REVERSE PHASE HIGH SPEED LIQUID CHROMATOGRAPHY OF ANTIBIOTICS*

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Reverse phase high speed liquid chromatographic methods are presented for the separation and detection of cephalosporins, penicillins, tetracyclines and other miscellaneous antibiotics. The reverse phase approach is superior to ion-exchange liquid chromatography and spectrophotometric, chemical and microbiological procedures currently in use. In addition to being simple and easy to control, the technique is rapid, convenient and precise and provides the basis for the direct analysis of pure compounds, stability samples, complex mixtures and dosage forms of all types. Preparative chromatography has been used in our laboratory for the separation and isolation of up to 500 mg of antibiotics. Using this approach, we have separated and isolated small impurities as well as pure reference compounds. The methodology reported here can be extensively applied to the separation, quantitation and isolation of both naturally occurring and synthetically produced antibiotics in a variety of media including physiological fluids.

The quantitative determination of antibiotics is one of the more difficult areas of pharmaceutical analysis. The most serious disadvantage of the microbiological, spectrophotometric and chemical methods in current use is their lack of specificity. This deficiency has prompted the investigation and study of alternative methods which are fast, simple and specific, including gas chromatography and high speed liquid chromatography.

Gas chromatography has been employed for the determination of chloramphenicol,[1,2] penicillins,[3] erythromycin,[4] cephaloridine[5] and aminoglycosides.[6,7] While this technique is fast and specific, it is only applicable to those antibiotics which are thermally stable after prior derivitization.

High-speed liquid chromatography is a very powerful technique which has enjoyed much success in recent years for the analysis of many different types of compounds. However, the application of this technique to antibiotics has been limited to sulfa drugs,[8] tetracyclines,[9,10,11] cephalosporin C[12] and a few miscellaneous compounds.[13~16] Further, most of this work has been carried out using ion-exchange chromatography. This is a natural choice, since many antibiotics are highly polar ionic compounds. However, reverse phase chromatography offers an attractive alternative. The only reverse phase work reported has been on sulfa drugs[8] and tetracyclines.[9,11] This paper describes the conditions for the separation and detection of cephalosporins, penicillins, tetracyclines and other miscellaneous antibiotics by reverse phase high speed liquid chromatography.

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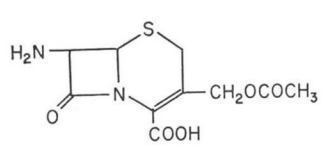
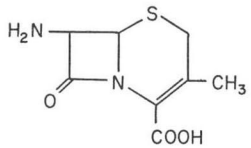


(I)



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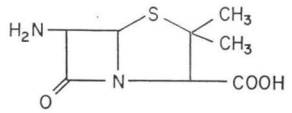

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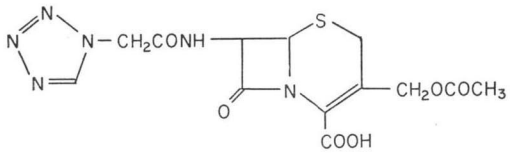

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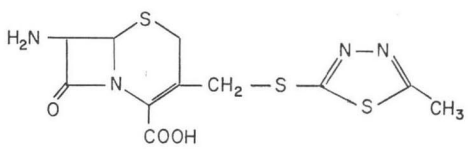

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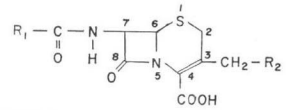
Reverse phase chromatography is normally used to separate relatively non-polar compounds such as fused ring aromatics, steroids, vitamins, pesticides, herbicides, *etc.* However, most investigators have overlooked the fact that reverse phase chromatography is also applicable to many moderately and even highly polar ionic compounds such as antibiotics.

