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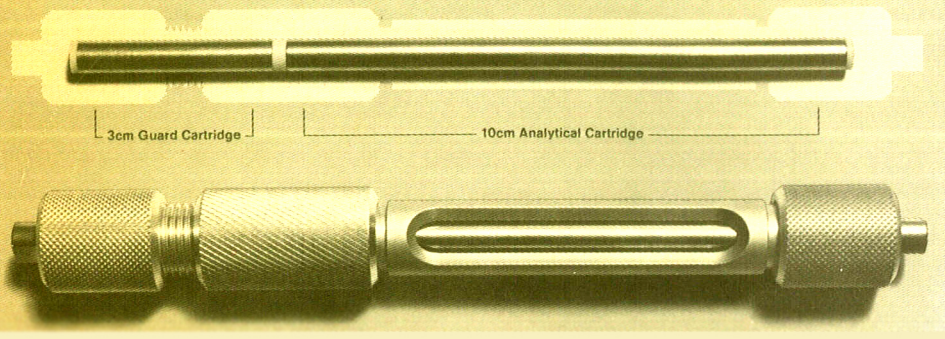

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JOURNAL OF LIQUID CHROMATOGRAPHY

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JOURNAL OF LIQUID CHROMATOGRAPHY

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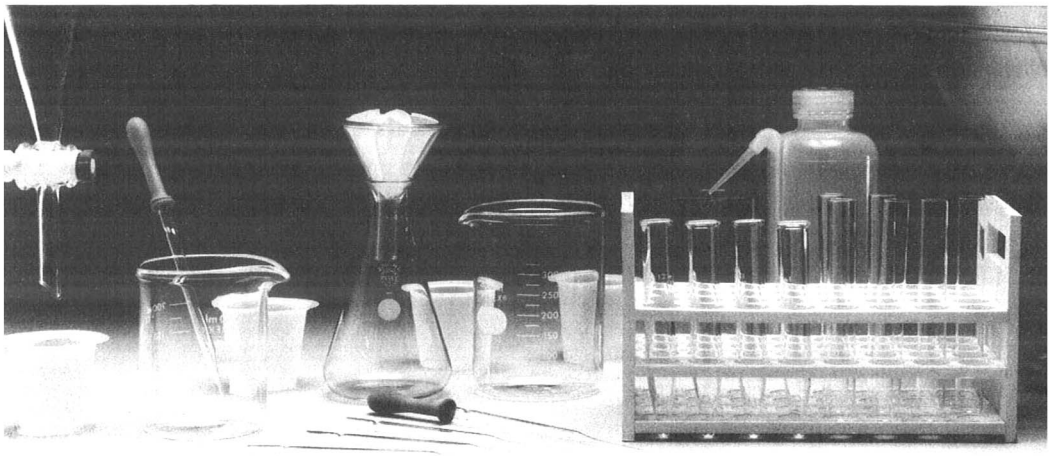

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(Suppl. 2), 179-221 (1982)

SUPERCRITICAL FLUID CHROMATOGRAPHY: METHODS AND PRINCIPLES

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ABSTRACT

Supercritical fluid chromatography has undergone much development in the past ten years. Important to this progress has been an understanding of the principles and problems involved. This review covers many of these principles, drawing on past work to illustrate how variables such as viscosity, temperature, pressure, and others, affect resolution. Overviews of the technical problems in instrumentation and column technology are also given.

INTRODUCTION

Supercritical fluid chromatography (SFC) is chromatography in which the mobile phase is a highly compressed gas. This condition is achieved in circumstances in which the operating temperature is higher than the critical temperature of the mobile phase and the operating pressure is sufficient to impart a solvating ability to the mobile phase. Pressures typically used vary from about one-half to several times the critical pressure of the mobile phase.

There are several features which make SFC desirable. The properties of a supercritical fluid are intermediate to those of gases and liquids. Two properties of primary importance in chromatography are solute diffusion coefficients and viscosity. Solute diffusion coefficients are higher in supercritical fluids than those in liquids, but they are lower than those in gases. As a result, optimum mobile phase flow rates are highest for gases, lowest for liquids, and intermediate for supercritical fluids. Analysis speeds are expected to increase in the sequence liquid chromatography, supercritical fluid chromatography, and gas chromatography.

The mobile phase viscosity determines the pressure drop across a column necessary to achieve the desired flow rate. Column pressure drops are about the same for gases and supercritical fluids, but are between 10 and 100 times greater when liquids are used. This is consistent with the fact that viscosities of gases and supercritical fluids are about the same, while liquids have viscosities which are about one hundred times greater than either gases or supercritical fluids.

Another advantage of using supercritical fluids in chromatography is their ability to handle high molecular weight and thermally labile compounds which cannot be analyzed by gas chromatography. Increasing the density of a supercritical fluid increases its ability to dissolve larger compounds. By controlling the density of the supercritical fluid through pressure control,

another advantage is obtained, since now the solvating ability of the mobile phase can be adjusted. Progressively increasing the mobile phase density throughout a chromatographic run causes higher molecular weight materials to be eluted. This is similar in many respects to temperature programming in gas chromatography and solvent programming in liquid chromatography. The molecular weight range capable of being analyzed by SFC in some cases extends over the full range of both gas and liquid chromatography.

The variety of mobile phases possible for use in SFC adds the dimension of mobile phase selectivity as a factor which can aid in obtaining separations. Liquid chromatography is the most flexible in this regard since many more mobile phases are available for use. The variety of mobile phases available for SFC is limited by the critical temperatures and thermal stabilities of the fluids considered. In gas chromatography, the mobile phase has no selectivity, making variations in selectivity limited to modifications in only the stationary phase.

Capillary columns achieve their usefulness in SFC exactly for the same reasons that make them useful in gas chromatography. Packed columns have many possible mobile phase flow paths which add to band broadening. This is not the case with capillary columns. Also, capillary columns can be made much longer than packed columns, making it possible to generate very high numbers of theoretical plates.

HISTORICAL

The solvating ability of supercritical fluids was not discovered until 1879 when Hannay and Hogarth (1) studied the solubilities of cobalt and ferric chlorides in supercritical ethanol. They found the concentrations of the metal chlorides to be much higher than vapor pressures alone would predict. Absorption spectra of the chlorides were essentially the same as found in the corresponding liquid solution.

SFC was first reported by Klesper, Corwin, and Turner in 1962 (2). Using various chlorofluoromethanes, they demonstrated the separation of nickel porphyrin derivatives from each other. Work by Karayannis et al. continued this research with studies of the separation of a number of porphyrins (3,4) and also metal chelates (5,6) with dichlorodifluoromethane as the mobile phase.

A number of excellent and indepth studies using carbon dioxide, isopropanol, and pentane as mobile phases were conducted by Sie and Rijnders (7-11). In addition to demonstrating various applications of SFC, they presented a good evaluation of many theoretical aspects of this technique.

