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PREPARATIVE COLUMNS IN HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

When high-speed liquid chromatography is used to obtain pure material for subsequent investigation, it can correctly be called preparative, regardless of the sample weights employed. In this paper, the requirements for scaling up from a small-diameter analytical column to larger-diameter preparative columns are presented. An example of the use of high-speed preparative liquid chromatography to isolate and identify the components of a complex mixture is presented. General requirements of sample size for subsequent investigation are discussed.

INTRODUCTION

High-speed liquid chromatography (LC) may be considered to be preparative in nature any time a component is collected, regardless of the amount of material placed on the column or isolated in a fraction. The amount of material that must be collected will vary widely, depending upon the subsequent treatment intended for the compound[1].

Recent developments in LC column technology and hardware have led to the availability of high-speed preparative LC. Most LC separations have been carried out using columns which may be considered narrow bore (2–3 mm I.D.). These columns have been optimized for analytical work and represent a compromise between efficiency, capacity and amount of packing required. Recently, studies have shown that increased efficiency can be obtained using larger-diameter columns[2–5] and that a direct scale-up from analytical to preparative liquid chromatography can be achieved without loss of speed or resolution[1,6]. Separations can be performed on a preparative scale in the same length of time required for an analytical investigation by simply increasing the flow-rate in proportion to the increase in the cross-sectional area of the column to maintain the same linear velocity.

It is obviously desirable to determine conditions required for a separation (*i.e.*, column type, mobile phase composition, etc.) using a small-diameter analytical column then translate these conditions to a preparative column packed with the same material. In this manner, large quantities of sample and mobile phase are not wasted.

After the preparative experiment, purity of collected fractions can be determined on the analytical column.

The purpose of this paper is to describe the methodology involved in scaling-up from analytical to preparative high-speed LC and to illustrate general requirements of sample size for subsequent investigation.

EXPERIMENTAL

Apparatus

A DuPont Model 830 liquid chromatograph equipped with an ultraviolet photometer detecting at 254 nm, gradient elution, and forced air oven was used. The Model 830 is equipped with a pneumatic amplifier pump which is capable of delivering mobile phase at flow-rates greater than 100 ml/min. This flow-rate versatility allows the use of the same instrument for both analytical and preparative separations. When a 23-mm-I.D. column was used at elevated temperature, a 2-m coil of 0.030-in.-I.D. tubing was installed in the oven before the injection port to allow temperature equilibration of the mobile phase.

Narrow-bore analytical columns were packed with Permaphase® ODS and "Zorbax"*-SIL. Larger-diameter preparative columns were packed with Permaphase® ODS and "Spherosil"® XOA-400. The column end fittings and frit design were as previously described[6]. Samples were deposited by syringe (Precision Sampling Corp., Baton Rouge, La., U.S.A.) or a loop valve (Valco, Inc., Houston, Texas, U.S.A.) on to the frit rather than into the column packing. All columns used in this study are commercially available (DuPont, Instrument Products Division, Wilmington, Del., U.S.A.).

Collected fractions were investigated by mass spectroscopy (DuPont Model 21-110B, high resolution, double focusing), nuclear magnetic resonance (Varian 220 MHz, Fourier Transform), and infrared spectroscopy (Perkin-Elmer Model 21) where ethanolic solutions of the samples were spread over a KRS-5 plate and the solvent allowed to evaporate. The samples were then analyzed by the attenuated total reflectance (ATR) technique.

Chemicals

Cholesteryl phenylacetate was obtained from Aldrich Chemical Co., Inc.; progesterone from Nutritional Biochemicals Corp.; and a World Standard Pyrethrum Extract was obtained from the Pyrethrum Bureau, Kenya, Africa, as a 23% solution of pyrethrins in hydrocarbons. Solvents which were used as mobile phases for LC were reagent grade or spectral quality.

RESULTS AND DISCUSSION

In preparative high-speed LC, it is desirable to collect the maximum amount of pure material in the shortest possible time. Therefore, one should place sample amounts on the column which exceed the linear capacity of the column packing, *i.e.*, operate with the column slightly overloaded. The largest quantities of pure component can then be obtained by collecting center-cuts of the overloaded peak(s).