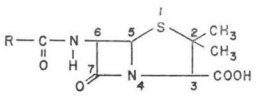
There are many disadvantages to ion-exchange chromatography. It is one of the more difficult chromatographic modes to control because of the many variables involved, including pH, ionic strength, temperature and the nature of the counter ion. On the other hand, reverse phase chromatography, in addition to being relatively simple, has several important advantages: (1) mobile phases are simple—usually aqueous solutions of methanol, acetonitrile, tetrahydrofuran or dioxane; (2) no corrosive salts are used; (3) retention times are easily adjusted by control of the organic modifier; (4) separations can be carried out at ambient temperature; (5) it is ideally suited for preparative separations; and (6) a wide variety of permanently bonded packings are commercially available.

Table 1. Cephalosporins.



R_1	R_2	Common name
HOOC-CH-$CH_2CH_2CH_2$- NH_2	-OC-CH_3 O	Cephalosporin C
		Cephaloridine
	-OC-CH_3 O	Cephaloglycin
	-OC-CH_3 O	Cephalothin
		Cefazolin
	-H	Cephalexin
	-H	Cephradine

Table 2. Penicillins.



R	Common name	R	Common name
HOOC-CH-$CH_2CH_2CH_2$- NH_2	Penicillin N		Methicillin
	Penicillin G		Phenethicillin
	Penicillin V		Nafcillin
	Ampicillin		
	Oxacillin		Dicloxacillin

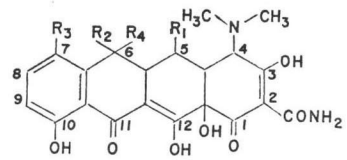
Classification of Antibiotics Studied

Cephalosporins, penicillins and tetracyclines are the three major classes of antibiotics studied. In addition, conditions are given for the separation of several miscellaneous antibiotics.

Cephalosporins: The cephalosporins constitute a relatively new and important class of antibiotics.[17] The structure for cephalosporin C, a naturally occurring material, and the structures for several of the better known synthetic cephalosporins are given in Table 1.

The two most commonly used starting materials for the synthesis of cephalosporin antibiotics are 7-aminopenicillanic acid (7-ACA) (**I**) and 7-aminodeoxycephalosporanic acid (7-ADCA) (**II**).

Table 3. Tetracyclines.



R_1	R_2	R_3	R_4	Common name
H	CH_3	H	OH	Tetracycline
OH	CH_3	H	OH	Oxytetracycline
H	CH_3	Cl	OH	Chlortetracycline
H	H	H	H	Minocycline
OH	$=CH_2$	H	—	Methacycline
H	H	Cl	OH	Demeclocycline
OH	CH_3	H	H	Doxycycline

Penicillins: The penicillins constitute a more familiar, but nonetheless very important, class of antibiotics.[17] The structures of some of these compounds are given in Table 2. One of these, 6-aminopenicillanic acid (6-APA) (**III**), is the basic starting material for the synthesis of the semi-synthetic compounds.

Tetracyclines: A third class of important antibiotics is the tetracyclines.[18] The structures of several of the better known tetracyclines are given in Table 3.

Experimental

Apparatus

The following types of liquid chromatographs were used in this study: DuPont Models 820, 830 and 840 (E. I. duPont de Nemours and Co., Inc., Instrument Products Division, Wilmington, Delaware); Chromatronix 3100 (Chromatronix 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 (Chromatronix Division of Spectra-Physics, Berkeley, California). All instruments were equipped with UV detectors (254 nm). Most samples were syringe-injected with a 10 μl syringe (Catalog No. 160022, C-160 Syringe, Precision Sampling Co., Baton Rouge, Louisiana). However, all instruments are easily modified for valve and loop injection. All separations were carried out at room temperature.

Reagents

The water used in the mobile phases was de-ionized and distilled. Other reagents used in the mobile phases were reagent-grade quality and were obtained from Arthur H. Thomas Co., Philadelphia, Pennsylvania.

Materials

The antibiotics used in this study were obtained from commercial sources.

Column Packings

All of the column packings used in this study were permanently bonded.

Octadecylsilyl (ODS) Sil-X-II was obtained from Perkin-Elmer Corporation, Norwalk, Connecticut.

Vydac Reverse Phase (RP) was obtained from the Separations Group, Hesperia, California.

C18/Porasil, C18/Corasil and Phenyl/Corasil were obtained from Waters Associates, Milford, Massachusetts.