Part of the usefulness of SFC comes from its ability to handle high molecular weight and thermally labile compounds. This usefulness is shown in part by the types of compounds which have been separated using various mobile phases. Classes of compounds which have been chromatographed include paraffins (7,12), alkyl-

bromides (13), aromatic hydrocarbons (11,14), metallo-organic compounds (13), polystyrenes (15-17), phospholipids and epoxy resins (18), various natural products (19,20), sugars, amino acids and proteins (21), polysiloxanes (22), and alcohols and acids (23).

Several reviews have appeared which are excellent treatments of SFC. Van Wasen et al. (24) reviewed SFC in terms of physicochemical properties and instrumental considerations. Klesper (25) reviewed the principles of SFC, column performance, instrumentation, and applications. Reviews by Gouw and Jentoft (26-28) cover all aspects of SFC, including many applications and practical considerations.

VISCOSITY EFFECTS

The viscosity of a supercritical fluid is approximately 10^{-4} to 10^{-3} g/(cm-sec). This is only slightly higher than that of gases, and a factor of 10 to 100 times lower than that of liquids (24,25). Because of this, column pressure drops are about an order of magnitude smaller than those observed with liquid mobile phases. Nevertheless, column pressure drops are more important in SFC than in gas or liquid chromatography because of the density changes that they create in the mobile phase. These density changes have effects on selectivity (8,22), retention (29,30,31), and column efficiency (30,32).

Considerable data have been published on the viscosity of various substances under supercritical conditions by Stephan and Lucas (33). Viscosity increases with pressure, but is related more accurately to the molar density of the mobile phase. The change in viscosity roughly follows the relationship:

$$1/\eta = B(V-V_o)/V_o \qquad [1]$$

where η is the viscosity, V the molar volume, V_o the molar volume where η becomes infinite, and B is a constant which varies for different solvents (34). With sufficient compression, it is possible to bring the viscosity of a dense gas to values equal to those of the liquid mobile phase. At this point the gas density is equivalent to liquid mobile phase densities, and many of the advantages of the dense gas state over the liquid state are lost (34).

Flow profiles in supercritical fluid chromatography are much more likely to have a turbulent character than in gas or liquid chromatography. The extent of this turbulence is often indicated by a quantity known as the Reynold's number. This is a dimensionless number given by $R_e = \rho v d_p/\eta$, where ρ is the fluid density, v the velocity, d_p the particle (or tube) diameter, and η the viscosity. Higher Reynold's numbers indicate more turbulence. In packed columns, turbulence increases its role as R_e varies from one to one hundred. Open tubular columns require R_e values around 2100 for turbulence to occur (35). In supercritical fluids, densities are greater than in gases, and viscosities are

similar to those found in gases. Because of this, the ratio ρ/η is larger, making turbulence more prevalent in SFC (8,36). Reynold's numbers in the dense gas region are found to be one thousand times larger than those in gas chromatography and about ten times larger than those in liquid chromatography. This can be beneficial to the chromatographic process if stationary phase mass transfer effects are small enough (37).

The effect of pressure drop on column efficiency has been studied by several workers. From the assumption that the diffusion coefficient times the mobile phase density is constant and that for an ideal gas the product PV is a constant, it has been shown that pressure drops should not adversely effect the efficiency of a chromatographic system (38,39). This has been found to hold true for up to about 80% of the critical pressure (40), but not at higher pressures (30). SFC, however, is not a chromatographic system in which ideal gas behavior is followed. For gases, detailed relationships show viscosity to have significant effects on diffusion (41,42). In liquids, diffusion coefficients decrease with increasing viscosity. In fact, solvent viscosity is the most important factor affecting solute diffusibility (43). Because viscosity also plays a role in determining diffusion coefficients, the product between the diffusion coefficient and the mobile phase density, rather than being a constant, is found to vary with pressure. This is especially true near the critical point (24,44). Since the solute diffusion coefficient

varies along the column length due to mobile phase viscosity and density changes, larger theoretical plate heights are often obtained than are theoretically predicted. However, this can be totally compensated for by the increased mass transfer due to turbulence in the mobile phase (40,45).

Density changes along the column length cause solute retention to vary along the column length. The mobile phase linear flow rate has a direct effect on the magnitude of this column density drop. The combination of these two effects results in changing k' values when column flow rates are varied (31). Selectivity has also been observed to be affected by density changes along a column (30). Losses in resolution due to these effects have caused some workers to use columns packed with larger particles and operate at higher temperatures in order to minimize density gradients (30,31). Capillary columns present an advantage in light of these concerns since column pressure drops are very low and, therefore, density changes along the column length are minimized (46,47). An alternate approach is to work at higher pressures where the variation of viscosity and density with pressure is smaller. Under these conditions efficiency losses are minimal, even with small particle columns, and very good results can be obtained (19,32). This is demonstrated in figure 1 where several polycyclic aromatic hydrocarbons have been separated on a 3 μm particle diameter packed column using CO_2 as the mobile phase (32).

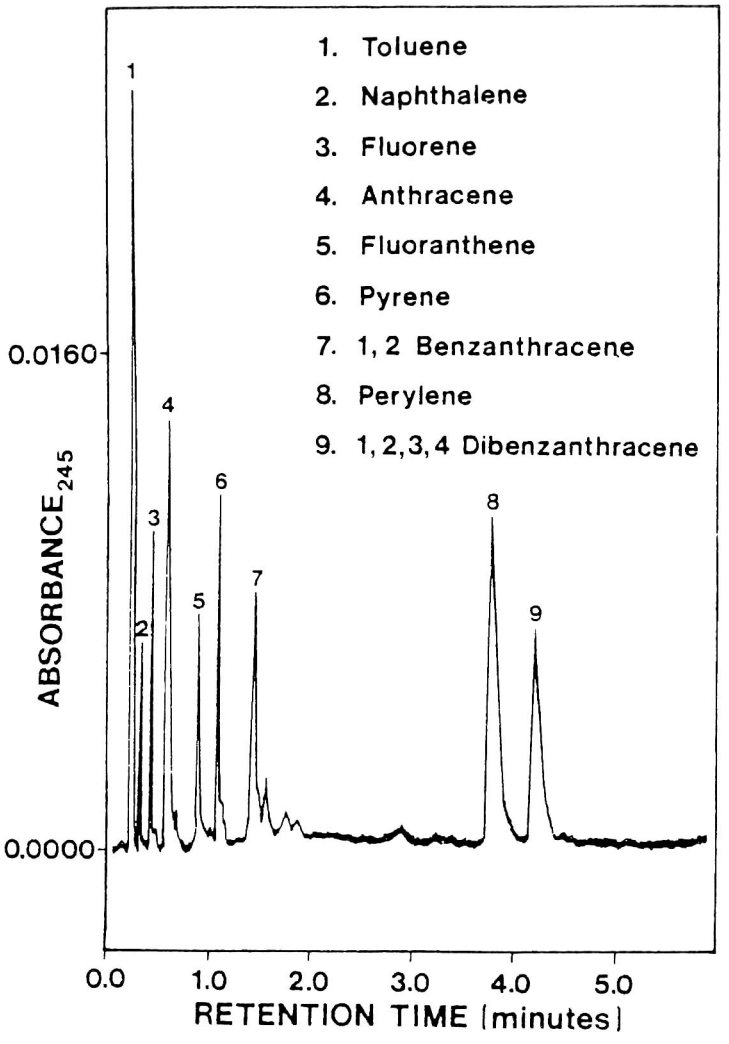

FIGURE 1. Nine polycyclic aromatic hydrocarbons separated on a 3 μm particle diameter packed column with CO_2 at 33°C as the mobile phase; inlet pressure, 400 bar; outlet pressure 243 bar (ref. 32).

