was shown how significant improvements in the separations were made by the use of high efficiency, small particle (~ 10 μm) columns. By using ultra high efficiency 5 μm C8 and C18 columns, dramatic improvements in these separations are achieved. In addition, with the use of ion pairing techniques, as necessary, additional separations have been obtained which were previously either very difficult or nearly impossible to achieve by conventional reverse phase methods.

Separation of Cephalosporin Mixtures

The separation of a mixture of cephalosporins carried out on a 30 cm, 10 μm C18/Lichrosorb column is shown in Fig. 1A. The resolution of cephalexin and cefazolin is incomplete, but the overall separation is quite adequate for most purposes.

Fig. 1B shows a much improved separation on an ultra high efficiency 15 cm, 5 μm C8/Lichrosorb column. Baseline resolution of all compounds has been achieved. The 5 μm column is approximately two to three times more efficient than the 10 μm column and, as a result, the peaks are much sharper and the sensitivity is greater by about a factor of two. C8 rather than C18 was used for this separation because it was noticed that the former material gave better efficiencies. This is probably due to the fact that C8 is somewhat less hydrophobic than C18, and probably more compatible with highly polar compounds such as antibiotics. This column gives much greater resolution at long retention times than does the 10 μm C18 column. In fact, the column was so retentive and the resolution was so great that it was necessary to apply a step gradient of 15~25% methanol in order to elute the last three compounds.

Fig. 1A. Separation of a mixture of cephalosporins.
Column: 30cm×4.6mm i.d., C18/Lichrosorb 10μm
Mobile phase: 17% methanol - 83% 0.01 M sodium dihydrogen phosphate
Pressure: 70 kg/cm²
Flow: 1.3 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.04 AUFS
Sample solvent: Mobile phase
Sample size: 10 μl

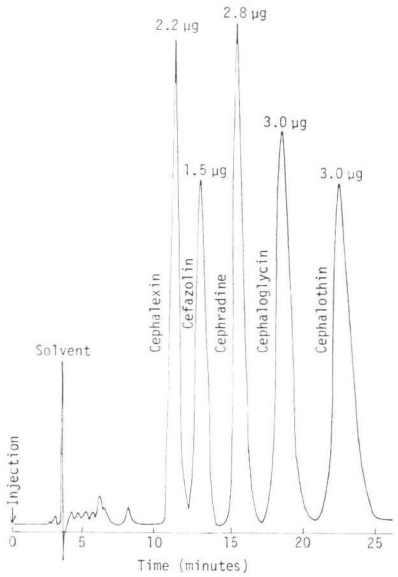
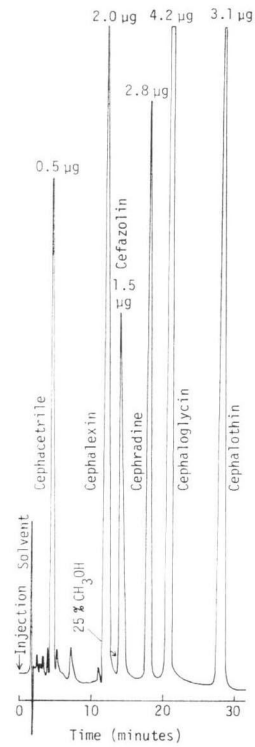


Fig. 1B. Separation of a mixture of cephalosporins.
Column: 15 cm×4.6 mm i.d., C8/Lichrosorb 5 μm
Mobile phase: 15% methanol - 85% 0.01 M sodium dihydrogen phosphate
Pressure: 91 kg/cm²
Flow: 1.0 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.08 AUFS
Sample solvent: water
Sample size: 20 μl



Cephalosporin C Fermentation Broths

Fermentation broths can be analyzed after a very simple pretreatment. Samples are centrifuged to get rid of particulate matter, then the clear solutions are simply diluted with varying amounts of water, depending on the level of cephalosporin C expected.

Fig. 2A shows the analysis of a fermentation broth for cephalosporin C and desacetylcephalosporin C on a 10 μm Spherisorb ODS column. It should be noted that desacetylcephalosporin C is separated only slightly from the solvent front which makes quantitation very difficult and inaccurate. In addition, there is evidence of another component on the tail of the cephalosporin C peak.