The weight of individual components placed on the column is usually of more

* DuPont trade-mark.

importance than the total weight of sample in determining column capacity. This is due to the fact that as components are distributed on the column they are no longer in competition for the same stationary phase sites on the column packing. This argument assumes that the total number of compounds is not too large and that the compounds are reasonably well resolved. For most practical purposes, column capacity can be approximated as 1 mg of solute per gram of totally porous packing and 0.1 mg of solute per gram of superficially porous packing in the column[7]. For example, a column containing 100 g of porous silica gel would be able to accomodate up to 100 mg of solute without serious overload.

Other types of overloading which can occur are mobile phase overload and detector overload. Conditions for mobile phase overload exist when the solubility of the solute in the sample solvent is much greater than in the mobile phase and small volumes of concentrated solute are applied. When overload occurs, the solute precipitates out at the head of the column and redissolves as it is exposed to fresh mobile phase. This results in broad tailing peaks as the solute passes through the column. Mobile phase overload is common in partition chromatography with superficially porous packings and can be reduced by increasing the sample volume injected while maintaining the same solute weight and by changing to a higher operating temperature or a different mobile phase. Mobile phase overload can frequently be identified by injecting a sample volume into a small amount of mobile phase and observing the formation of a precipitate. Detector overload occurs when the linear dynamic range of the detector is exceeded. This type of overload results in peaks which are still reasonably symmetrical but rounded off at the top. Detector overloading is not a serious drawback in preparative high-speed LC as it results only in rounded peaks and has nothing to do with the efficiency of the column.

The introduction of large sample volumes on to the column will normally be required in preparative LC. It has been shown[4] that introduction of large volumes of dilute solution gives greater efficiency than a smaller volume of more concentrated solution. This is apparently due to localized overloading at the head of the column when the more concentrated solution is applied. In many cases, the sample volume to be placed on the column will be dictated by the solubility of the solute in various solvents. Sample introduction can be made by syringe (large sample volumes can be accumulated by replicate injections made under stopped-flow conditions), loop valve, or by use of the pump. In the latter case, an infinite volume injection can be made,

TABLE I
HIGH-SPEED PREPARATIVE LC SAMPLE QUANTITY REQUIREMENTS

Objective	*Sample weight required (mg)*
Tentative identification by instrumental methods	< 1
Positive identification by instrumental methods, including NMR. Confirmation of structure by chemical reactions	1–100
Positive identification and subsequent use in research or synthesis	>100

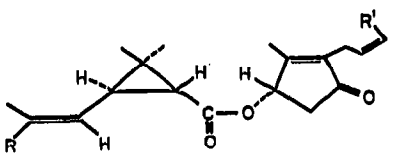
TABLE II
HIGH-SPEED PREPARATIVE LC QUANTITY REQUIREMENTS FOR PEAK IDENTIFICATION

Technique	*Requirement (mg)*
NMR (Fourier transform)	0.1
NMR (conventional)	1
IR	0.01
MS	0.001
Elemental analysis (C, H and N)	0.1

provided the sample can be dissolved in a solvent which will not elute it from the column (as is often the case in liquid–solid and ion-exchange chromatography). To load a sample with the pump, the solute is placed in the reservoir as a dilute solution and pumped on to the column, thereby concentrating it at the head of the column. The eluting solvent is then placed in the reservoir and pumped through the column to generate the chromatogram.

The amount of material that must be collected in a preparative experiment is dictated by the subsequent treatment intended for the sample. Table I lists approximate sample size requirements for various subsequent treatments. Table II gives minimum sample size requirements for component identification by instrumental methods. Often, prior knowledge of the sample nature allows a tentative identification to be made by comparing the chromatogram to that of a standard or "spiking" the sample with the component which is suspected to be present. When this can be done, it may suffice to collect only enough material for infrared or mass spectral confirmation.