DIFFUSION EFFECTS

Optimum linear velocities in chromatography are directly proportional to the mobile phase diffusion coefficient and inversely proportional to particle diameters (48). It is this direct dependence of optimum linear operating velocities on diffusion coefficients which predicts that the optimum mobile phase velocities in SFC are about one hundred times greater than those in liquid chromatography (since the diffusion coefficients are one hundred times greater). These higher diffusion coefficients provide another advantage in SFC. Mobile phase flow rates greater than the optimum can be used with less loss in column efficiency.

Increases in theoretical plate heights due to mobile phase velocity increases are directly proportional to the square of the particle diameter and inversely proportional to diffusion coefficients in the mobile phase. The combination of these two effects allows for very high speed chromatography to be performed. This has been shown by several workers (8,11,31,32). By using smaller particle diameters to minimize the increase in plate height with velocity, it has been possible to separate toluene and naphthalene in less than 20 seconds (32).

Diffusion coefficients in SFC are slow enough to make use of the infinite wall effect also seen in liquid chromatography. This has been shown in work by Semonian and Rogers (40) in which they used 0.53 cm i.d. columns.

TEMPERATURE AND PRESSURE EFFECTS ON RETENTION

Principally two properties are responsible for determining the retention of a solute in SFC. These are the volatility of the solute and its solubility in the mobile phase. Thus, it is possible to separate compounds having the same volatility based on solubility differences in the mobile phase. Less volatile compounds can even be made to elute before more volatile compounds. This occurs if the mobile phase is a better solvent for the less volatile compounds. An example of this effect has been shown by the separation of 1,1'-binaphthyl and anthracene (10). With n-pentane as the mobile phase at 40 kg/cm^2, anthracene elutes well before 1,1'-binaphthyl at 300°C because it is more volatile. At the same pressure, but at 200°C, 1,1'-binaphthyl elutes before anthracene because it is more soluble in the mobile phase.

At constant temperature and pressure, homologous series show predictable changes in solute retention. Retention in chromatography is often measured as a function of k', where $k'=(t_r-t_o)/t_o$. Here t_r is the retention time of the solute and t_o is the retention time of an unretained solute. As shown in figure 2, plots of log k' against carbon number for n-alkanes using CO_2 as the dense gas mobile phase are linear (7,24). This principle has also been demonstrated for series of alkyl phthalates (9) and polycyclic aromatic hydrocarbons (10).

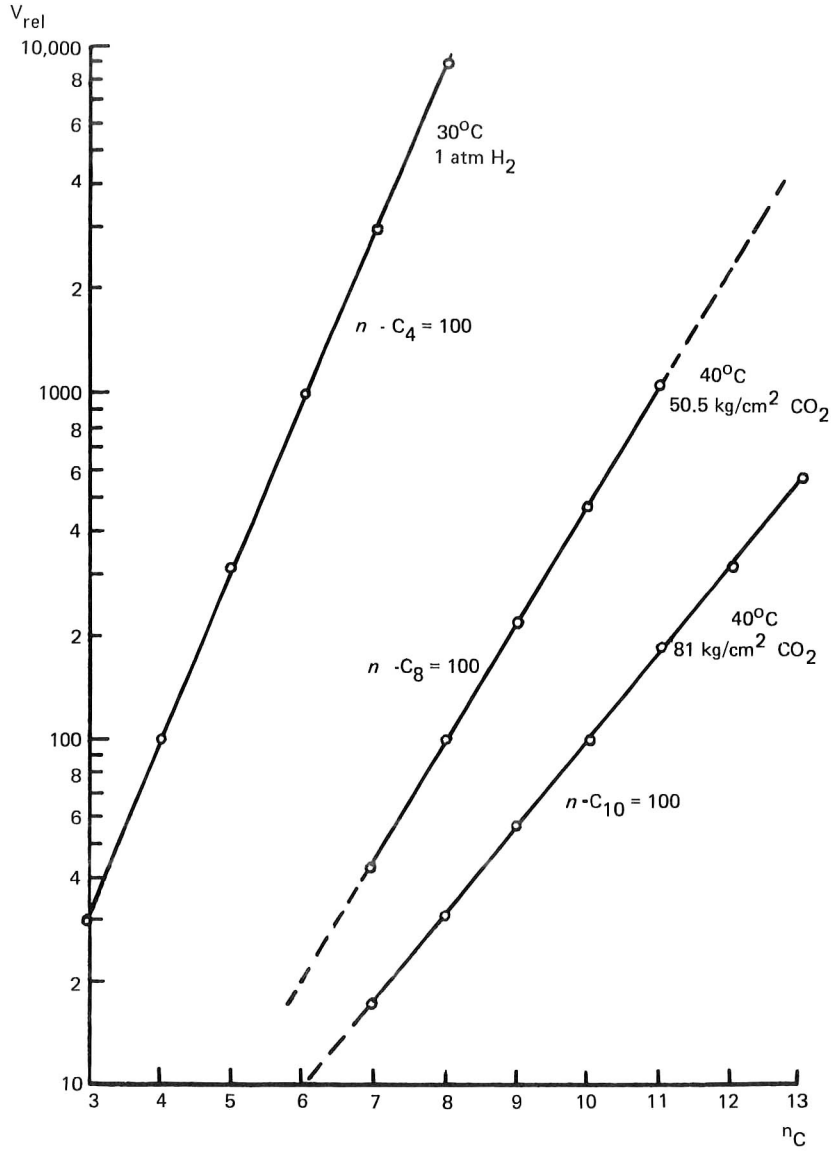

FIGURE 2. Relative retention of C_3-C_{13} n-alkanes using carbon dioxide as the mobile phase and squalane as the stationary phase (ref. 7).