"Permaphases" Octadecylsilyl (ODS) and Ether (ETH) were obtained from E. I. duPont de Nemours and Co., Inc., Instrument Products Division, Wilmington, Delaware.

All analytical columns used in this study were 1 m×2.1 mm i. d.×6.3 mm o. d. 316 stainless steel. They were packed in our laboratories using currently accepted dry pack procedures.[19] The columns for the DuPont 820 and the self-built instruments were straight, and the columns for the DuPont 830, 840 and Chromatronix 3100 were "U"shaped.

Results and Discussion

Reverse phase chromatography was found to be applicable to a wide variety of antibiotics. However, many variations of columns and mobile phases were necessary to gain the desired degree of separation.

Some columns were tried but were found not applicable to the antibiotics studied.

Fig. 1. Separation of 6-APA and 7-ACA.

Column: 1 m × 2.1 mm i. d., C 18/Porasil B; mobile phase, 0.1% ammonium carbonate; pressure, 200 psi; flow, 0.75 ml/min; detector, UV (254 nm); sensitivity, 0.08 AUFS.

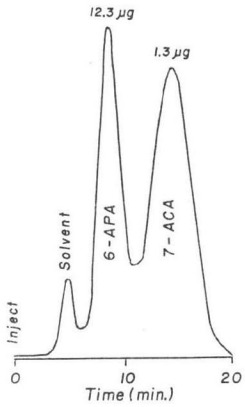


Fig. 2. Separation of some cephalosporin starting materials.

Column: 1 m×2.1 mm i. d., C 18/Porasil B; mobile phase, water; pressure, 500 psi; flow, 1.1 ml/min; detector, UV (254 nm); sensitivity, 0.08 AUFS.

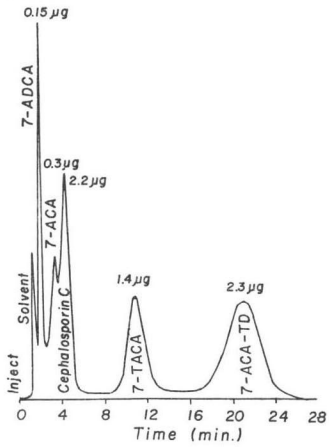


Fig. 3. Quantitation of cephradine in an oral suspension.

Column: 1 m × 2.1 mm i. d., ODS-Sil-X-II; mobile phase, 7% methanol, 93% 0.05 M ammonium carbonate; pressure, 1,000 psi; flow, 0.6 ml/min; detector, UV (254 nm); sensitivity, 0.08 AUFS.

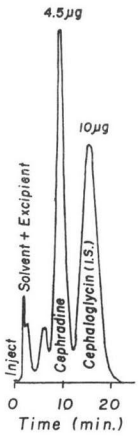
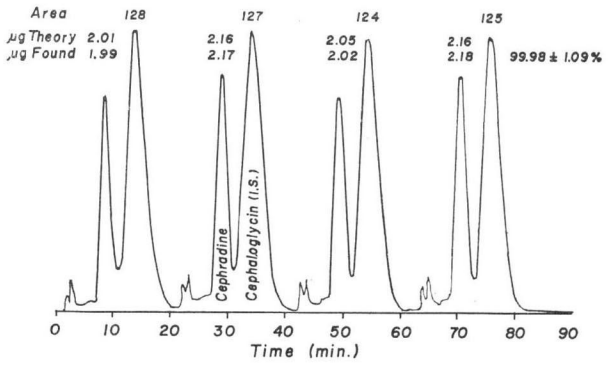


Fig. 4. Quantitation of cephradine.

Precision and accuracy. Conditions same as for Fig. 3.