The collected sample in a preparative experiment may be present in a large volume of solvent. The volume of the collected fraction will be determined primarily by the initial size of the sample, the size of the column and the efficiency of the column. In a typical LC experiment, the sample will be diluted by a factor of at least one hundred as it passes through the column. This means that a sample introduced in a 1-ml volume might be collected as a pure fraction in 100 ml of mobile phase. After collection of the peak it will usually be necessary to concentrate the sample before further use. This can easily be achieved by solvent evaporation when volatile organic solvents are used. However, when aqueous solvents are used it may be necessary to



	R	R'	Molecular weight
Pyrethrin I	$-CH_3$	$-CH=CH_2$	328.4
Jasmolin I	$-CH_3$	$-CH_2-CH_3$	330.4
Cinerin I	$-CH_3$	$-CH_3$	316.4
Pyrethrin II	$-COOCH_3$	$-CH=CH_2$	372.4
Jasmolin II	$-COOCH_3$	$-CH_2-CH_3$	374.4
Cinerin II	$-COOCH_3$	$-CH_3$	360.4

Fig. 1. Structures and molecular weights of the pyrethrins.

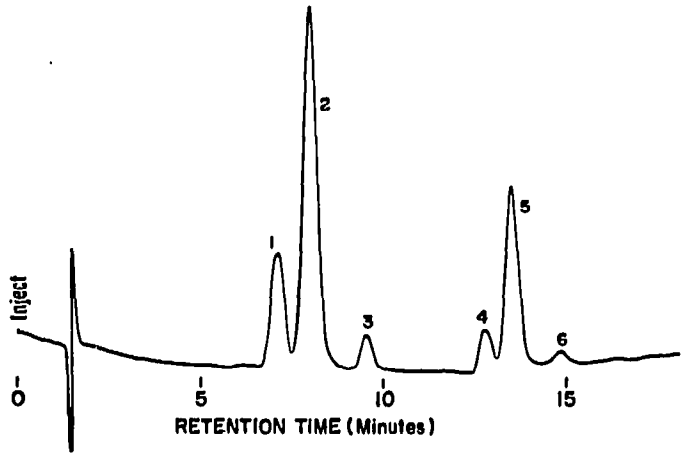


Fig. 2. Chromatogram of pyrethrum extract using an analytical column and gradient elution. Sample size, 10 μg; column, 1 m × 2.1 mm I.D. packed with Permaphase® ODS; mobile phase, linear gradient from 30% methanol–70% water to 100% methanol at 3%/min; temperature, 50°; column pressure, 1000 p.s.i.g.; detector sensitivity, 0.64 absorbance units full scale.

extract the sample into a more volatile, water-immiscible solvent such as chloroform or hexane.

Pyrethrins are a group of naturally occurring insecticides which have a low order of toxicity to warm-blooded animals[8]. This complex mixture of closely related compounds was chosen to illustrate how a separation achieved on a small-diameter analytical column can be scaled up to a large-diameter preparative column so that fractions could be collected in sufficient quantities for identification. Fig. 1 shows the general structure reported[9] for the six insecticidally active esters of pyrethrum. Fig. 2 shows a chromatogram of pyrethrum extract obtained using an analytical column

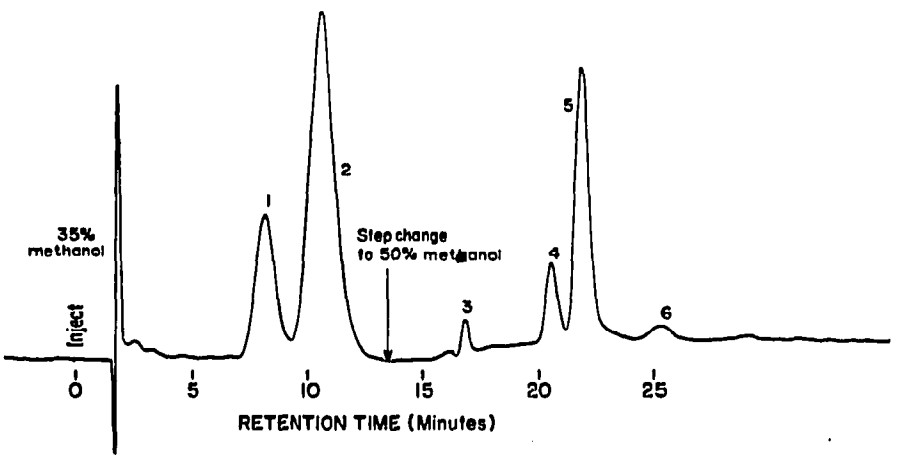


Fig. 3. Chromatogram of pyrethrum extract using an analytical column and step elution. Sample size, 10 μg; column, 1 m × 2.1 mm I.D. packed with Permaphase® ODS; mobile phase, step elution from 35% methanol–65% water to 50% methanol–50% water; temperature, 50°; column pressure, 1000 p.s.i.g.; detector sensitivity, 0.16 absorbance units full scale.