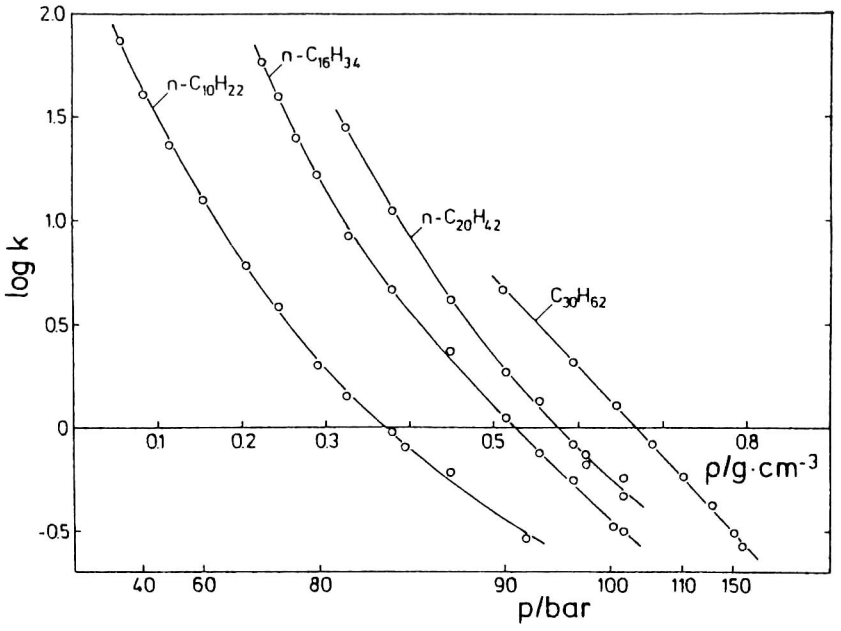

FIGURE 3. Log_{10} k' of decane, hexadecane, eicosane and squalane plotted against the density and pressure of carbon dioxide at 40°C using Carbowax 400 on 100-120 mesh Porasil C as the stationary phase (ref. 24).

For non-volatile compounds the mobile phase density plays the most important role in determining k' values. Several workers have shown that plots of log k' against the density of the mobile phase for various solutes are almost linear (21,22,24,32,40,49). Figure 3 illustrates this phenomenon for alkanes with plots of log k' against CO_2 density using Carbowax 400 as the stationary phase. Since both pressure and temperature play important roles in controlling mobile phase density, changes in either parameter should affect retention. This has been shown to be the case with

naphthalene in CO_2 at various temperatures and pressures (24). When log k' of naphthalene is plotted against the density, rather than pressure of the mobile phase, data obtained at 35°C, 40°C, and 50°C are all found to lie practically on the same line. Basing solubility on mobile phase density alone is to some extent oversimplified, but still serves as a very important guide for retention in SFC. Decreases in k' values obtained by lowering operating temperatures can be attributed to the increased density and, therefore, solvating ability of the mobile phase at the lower temperatures (9,22,31,40,45). Increases in k' values obtained when temperatures are increased, but pressures are held constant, are likewise a result of decreasing the mobile phase density.

It is a well known fact that the solubilities of solutes in liquids generally increase with temperature (although they can also decrease). This is also the case with supercritical fluids. At higher pressures where the density of the supercritical fluid does not change as much with temperature or pressure, the effect of temperature on solubility would be expected to become more noticeable. An example of this is shown in figure 4 where an increase in the solubility of naphthalene in ethylene is observed with temperatures increasing from 7° to 57°C at both 127 and 304 bar (50). This can also be seen in log k' versus density plots of polycyclic aromatic hydrocarbons in CO_2 (32) where, with a

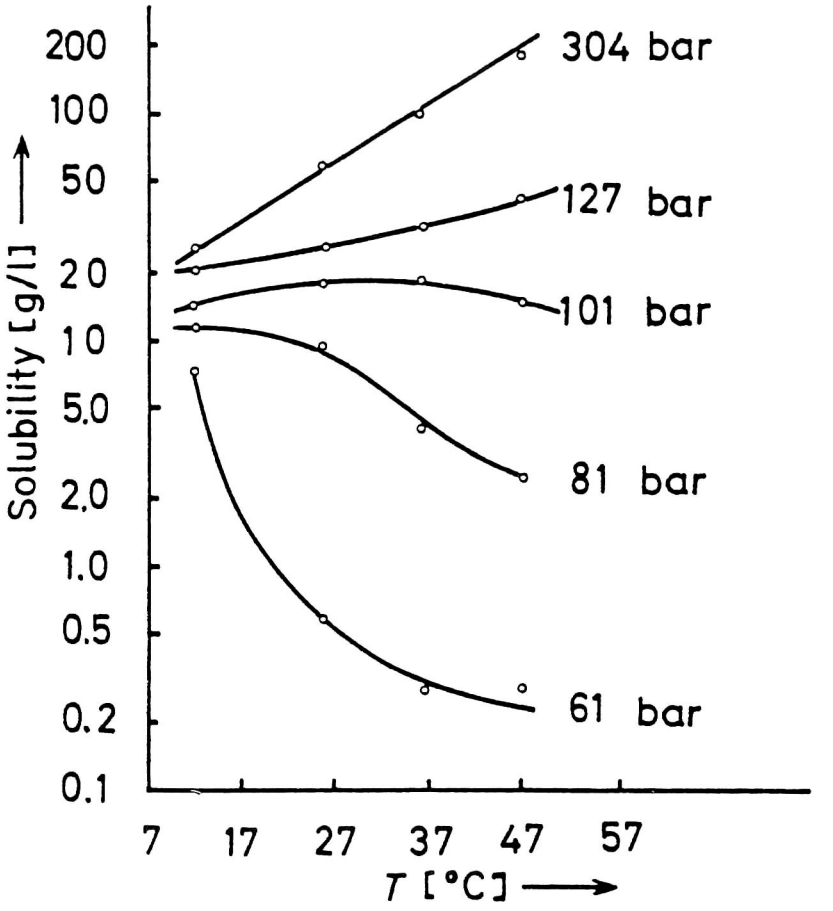

FIGURE 4. The solubility of naphthalene in ethylene as a function of pressure and temperature (ref. 49).

constant mobile phase density, higher temperatures gave smaller values for k'.

In constant temperature work, the mobile phase density is the controlling factor in solute solubility. This was expressed

in early work in terms of threshold pressures. The concept of threshold pressures was introduced by Giddings when it was observed that certain minimum pressures were required to obtain the chromatographic migration of macromolecules (20,21,51). These minimum pressures both increased and became more abrupt with increasing molecular weight. To describe this effect and to better understand the eluting power of various supercritical fluids, a solubility parameter δ was defined (21,52) as:

$$\delta = 1.25\ P_c^{\frac{1}{2}}(\rho/\rho_{liq}) \qquad [2]$$

Here P_c is the critical pressure of the mobile phase, ρ is the density of the mobile phase, and ρ_{liq} is the density of the mobile phase in the reference liquid state. Qualitatively, the closer the solubility parameters of the solute and mobile phase are to each other, the better is the ability of the mobile phase to act as a solvent for that solute. This means that adjustments of the mobile phase density in order to bring its solubility parameter to the same value as that of the solute will cause chromatographic migration. Other factors are also important in predicting chromatographic migration (25,51), but the solubility parameter can still serve as a guideline in understanding mobile phase and solute interactions.