This separation can be greatly improved by using an ultra high efficiency 5 μm C8 column. This is illustrated in Fig. 2B where it is shown that many more components from the broth are resolved and the cephalosporin C is now separated away from all other interfering substances. However, the situation with desacetylcephalosporin C has improved only slightly. It is still too close to the solvent front for accurate quantitation and there is no way to increase the k′ value, since the mobile phase—essentially water—is already as weak as it can possibly be made.

The solution to this problem is ion-pairing. This is illustrated in Fig. 2C, where a mobile phase containing 10% methanol and 90% 0.01 M tetrabutylammonium hydroxide is effective in moving the desacetylcephalosporin C peak sufficiently far away from the solvent front to be separated from other broth constituents. In fact, now there is even room between the solvent front and the desacetylcephalosporin C peak for several other separated components. The important thing that is illustrated in this

Fig. 2A. Fermentation broth on medium efficiency column.
Column: 30cm×4.6mm i.d., ODS/Spherisorb 10μm
Mobile phase: 0.2% sodium dihydrogen phosphate
Pressure: 35 kg/cm²
Flow: 0.9 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.16 AUFS
Sample solvent: water
Sample size: 25 μl

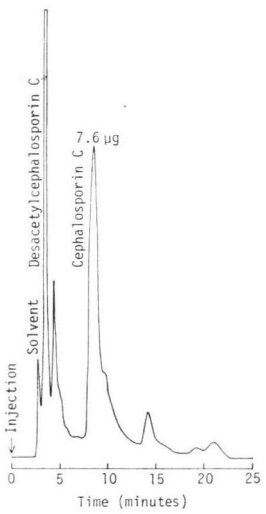


Fig. 2B. Fermentation broth on ultra high efficiency column.
Column: 15 cm×4.6 mm i.d., C8/Lichrosorb 5 μm
Mobile phase: 0.2% sodium dihydrogen phosphate
Pressure: 91 kg/cm²
Flow: 1.0 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.16 AUFS
Sample solvent: water
Sample size: 20 μl

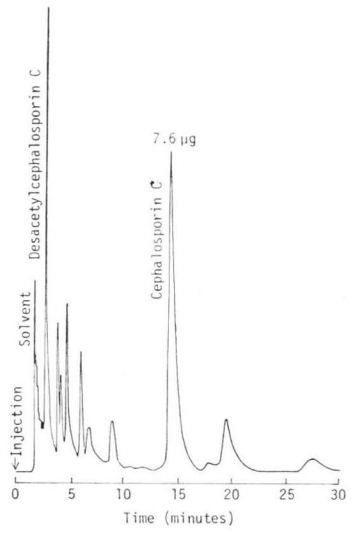


Fig. 2C. Fermentation broth on ultra high efficiency column with ion-pairing.
Column: 15 cm×4.6 mm i.d., C8/Lichrosorb 5 μm
Mobile phase: 10% methanol - 90% 0.01 M tetrabutylammonium hydroxide, adjusted to pH 7.4 with phosphoric acid
Pressure: 134 kg/cm^2
Flow: 1.0 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.08 AUFS
Sample solvent: water
Sample size: 20 μl

Fig. 3. Ion-pair separation of cephalosporin starting materials.
Column: 15 cm×4.6 mm i.d., C8/Lichrosorb 5 μm
Mobile phase: 10% methanol - 90% 0.01 M tetrabutylammonium hydroxide adjusted to pH 7.4 with phosphoric acid
Pressure: 105 kg/cm^2
Flow: 1.1 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.02 AUFS
Sample solvent: 0.05 M sodium bicarbonate
Sample size: 25 μl

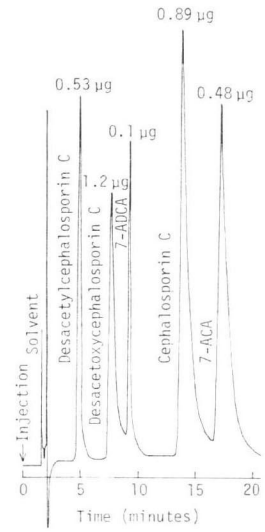


figure is that by the addition of the pairing agent, there is now complete control of the separation. For example, by changing the concentration of the methanol in the mobile phase, the resolution of the desacetylcephalosporin C peak from other broth constituents can be varied as desired.

Cephalosporin Starting Materials

Fig. 3 shows the ion-pair separation of some other related cephalosporin starting materials which are poorly retained under conditions of conventional reverse phase chromatography. Besides desacetylcephalosporin C and cephalosporin C, desacetoxycephalosporin C, 7-ADCA and 7-ACA are separated as ion-pairs with tetrabutylammonium hydroxide.