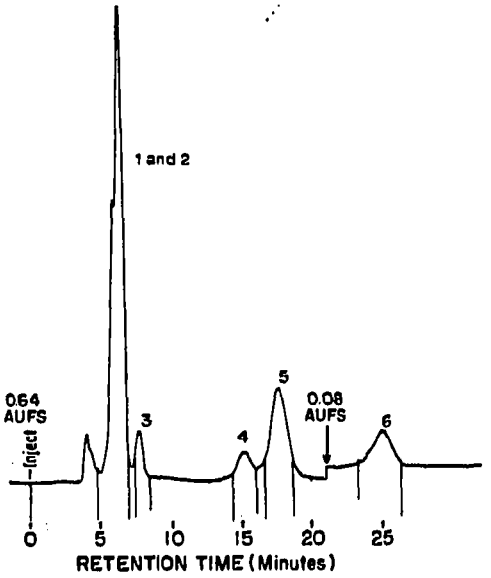


Fig. 4. Chromatogram of pyrethrum extract using a larger-diameter preparative column. Sample size, 2 mg; column, 0.5 m × 23 mm I.D. packed with Permaphase® ODS; mobile phase, methanol–water (50:50); temperature, 50°; column pressure, 1200 p.s.i.g.; flow-rate, 25 ml/min. Vertical lines below the baseline indicate start and finish of fraction collection. AUFS = Absorbance units full scale.

packed with Permaphase® ODS and gradient elution. Due to difficulties in mixing solvents at the high flow-rates frequently required for preparative work, gradient elution is usually not convenient and is seldom required. Conditions for achieving comparable separation of the pyrethrum extract by step elution were determined and are shown in Fig. 3. In this manner, six major components of the sample were separated.

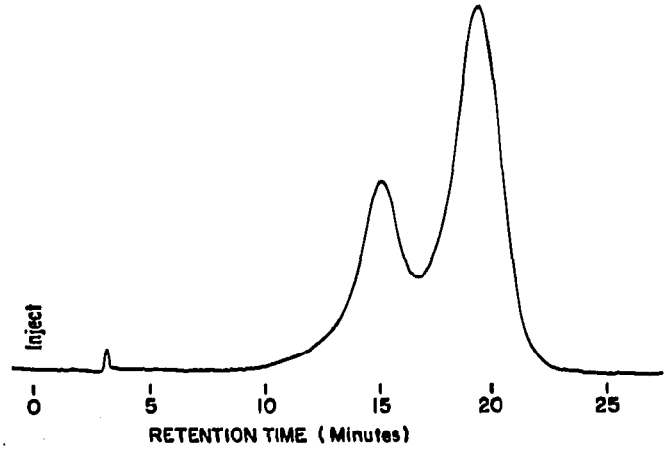


Fig. 5. Chromatogram of pyrethrum extract fractions 1 and 2. Column, 0.5 m × 23 mm I.D. packed with Permaphase® ODS; mobile phase, methanol–water (35:65); temperature, 50°; column pressure, 1500 p.s.i.g.; flow-rate, 30 ml/min.

TABLE III
STRUCTURE ASSIGNMENT OF PYRETHRUM EXTRACT FRACTIONS

Fraction	*Molecular ion*	*NMR* and IR (ATR)*		*Assignment*
		R	*R'*	
1	360	$-COOCH_3$	$-CH_3$	Cinerin II
2	372	$-COOCH_3$	$-CH=CH_2$	Pyrethrin II
3	374	$-COOCH_3$	$-CH_2-CH_3$	Jasmolin II
4	316	$-CH_3$	$-CH_3$	Cinerin I
5	328	$-CH_3$	$-CH=CH_2$	Pyrethrin I
6	330	$-CH_3$	$-CH_2-CH_3$	Jasmolin I

* 220 MHz, Fourier transform.