Based on the thermodynamic properties of gases, the following relationship (49) has been derived:

$$\left(\frac{\partial \ln k'}{\partial \rho_{mob}}\right)_T = \frac{V_i^{\infty mob} - V_i^{\infty stat}}{RT}\left(\frac{\partial P}{\partial \rho}\right)_T - \frac{1}{\rho_{mob}} \qquad [3]$$

where $V_i^{\infty mob}$ and $V_i^{\infty stat}$ are the partial molar volumes of a test substance i at infinite dilution in the mobile and stationary phases. An integration of this equation predicts plots of log k' against log ρ to be linear. This has been shown to be the case with various types of compounds in CO_2 (24,52). This relationship between mobile phase density and retention is much like that found for relationships of retention and solvent composition in liquid chromatography where plots of log k' against the solvent composition or the log of the solvent composition have been reported (53,54). This serves to further emphasize the similarity of SFC to liquid chromatography.

The roles of pressure and temperature in determining solute solubility in the mobile phase are further treated in studies of phase diagrams and phase equilibria (44,55,56). In the simplest case, the transition from two immiscible phases to the single phase supercritical fluid state is found along a curve connecting the critical pressures and temperatures of the substances involved as shown in figure 5. In this case, chromatographic migration of solutes can be achieved by raising the pressure of the system sufficiently. More complex equilibria are possible when the phase transition to a single phase system does not lie on a curve

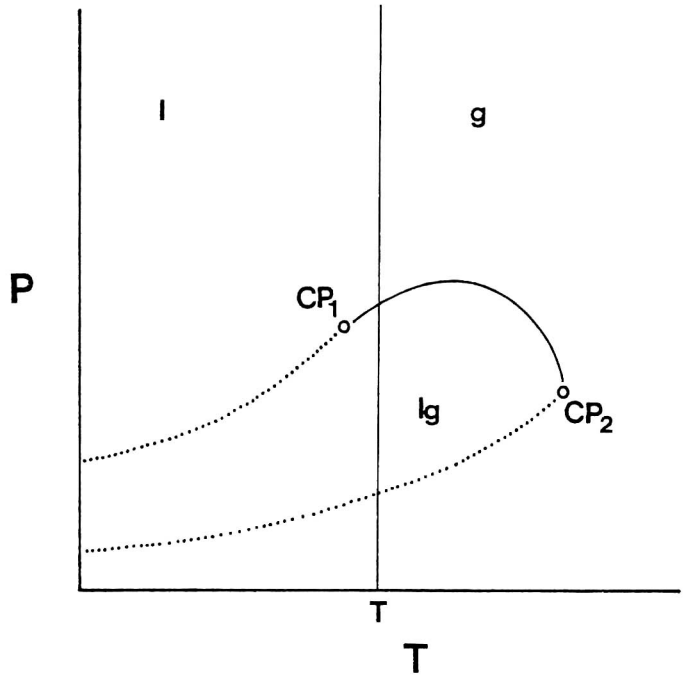

FIGURE 5. Phase diagram of two mutually miscible compounds in the critical region. (——) critical curve for mixtures of components 1 and 2; (....) vapor pressure curves for components 1 and 2; CP_1 and CP_2 are the critical points; l, g, and lg represent regions where the liquid, gas, and both liquid and gas phases are present. Raising the temperature T between CP_1 and CP_2 will cause the liquid component 2 to become miscible with component 1 in the gas phase.

connecting the critical points of the solute and solvent. This is shown in figure 6. In this situation, it is possible to never reach (at any pressure) adequate solubility for chromatographic migration at a selected temperature T'. A higher temperature T", however, does allow miscibility to occur as the pressure is

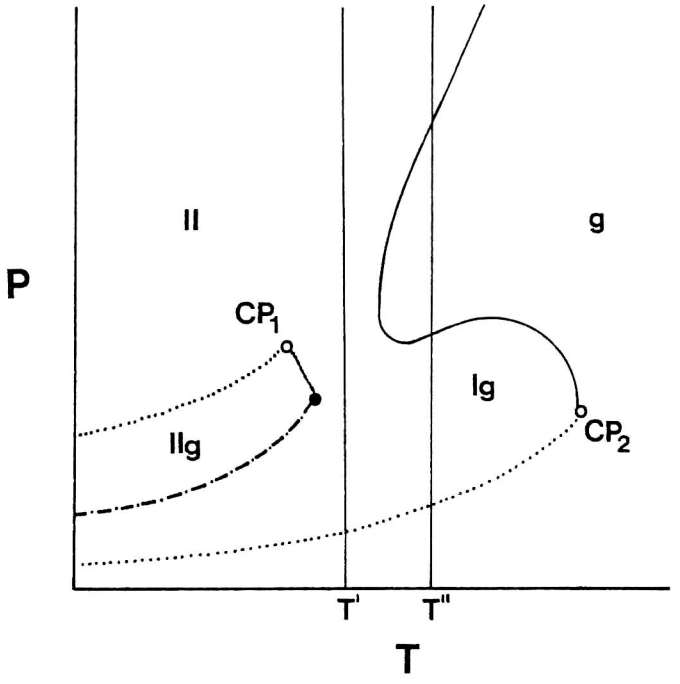

FIGURE 6. Phase diagram of two compounds not exhibiting total miscibility in the critical region. (-···-) vapor pressure curve for the two immiscible liquids, 11; two immiscible liquids and a gas phase, 11g; other symbols are as in figure 5. At temperature T' total miscibility never occurs with increasing pressure. Increasing the pressure at temperature T'' first causes miscibility, and then as even higher pressures are used, immiscibility.

increased. Figure 6 also demonstrates the possibility of decreasing miscibility with increasing pressure. This has been demonstrated in some very high pressure work with CO_2 in which the solubilities of stearic acid and octadecanol were shown to decrease in the pressure region from 270 atmospheres to 2000 atmospheres (29,57).

These types of phase equilibria show a parabolic dependence of solubility on the density of the mobile phase (50,51). This allows retention data to be fit to an equation of the form:

$$\ln k' = a\rho^2 - b\rho + c \qquad [4]$$

At lower pressures, the magnitude of the $a\rho^2$ term is usually insignificant, resulting in the almost linear plots of $\ln k' = c - b\rho$ previously described (see figure 3). A parabolic dependency of ln k' on the volume fraction composition of the mobile phase has also been described in liquid chromatography (58). This serves to give further support to the idea that retention mechanisms for SFC and liquid chromatography are quite similar to each other.

At lower pressures, second virial coefficients between the mobile phase and solute can be used to describe retention changes with pressure (7,24). Theory predicts k' to vary with pressure according to the relation:

$$\ln k' = \ln k'_{P_o} + \frac{P - P_o}{RT}(2B_{1,2} - V_2) \qquad [5]$$

Here P_o is the reference pressure, P is the pressure, V_2 is the partial molar volume of the solute in the stationary phase at infinite dilution, and $B_{1,2}$ is the second virial coefficient of the interaction between the mobile phase and the solute. This equation predicts a straight line for plots of ln k' against pressure having slopes of $2B_{1,2}/RT$. Experimental results

(7,24,40) have shown this relation to hold with pressures up to 70% of the critical pressure of the mobile phase.