"Permaphases" ODS and ETH and Phenyl/Corasil could not be used. These packings gave columns with little or no retentive power, with the result that all compounds eluted at the solvent front (k′=0). Apparently, "Permaphase" ODS does not have a sufficiently heavy liquid loading. "Permaphase" ETH and Phenyl/Corasil are both too polar for most antibiotics. Similarly, some batches of Vydac-RP and ODS-Sil-X-II gave non-retentive columns. Again, this is probably due to insufficient liquid loading which is a result of batch to batch non-uniformity in manufacturing. Apparently, this problem has not been fully solved by the manufacturers. Unfortunately, until it is, there is no assurance that a specific commercial packing will be satisfactory for the user's application. However, in most cases, the manufacturers will supply a new batch of packing when problems of this type are encountered.

In some cases, it is necessary to use a body porous packing material (C18/Porasil B) with a heavier loading of stationary phase to obtain sufficient retention.

With regard to the mobile phase, ammonium carbonate is used at various concentrations in most of the separations given here. There are several important reasons for including it in the aqueous phase: (1) it acts as an ionization suppressant for primary amine groups; (2) it reacts with acidic groups to form ammonium salts which tend to partition nicely on reverse phase columns; (3) it saturates the active polar sites on the stationary phase to give, in effect, a very non-polar column; and (4) it increases the polarity of the mobile phase. The net effect of all of these is an improvement in the separation process. Specifically, the addition of ammonium carbonate usually increases retention and in many cases improves selectivity and peak shape. In general, retention times increase with increasing ammonium carbonate concentration. In many cases the presence of ammonium carbonate is the difference between separation and no separation.

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. 1 shows the separation obtained with 0.1 % ammonium carbonate solution as the mobile phase. Ammonium carbonate is necessary to obtain suf-

Fig. 5. Decomposition of a cephalosporin injectable solution, percent remaining as a function of time.

Column: 1 m×2.1 mm. i. d., Vydac-RP; mobile phase, 5 % methanol, 95 % 0.05 M ammonium carbonate; pressure, 700 psi; flow, 0.7 ml/min; detector, UV (254 nm); sensitivity, 0.08 AUFS.

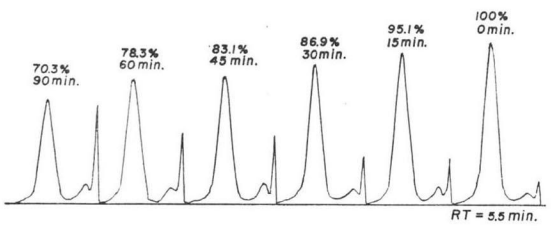
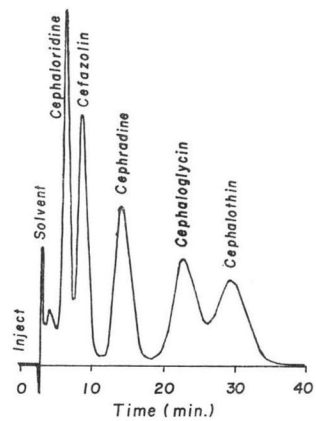


Fig. 6. Separation of a mixture of cephalosporins.

Column: 1 m × 2.1 mm i. d., ODS-Sil-X-II; mobile phase, 5 % methanol, 95 % 0.05 M ammonium carbonate; pressure, 800 psi; flow, 0.5 ml/min; detector, UV (254 nm); sensitivity, 0.08 AUFS.



ficient retention. Even though 6-APA is reported to have only end absorption,[20] the absorption at 254 nm (*ca.* one-tenth that of 7-ACA) is sufficient for detection of μg quantities of material.

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 some of these compounds is shown in Fig. 2 where 7-ADCA, 7-ACA, cephalosporin C, 7-tetrazolyl-ACA (7-TACA) (**IV**) and 7-ACA thiadiazol (7-ACA-TD) (**V**) are separated on a C18/Porasil B column with plain water as the mobile phase.

Quantitation of Cephradine in an Oral Suspension

An example of the use of reverse phase chromatography is shown in Fig. 3, in which cephradine is separated and quantitated in an oral suspension. This separation is also feasible by ion-exchange, but it is easier and more convenient by this reverse phase method. Of considerable importance is the fact that another cephalosporin, in this case cephaloglycin, is used as the internal standard. In reverse phase chromatography it is usually possible to choose the internal standard from the same class of compounds. This is generally not the case in ion exchange chromatography. The example cited here is an oral suspension, but other pharmaceutical forms such as tablets, capsules, intravenous solutions and stability samples can be handled with equal ease.