For the scale-up to a larger-diameter column, the mobile phase composition was chosen so that the two components eluting first could be collected as one fraction and the four later eluting components collected separately, as shown in Fig. 4. Fractions 1 and 2 were then re-chromatographed, as shown in Fig. 5, using a different mobile phase composition. Alternatively, one could collect fractions 1 and 2 using a mobile phase composition of 35% methanol then increase the methanol concentration in a stepwise fashion to decrease the retention times of fractions 3–6, as was shown in

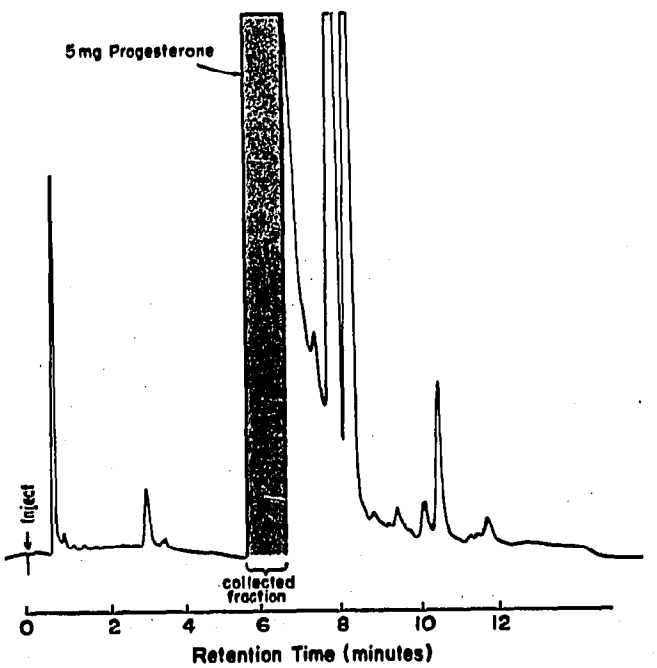


Fig. 6. Chromatogram of impure progesterone. Sample size, 5 mg; column, 0.25 m × 2.1 mm I.D. packed with Zorbax-SIL; temperature, ambient; mobile phase, linear gradient from 100% dichloromethane to 90% dichloromethane–10% ethanol at 2%/min; column pressure, 1500 p.s.i.g.; detector sensitivity, 0.02 absorbance units full scale.

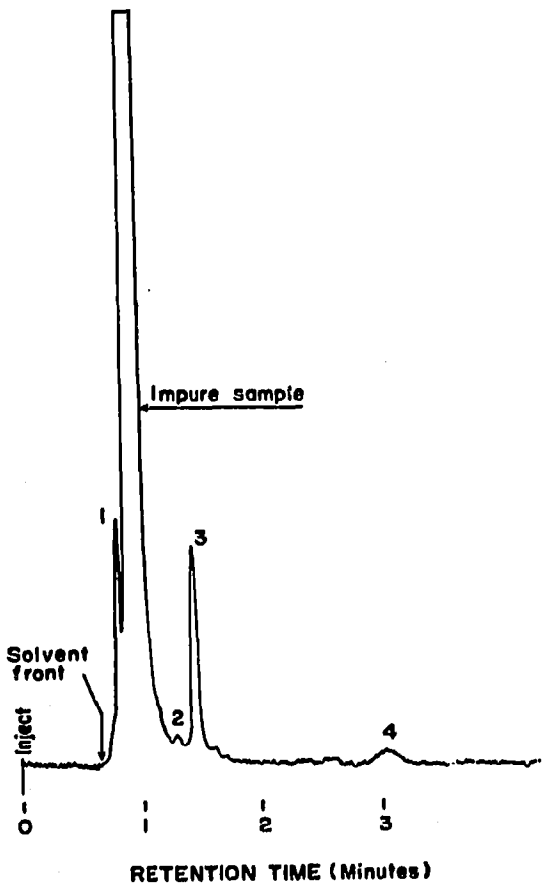


Fig. 7. Chromatogram of a 500-μg sample of cholesteryl phenylacetate. Column, 0.25 m × 2.1 mm I.D. packed with Zorbax-SIL; mobile phase, dichloromethane containing 0.1 % methanol; temperature, ambient; column pressure, 1500 p.s.i.g; detector sensitivity, 0.02 absorbance units full scale.