The increase in solubility with increasing pressure gives rise to a technique similar to temperature programming in gas chromatography or solvent programming in liquid chromatography. By gradually raising the pressure during a chromatographic run, it is possible to elute compounds over a wide molecular weight range. This was first shown by Sie and Rijnders (10) in the separation of components in a polyphenyl tar. Giddings mentioned this possibility in his work (29), but the first real demonstration of the potential of this technique was shown by Jentoft and Gouw (36) in separations of polycyclic aromatic hydrocarbons, polystyrene oligomers, and polyphenyl ethers with pentane containing 5% methanol as the mobile phase. Separations of polystyrene oligomers are shown in figures 7 and 8. Pentane containing 5% methanol and a packed column containing Porasil C (120-150 μm) with bonded n-octyl groups were used for the separation in figure 7. The separation in figure 8 resulted from the use of pure pentane as the mobile phase and a capillary column containing a nonextractable 50% phenyl polymethylphenylsiloxane stationary phase. The improved resolution shown in figure 8 resulted from the higher number of theoretical plates provided by the capillary column.

Several workers have studied the effects of different pressure programming parameters on the quality of the resulting

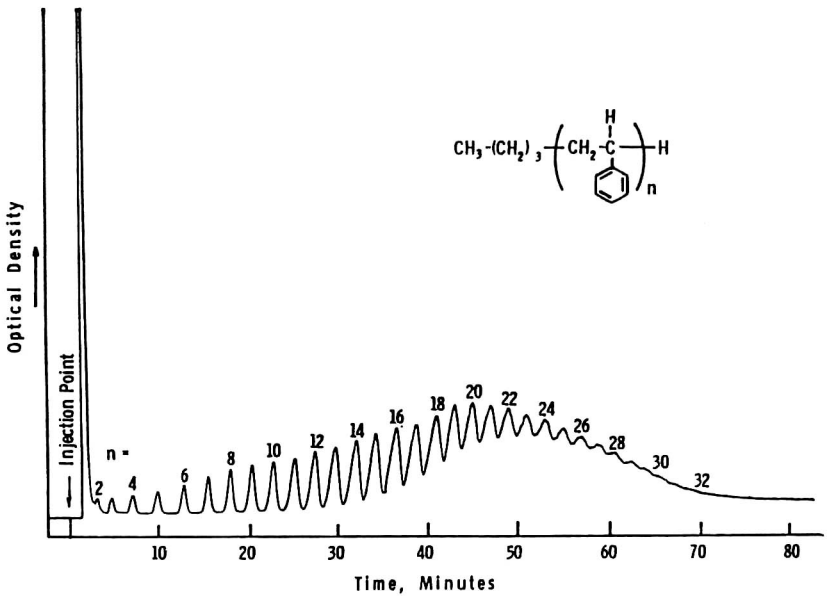

FIGURE 7. 2100 MW polystyrene sample separated on a four meter packed column (ref. 36).

separations. It is generally recognized that the slowest programming rates give the best results (22,30,59,60). This is not unreasonable since the slowest temperature programming rates in gas chromatography also give the best results. Higher temperatures have also given better results because the pressure-density isotherms are more linear (22,45). Hence, it is not the pressure programming rate which is of direct importance, but the rate at which the mobile phase density is increasing as compared to its flow rate. The presence of a large pressure drop across a column can make pressure programming useless for the separation of very wide molecular weight range materials such as polystyrenes (30).

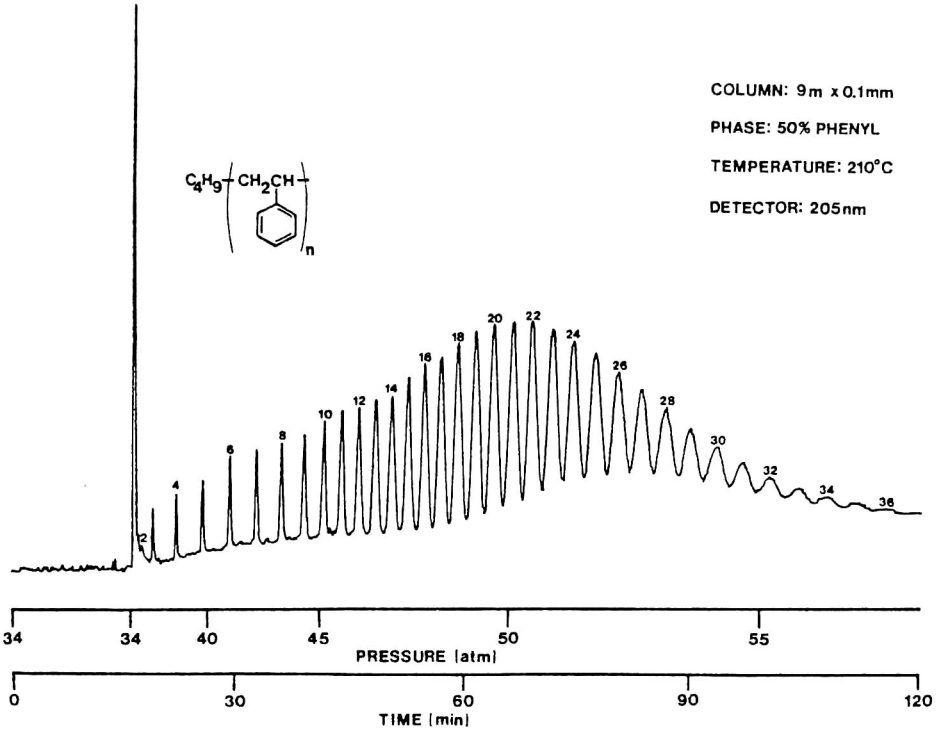

FIGURE 8. 2100 MW polystyrene sample separated on a nine meter capillary column.

Small particles cause large pressure drops which, in turn cause compounds with different elution pressures to elute closer together from the column. Columns packed with larger particles (122 to 205 μm) have low pressure drops and therefore give the best results. The possibility of nonlinear pressure programming to compensate for nonlinear pressure-density profiles is also available (22,45). This also becomes desirable because linear density programming causes compounds in homologous series to

gradually elute closer to each other (60). An ideal pressure programming system would allow members of homologous series to be eluted at regular time intervals (61,62).

RESOLUTION

Increasing pressure can have both positive and negative effects on the resolution of sample components. The first treatment of this was given by Giddings in a study of the effect of pressure on selectivity in chromatography (63). Theoretically, he demonstrated that pressure increases to about 500 atmospheres or higher could cause equilibrium shifts large enough to resolve isomers.

The elution properties of various homologous series at different pressures have been studied by a number of workers (7,8,24). It was observed that the slope of log k' versus the carbon number of homologous series decreases at higher pressures (7,25) and at temperatures closer to the critical temperature of the mobile phase (22,45). This gives a net loss of selectivity as higher mobile phase densities are used. While the selectivity within a homologous series decreases with pressure, compound type selectivity still remains good. This can be very advantageous when class separations are desired in wide molecular weight range mixtures. Very wide molecular weight ranges of the same compound type can be made to elute together, but separate from other compound types. This is generally more difficult in gas and

liquid chromatography in which wide molecular weight range samples tend to exhibit considerable overlap with other compound types (7,26).