Penicillins

Fig. 4A shows the separation of a mixture of penicillins on a 10 μm C18 column. However, there is incomplete resolution of the compounds on this column.

Fig. 4B shows the separation of the same penicillins on a 15 cm 5 μm ultra high efficiency C8 column. Note that now there is baseline resolution of each compound. This improved separation is due to the increased efficiency of the column and the fact that C8 is more compatible with penicillins than is C18. Note that there is a reversal of methicillin and penicillin G on this column. Presumably, this is due to a slightly different selectivity of the column.

Fig. 4A. Separation of penicillins.
Column: 30cm×4.6mm i.d., C18/Lichrosorb 10μm
Mobile phase: 35% methanol - 65% 0.01 M sodium dihydrogen phosphate
Pressure: 91 kg/cm^2
Flow: 1 ml/minute
Detector: UV (225 nm)
Sensitivity: 0.04 AUFS
Sample solvent: Mobile phase
Sample size: 8 μl

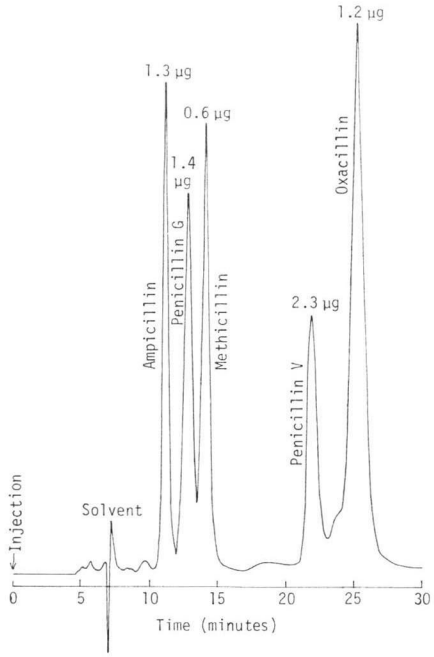
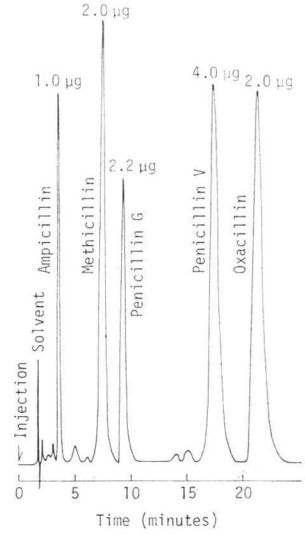


Fig. 4B. Separation of penicillins.
Column: 15 cm×4.6 mm i.d., C8/Lichrosorb 5 μm
Mobile phase: 35% methanol - 65% 0.01 M sodium dihydrogen phosphate
Pressure: 105 kg/cm^2
Flow: 1.0 ml/minute
Detector: UV (225 nm)
Sensitivity: 0.1 AUFS
Sample solvent: Mobile phase
Sample size: 20 μl



Aminoglycosides

Aminoglycosides are highly basic amino sugars. Kanamycins A and B have been separated by ion-exchange chromatography[23,24]. Gentamicin has been separated by reverse phase ion-pair chromatography[25] and by reverse-phase chromatography as the dansyl derivative[26]. However, in general, the aminoglycosides are very difficult to separate by conventional reverse phase chromatography. They can be eluted with mobile phases containing high concentrations of ammonia, but the ammonia has a deleterious effect on the column. Reverse-phase ion-pair chromatography is the best way to separate aminoglycosides. This is illustrated in Fig. 5, which shows the separation of four common aminoglycosides: streptomycin A (2.8 minutes), kanamycin A (4.1 minutes), gentamicin (7.9 minutes) and neomycin A (11.8 minutes), using camphorsulfonic acid as the pairing agent. The C_1, C_{1a} and C_2 components of gentamicin are not separated under these conditions. With the ion-pair approach, the mobile phase pH is on the acid side where the column is stable. Since these compounds have no UV chromophores it is necessary to use a refractive index detector. Because of the high column efficiency and sharp peaks which are obtained, concentrations in the 1~5 mg/ml range are measurable. In order to avoid ion-pair overload a relatively large concentration (0.05 M) of the pairing agent is used.