The precision and accuracy obtained with this method is illustrated in Fig. 4. Shown here are chromatograms of four replicate samples with the absolute peak areas and recoveries as indicated. The average recovery is 99.98%, and the relative standard deviation is 1.09%. This level of precision is quite typical of most assays carried out by liquid chromatographic techniques.

Decomposition Kinetics

Reverse phase liquid chromatography has proved to be useful for determining stabilities and studying decomposition kinetics of antibiotics. This is illustrated in Fig. 5. An experimental cephalosporin antibiotic was delib-

Fig. 7. Separation of penicillins G and V.
Column: 1 m × 2.1 mm i.d., C 18/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.

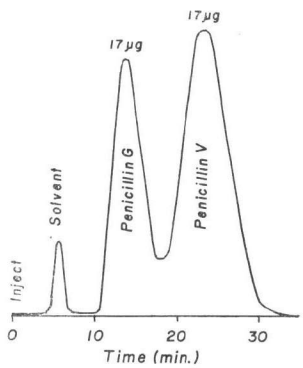
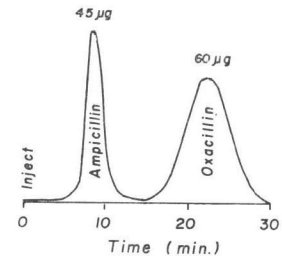


Fig. 8. Separation of synthetic penicillins. I.
Column: 1 m×2.1 mm i.d., C18/Porasil B; mobile phase, 30% methanol, 70% 0.05 M ammonium carbonate; pressure, 500 psi; flow, 0.5 ml/min; detector, UV (254 nm); sensitivity, 0.16 AUFS.



erately placed in an unstable medium (equimolar Na_2CO_3; pH 7.88), and the decomposition rate was followed by successive injections of sample at regular intervals of time. As shown by the decrease in peak areas, this particular antibiotic decomposed approximately 30% in one and one-half hours.

Separation of Cephalosporin Mixtures

There are several commercially available cephalosporin antibiotics, and they can be separated by reverse phase liquid chromatography. Shown in Fig. 6 is the separation of cephaloridine, cefazolin, cephradine, cephaloglycina nd cephalothin, which are five of the most well-known and effective compounds available. This particular separation was carried out on an ODS-Sil-X-II column, but it can also be done on Vydac-RP and C18/Corasil. However, the separation is not possible on "Permaphases" ODS and ETH, Phenyl/Corasil and on some batches of Vydac-RP. With these columns, all compounds elute at the solvent front because of reasons discussed earlier. 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. Subtle differences in selectivity are sometimes noticed when changing from one reverse phase column to another. For example, cefazolin is eluted bofore cephradine on ODS-Sil-X-II, but after cephradine on Vydac-RP.

Penicillins

The penicillins can be chromatographed by reverse phase methods, but a body porous packing, such as C18/Porasil B, is required. The pellicular packings, such as C18/Corasil, Vydac-RP, ODS-Sil-X-II, give badly tailing peaks. The tailing, which cannot be eliminated by the addition of salts or organic modifiers or by pH adjustments, is presumably due to the strong interaction of the compounds with the active sites of the silica surface through the thin C18 organic coating.

The separation two common penicillins, G and V, on C18/Porasil B with 30% methanol, 70% 0.05 M ammonium carbonate is shown in Fig. 7. Because of the relatively large particle size (37~75 μ) of the packing, rather broad

Fig. 9. Separation of synthetic penicillins. II.
Column: 1 m × 2.1 mm i.d., C18/Porasil B; mobile phase, 30% methanol, 70% 0.05 M ammonium carbonate; pressure, 500 psi; flow, 0.6 ml/min; detector, UV (254 nm); sensitivity, 0.16 AUFS.