A more general expression of the pressure and temperature effects on selectivity can be made by relating the selectivity to mobile phase density. At constant pressure for homologous series, it has been shown that (7):

$$\log k' = A + Bn \qquad [6]$$

where A is a constant, B is another constant giving the change of log k' per carbon unit, and n is the number of carbon units in the member of the homologous series being eluted. The following expression has also proven to be approximately true for most work (32,44):

$$\log k' = a - b\rho \qquad [7]$$

This relates mobile phase density to the retention of any one compound at constant temperature. The constant a may be regarded as the log k' of a component under gas chromatographic conditions at the same temperature, and b is simply the rate of change of log k' with respect to mobile phase density, ρ. Equation 7 can be used to derive an equation for the slope B in equation 6. The resulting expression has the form:

$$B = B_o - m\rho \qquad [8]$$

where B_o and m are new constants. Inserting this into equation 6, the following equation for retention as a function of mobile phase density and carbon number is obtained:

$$\log k' = A + B_o n - mn\rho \qquad [9]$$

Selectivity, α, is usually defined as $\alpha = k'_2 / k'_1$. Using equation 9 to define k' and solving for α between two members of a homologous series results in the relationship:

$$\log \alpha = B_o - m\rho \qquad [10]$$

By comparing equations 8 and 10 it can be seen that log α is numerically equivalent to the slope B for a homologous series at any given mobile phase density. By plotting log α against the mobile phase density and using data taken by Sie and Rijnders (7,9), the results shown in figure 9 are obtained. This demonstrates and helps confirm the linear relationship between log α and mobile phase density. As a practical consequence of this relationship, the maximum resolution between members of homologous series is achieved when elution is carried out at the lowest possible mobile phase density. This is demonstrated further in figure 10 in which the α between two different polysiloxanes each containing four phenyl groups is plotted against pressure using n-pentane as the mobile phase on derivatized and underivatized Porasil C. Selectivity decreases with increasing pressure. It is possible to raise the operating pressure sufficiently to cause all compounds of the same type to coelute. In the less selective system shown, coelution occurs at 32 atmospheres or higher. This does not occur in the more selective system until 37 atmospheres is reached. Also worth noting is the fact that the polysiloxanes are still retained when coelution

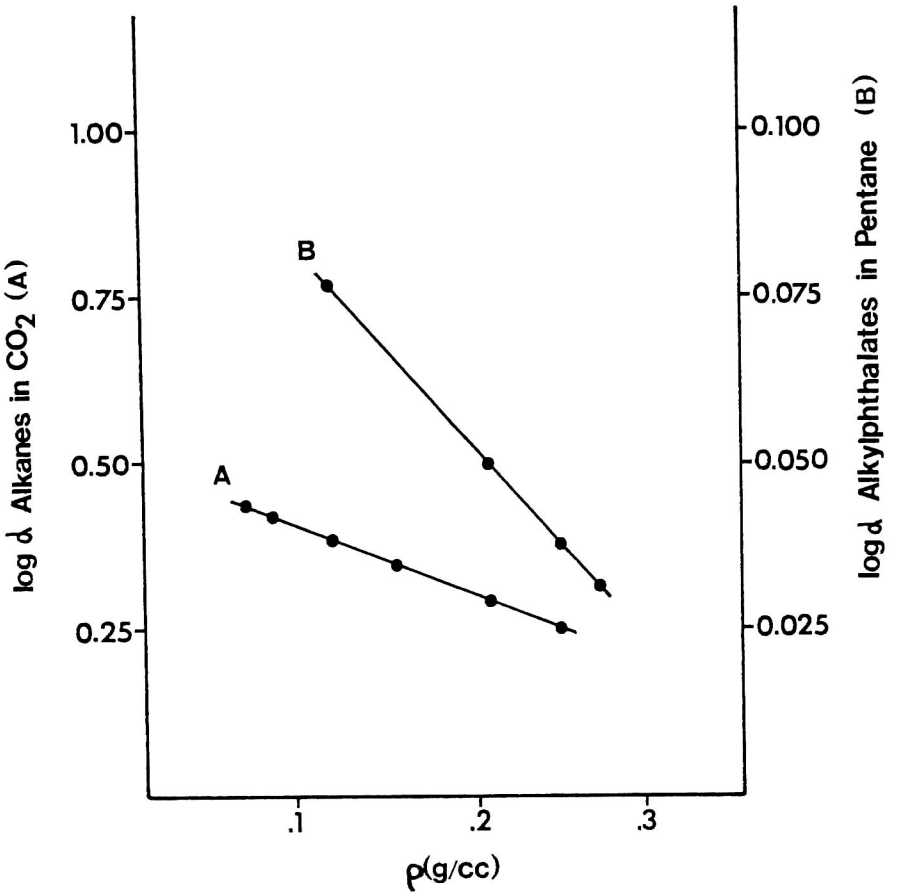

FIGURE 9. Plots of log α versus mobile phase density for alkanes in CO_2 and alkylphthalates in pentane. Data for these plots were taken from references 7 and 9.

occurs. This again points out the feasibility of separating different compound classes from each other with SFC. If coelution did not occur except when compounds were unretained, class separations would vary from difficult to impossible.

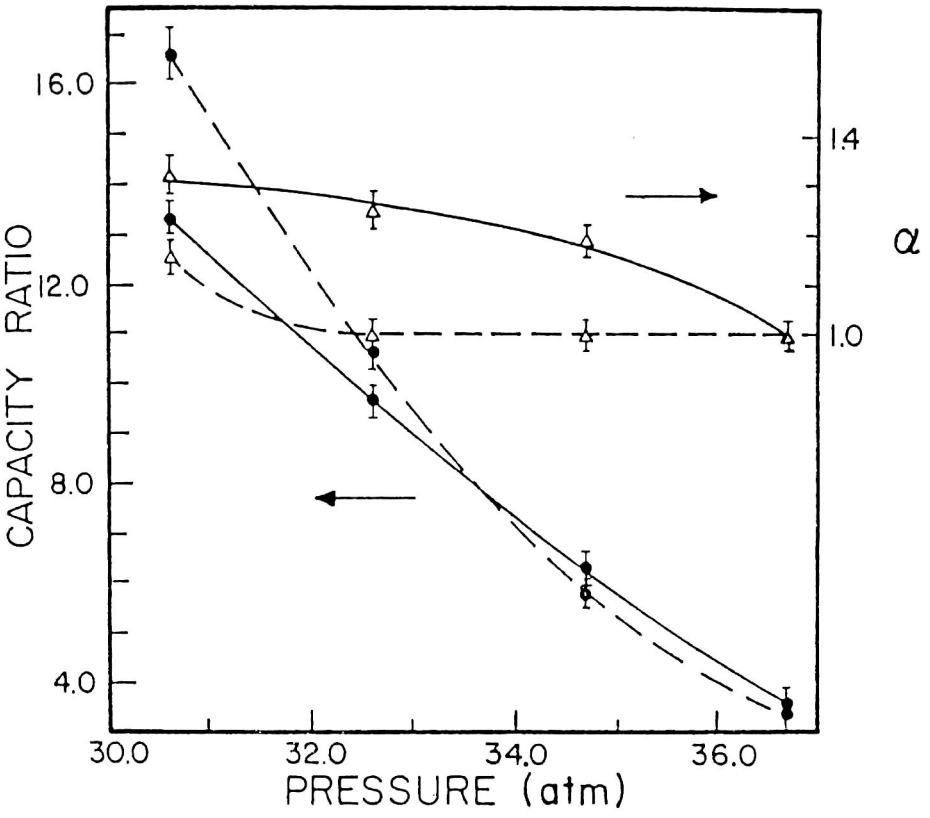

FIGURE 10. Comparison of capacity ratios for a cyclotetrasiloxane (•), and selectivity, α, between the cyclotetrasiloxane and a linear siloxane (Δ) on 100/150 mesh Porasil C (——), and on 100/120 mesh n-octyl bonded Porasil C (---) (ref. 22).