Fig. 3 on an analytical column. This latter approach is less desirable because a mobile phase change must be made after each injection to avoid contamination of subsequent samples by later eluting peaks. The approach illustrated in Figs. 4 and 5 is preferred because as many samples as desired may be injected to collect peaks 3 through 6 before a mobile phase change must be made so that peaks 1 and 2 can be re-chromatographed for collection.

In order to enhance the concentration of the components, each fraction was extracted from the water–methanol mobile phase into hexane. The more volatile hexane was then evaporated under nitrogen and the fractions were analyzed by mass (MS) and infrared (IR) spectroscopy, and by Fourier transform nuclear magnetic resonance (NMR). Samples were sealed in vials under nitrogen to prevent oxidation prior to analysis. Approximately 1 mg of each fraction was isolated for identification.

Table III lists the fraction assignments made on the basis of the IR, MS and NMR data. The 1-mg fractions were first investigated by NMR, a non-destructive

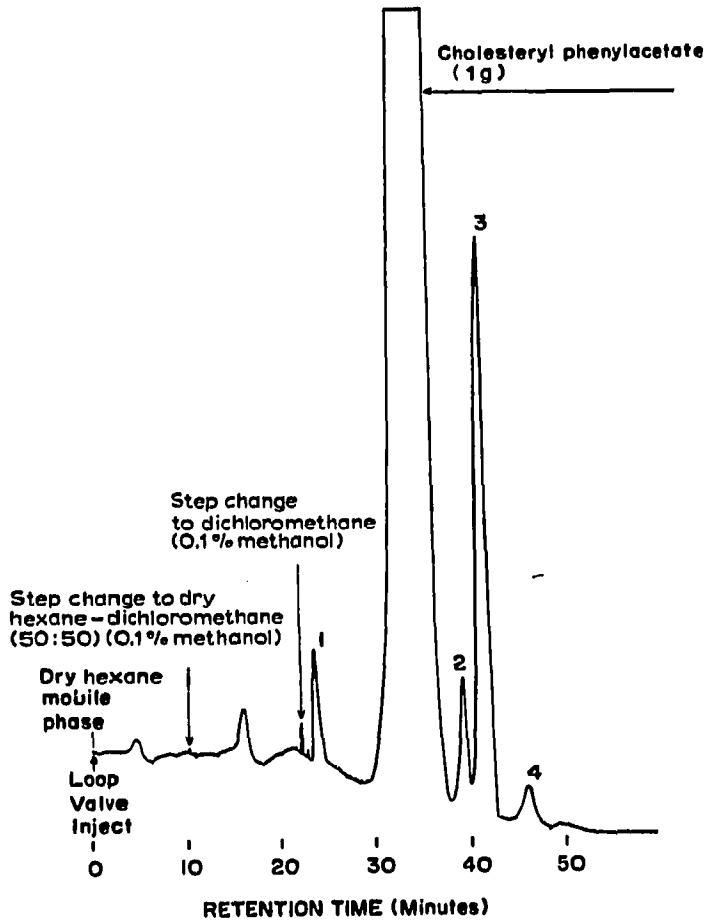


Fig. 8. Chromatogram of a 1-g sample of cholesteryl phenylacetate. Column, two 0.5 m × 23 mm I.D. columns packed with Spherosil® XOA-400 connected in series; mobile phase, step elution from dry hexane to dichloromethane containing 0.1 % methanol; temperature, ambient; column pressure, 1000 p.s.i.g; flow-rate, 30 ml/min; detector sensitivity, 0.32 absorbance units full scale.

technique, then a small portion of each fraction was removed for IR and MS analysis. Although it was not attempted on these fractions, the final step in the identification scheme would have been elemental analysis, a destructive technique which has relatively large sample size requirements. The IR spectra of the isolated fractions were compared at 3.40 μm (saturated C–H), 5.82 μm (>C=O), 6.07 μm (–C=C–), 7.25 μm (C–CH_3), 7.5–9 μm (ester, C–O–C), and 10.05 and 11.02 μm (CH_2=CH–). The NMR spectra were compared to previously published data[9].