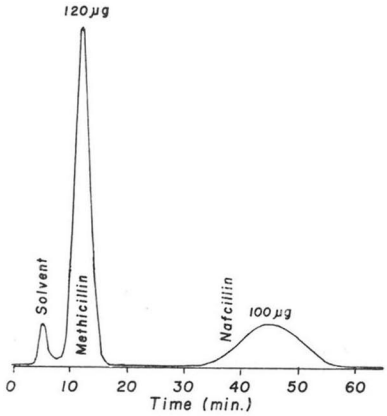
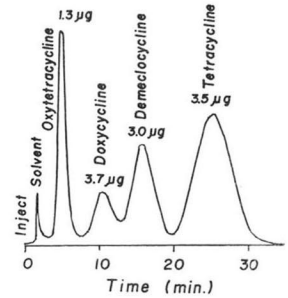


Fig. 10. Separation of tetracyclines.
Column: 1 m×2.1 mm i.d., ODS-Sil-II; mobile phase, 8% methanol, 92% 0.05 M ammonium carbonate, 0.005 M ethylene diamine tetra-acetic acid; pressure, 1,000 psi; flow, 0.56 ml/min; detector, UV (254 nm); sensitivity, 0.08 AUFS.



peaks are obtained. In spite of this, both separation and sensitivity are adequate.

The separation of two common synthetic penicillins, ampicillin and oxacillin, is shown in Fig. 8. Similarly, the separation of methicillin and nafcillin is shown in Fig. 9. The conditions used for separating these compounds are exactly the same as used for penicillins G and V.

Tetracyclines

Many tetracyclines have been separated by ion-exchange liquid chromatography.[10] However, they can also be separated by reverse phase chromatography. This is demonstrated in Fig. 10 where 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 ethylene diamine tetra-acetic acid in the mobile phase to prevent their complexing with the

Fig. 11. Separation of chloramphenicol.
Column: 1 m × 2.1 mm i. d., ODS-Sil-X-II; mobile phase, 100% 0.05 M ammonium carbonate; pressure, 750 psi; flow, 0.6 ml/min: detector, UV (254 nm); sensitivity, 0.08 AUFS.

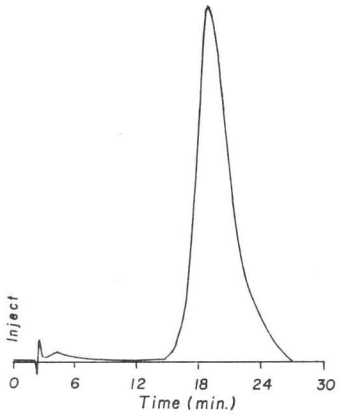


Fig. 12. Separation of usnic and helvolic acids.
Column: 1 m × 2.1 mm i. d., ODS-Sil-X-II; mobile phase, 30% methanol, 70% 0.05 M ammonium carbonate; pressure, 1,000 psi; flow, 0.06 ml/min; detector, UV (254 nm); sensitivity, 0.16 AUFS.

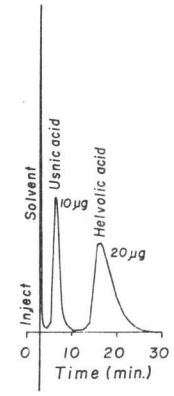


Fig. 13. Separation of griseofulvin.
Column: 1 m × 2.1 mm i. d., ODS-Sil-X-II; mobile phase, 30% methanol, 70% 0.05 M ammonium carbonate; pressure, 1,000 psi; flow, 0.6 ml/min; detector, UV (254 nm); sensitivity, 0.16 AUFS.