Fig. 5. Ion-pair separation of aminoglycosides.
Column: 15 cm×4.6 mm i.d., C18/Lichrosorb 5 μm
Mobile phase: 60% methanol - 40% 0.05 M camphorsulfonic acid, pH 1.7
Pressure: 148 kg/cm^2
Flow: 1.0 ml/minute
Detector: RI
Sensitivity: ×2
Sample solvent: water
Sample size: 10 μl

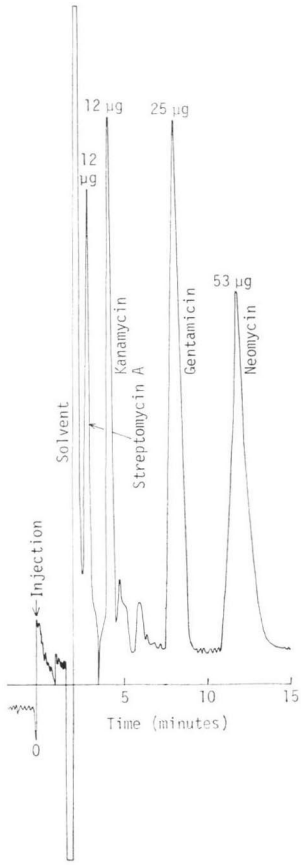
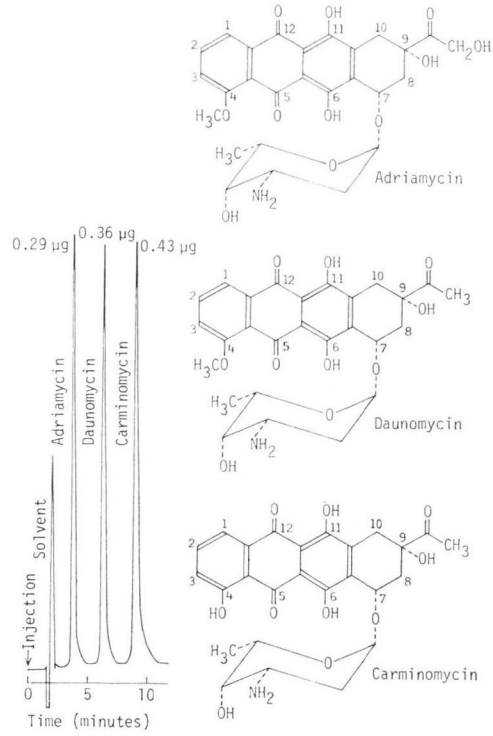


Fig. 6. Ion-pair separation of anthracycline antibiotics.
Column: 15 cm×4.6 mm i.d., C8/Lichrosorb 5 μm
Mobile phase: 50% acetonitrile - 50% 0.025 M camphorsulfonic acid adjusted to pH 3.8 with sodium hydroxide
Pressure: 127 kg/cm^2
Flow: 1.1 ml/minute
Detector: UV (254 nm)
Sensitivity: 0.08 AUFS
Sample solvent: water
Sample size: 25 μl



Anthracycline Antibiotics

Anthracycline antibiotics can be separated by high performance liquid chromatography. Daunomycin has been separated on silica[27,28] and also by reverse phase liquid chromatography[29,30]. Similarly, adriamycin has been separated by both chromatographic modes[31,32]. These compounds as well as carminomycin can be separated by reverse-phase ion-pair chromatography. This is illustrated in Fig. 6 where baseline resolution of all three compounds is obtained using camphorsulfonic acid as the pairing agent. The separation takes only about ten minutes and sharp, symmetrical peaks are obtained with high sensitivities. With this system any one of the three antibiotics would make an excellent internal standard for either or both of the other two compounds.

Conclusions

An investigation of cephalosporin, penicillin, aminoglycoside and anthracycline antibiotics by the use of ultra high performance 5 μm reverse phase columns shows considerable advantages over conventional reverse phase methods. The procedures are less time consuming, produce better resolution of compounds and minimize the possibility of hidden impurities in the main compound peak. These procedures may be applied to a large variety of antibiotics with resulting greater sensitivity to detect lower amounts of impurities. Ion-pairing techniques have been especially valuable in determining the composition of aminoglycoside and anthracycline antibiotics. However, ion-pairing is not needed for cephalosporin and penicillin antibiotics. The described methods have been successfully applied to a wide range of commercial and research antibiotics.

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