Whenever possible, system capacity may be increased through the use of totally porous chromatographic packings. Fig. 6 illustrates the separation of 5 mg of progesterone from impurities on a small-diameter column packed with Zorbax-SIL, a totally porous silica packing. The separation in Fig. 6 is noteworthy because a large sample weight of progesterone was injected. The amount of sample that can be placed on a column without serious overload can usually be maximized by introducing the

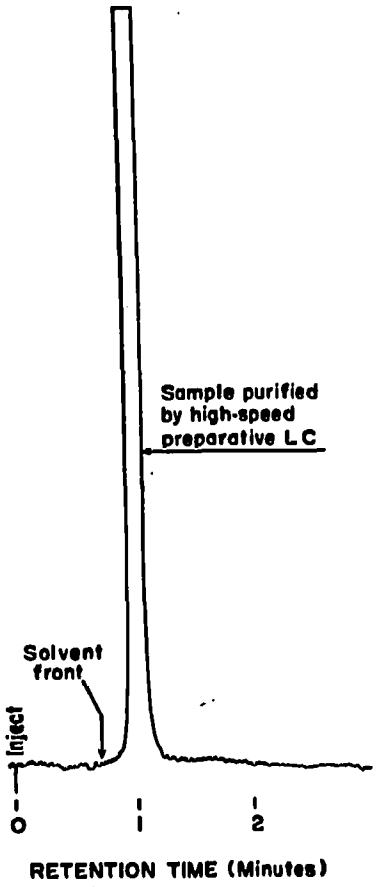


Fig. 9. Chromatogram of collected fractions of cholesteryl phenylacetate. For conditions, see Fig. 7.

sample under conditions where it is strongly retained and then eluting it with a continuous or stepwise gradient. In this manner, the sample spreads itself on the narrowest possible band at the head of the column. Under these same gradient conditions, as little as 10 ng of progesterone can be detected, so that the dynamic range of a 25-cm column packed with Zorbax-SIL is greater than 5×10^5 for progesterone. This means that not only milligram quantities of very pure compound can be collected but that impurities which have a molar extinction coefficient at 254 nm equal to that of progesterone can be detected at the 0.001% (w/w) level.

Fig. 7 shows a chromatogram of 500 μg of cholesteryl phenylacetate on Zorbax-SIL showing impurities which represent less than 1% of the total sample weight. In order to purify gram quantities of the sterol ester, 1-in. columns were packed with Spherosil® XOA-400. Since both Zorbax-SIL and Spherosil® XOA-400 are silica packings with similar pore sizes and large surface areas, they have similar chromatographic retention properties. Separations carried out on Zorbax-SIL can often be transferred to Spherosil® XOA-400, which is ideal for large-scale preparative work because it is reasonably efficient and relatively inexpensive.

Fig. 8 shows the purification of 1 g of cholesteryl phenylacetate on two 0.5 m × 23 mm I.D. (about 1 in.) columns packed with Spherosil® XOA-400 and connected in series. Impurities 1 through 4 are the same impurities shown in Fig. 7 on Zorbax-SIL. A step gradient from dry hexane to dichloromethane (0.1 % methanol) was employed on the 1-in. preparative columns to maximize the weight of sample that could be placed on the column without serious loss of resolution. Under constant mobile phase composition, only a few hundred milligrams could be placed on the preparative columns before serious overload was observed.

It is important to note that the final mobile phase composition in the purification of the 1-g sample shown in Fig. 8 is the same as the mobile phase employed in the analytical chromatogram on Zorbax-SIL shown in Fig. 7. Fig. 9 shows an analytical chromatogram of the purified cholesteryl phenylacetate under the same conditions as in Fig. 7 to show that impurities 1 through 4 have been removed from the 1-g sample. In order to achieve this purity, a center-cut from the peak in Fig. 8 was collected. Approximately 0.5 g of very pure compound was obtained in a single pass through the column.

CONCLUSIONS

By taking advantage of existing column packing technology, preparative-scale high-speed LC can prove to be a very useful technique for separating and isolating components present in mixtures. The use of a versatile pumping system together with the increased efficiency of larger-diameter columns allows the direct scale-up from analytical to preparative conditions on the same instrument. Separations that require gradient elution on an analytical scale can be duplicated on a preparative scale by step elution.

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