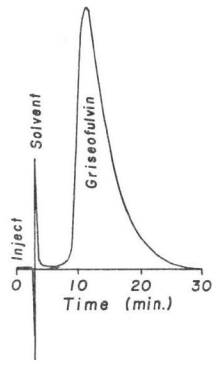
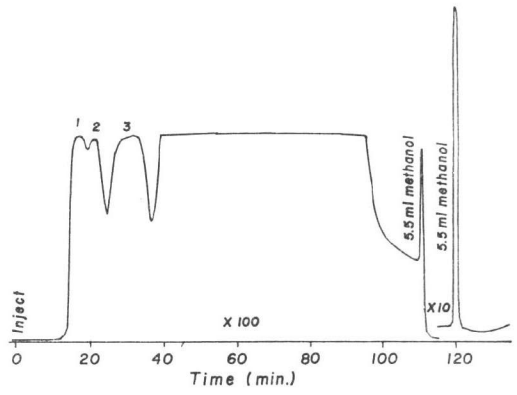


Fig. 14. Preparative level separation of cefazolin.
Column: 1.22 m × 9.5 mm o. d. × 8 mm i. d., C18/porasil B; mobile phase, water; pressure, 150 psi; flow, 2.5 ml/min; detector, UV (254 nm); sensitivity, 1.28 AUFS.



metal tubing. Other tetracyclines can be separated with minor mobile phase adjustments.

Miscellaneous Antibiotics

Chloramphenicol is a very common antibiotic, and it can be separated by reverse phase chromatography. To accomplish the separation, it is necessary to employ a column with high retentive power. As shown in Fig. 11, ODS-Sil-X-II is suitable with 100% 0.05 M ammonium carbonate as the mobile phase. C18/Corasil and Vydac-RP can also be used. However, "Permaphases" ODS and ETH and Phenyl-Corasil are unsuitable because of insufficient retention.

Two other antibiotics, usnic and helvolic acids, are shown separated on an ODS-Sil-X-II column in Fig. 12. It should be noted that a relatively high concentration of methanol in the mobile phase is necessary to elute these compounds. This, of course, is indicative of the fact that these materials are relatively non-polar as opposed to most antibiotics which are, for the most part, highly polar compounds.

An important antifungal antibiotic is griseofulvin. It, also, is relatively non-polar and thus requires a fairly high solvent strength for elution. With 30% methanol, it elutes from an ODS-Sil-X-II column as shown in Fig. 13.

Preparative Applications

One of the more important uses of liquid chromatography is preparative chromatography. In particular, reverse phase chromatography is especially useful, since high capacity packings are easily obtainable and moderately priced. Furthermore, since the mobile phases usually do not contain troublesome salts, sample recovery is greatly simplified. The technique has proved to be very useful in the isolation of trace impurities from cephalosporin type antibiotics. An example of such an application is given in Fig. 14. Shown here is a preparative separation of a crude sample of cefazolin. By injecting 500 mg of material on a C18/Porasil B column (1.22 m × 9.5 mm o. d. × 8 mm i. d.), sufficient quantities of impurities 1, 2 and 3 were obtained for identification by other techniques (NMR, MS, UV, *etc.*). In addition, cefazolin was obtained in high purity. At the time this work was done the only detector available to us was the UV (254 nm) which obviously was heavily overloaded in this application. A more accurate description of the separation process is obtained with a detector which does not suffer from this shortcoming. Such a detector is the refractive index which is recommended and is now used routinely for preparative separations. As mentioned earlier, sample recovery is usually quite easy in reverse phase chromatography, and the present example illustrates this. Since only water was used as the mobile phase, individual collected fractions were recovered simply by vacuum distilling off the water on a "roto-vap." A simple technique which is used frequently in preparative chromatography and which is worth pointing out pertains to column clean-up after a separation is completed. In many cases impurities remain on the column after all components of interest have been eluted. These are removed quite easily and quickly by making one or two injections of a stronger solvent. In the present case, two 5.5-ml injections of methanol cleaned the column and readied it for the next preparative separation.

Conclusions

The results given in this paper demonstrate that reverse phase liquid chromatography can be used to separate nearly any type of antibiotic. Others which have been separated, but which are not reported on here, are polypeptides, aminoglycosides and other miscellaneous

compounds. The methods presented here are now used routinely by us for the separation, quantitation and isolation of both naturally occurring and synthetically produced antibiotics, as well as many other miscellaneous pharmaceutical compounds.

The work reported here was performed on state of the art columns with relatively large particles. Work is in progress to improve the separations even further by the use of high efficiency small (5~10 μm) particle columns.

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