The change in selectivity with changing mobile phase density which results from large column pressure drops can also be disadvantageous. The higher mobile phase density present throughout the first part of a column gives a lower net α than if the whole column were closer to the same density.

Selectivity in SFC is also greatly affected by the choice of mobile and stationary phases. A main prerequisite for the use of any substance for a mobile phase in supercritical fluid chromatography is that it be chemically stable under the conditions to which it will be subjected. Many possible mobile phases have been evaluated in this regard by Asche (64). Chlorinated and fluorinated hydrocarbons usually give some acidic decomposition products, butanone is partially oxidized, and acetonitrile, when used with alumina, gives off ammonia. The following fluids are sufficiently stable for normal use at temperatures above 200°C: n-pentane, diethylether, methanol, 2-propanol, n-heptane, cyclohexane, diisopropylether, benzene, tetrahydrofuran, and ethylacetate. In evaluating the potential uses of these mobile phases, the polarity of the mobile phase was found to be important in the quality of the separations obtained.

The choice of mobile phase should be dependent on the types of compounds to be analyzed. Similar to the fact that some liquids are better solvents for various types of materials than others, some supercritical fluids are also better for certain types of compounds. Examples of this are seen in comparisons of CO_2 and NH_3 as solvents (20,29,51). Carbon dioxide was shown to be better for purines than NH_3, while NH_3 was a better solvent for carbohydrates and nucleosides. Supercritical CO_2 has also been shown to be a good solvent for various natural products (50). In a comparison between supercritical CO_2 and N_2O as

solvents for a wide variety of polar natural products, N_2O was generally found to be the better solvent.

Both the mobile and stationary phases play important roles in selectivity. Sie and Rijnders, in their early work, studied various combinations of polar and nonpolar stationary and mobile phases. They used n-pentane or 2-propanol as the mobile phase and polyethylene or polyethyleneglycol as the stationary phase (9), and were able to demonstrate an increase in selectivity on the basis of molecular weight or degree of alkyl carbon number by the proper choice of mobile and stationary phases. Alkyl phthalates were separated most effectively with the polar mobile phase and nonpolar stationary phase. Using the nonpolar mobile phase along with the polar stationary phase gave poor selectivity for the alkyl phthalates, but an improved separation of the polycyclic aromatic hydrocarbons on the basis of the number of aromatic rings in the compound. This corresponds well to work by Jentoft and Gouw (14) in which separations of polycyclic aromatic hydrocarbons were obtained on the basis of structure when using CO_2 as the mobile phase with a polar stationary phase. When a nonpolar stationary phase was used, separations were obtained on the basis of alkyl substitution and molecular weight. These effects are shown in figures 11 and 12, respectively.

It is also possible to mix different mobile phases to improve selectivity. Several workers have shown that the addition of methanol or 2-propanol to pentane improves the selectivity in the

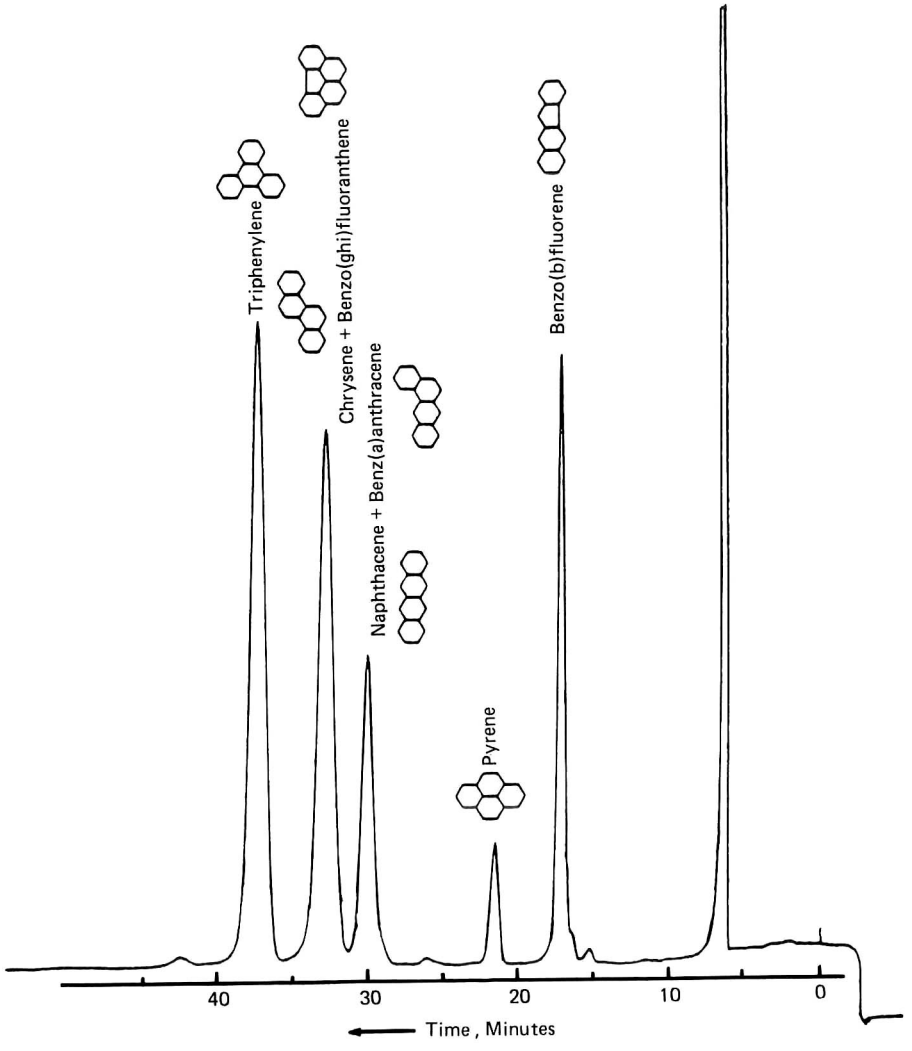

FIGURE 11. Chromatogram of seven PAH on Permaphase-ETH using CO_2 as the mobile phase at 33°C and 1500 psi (ref. 14).

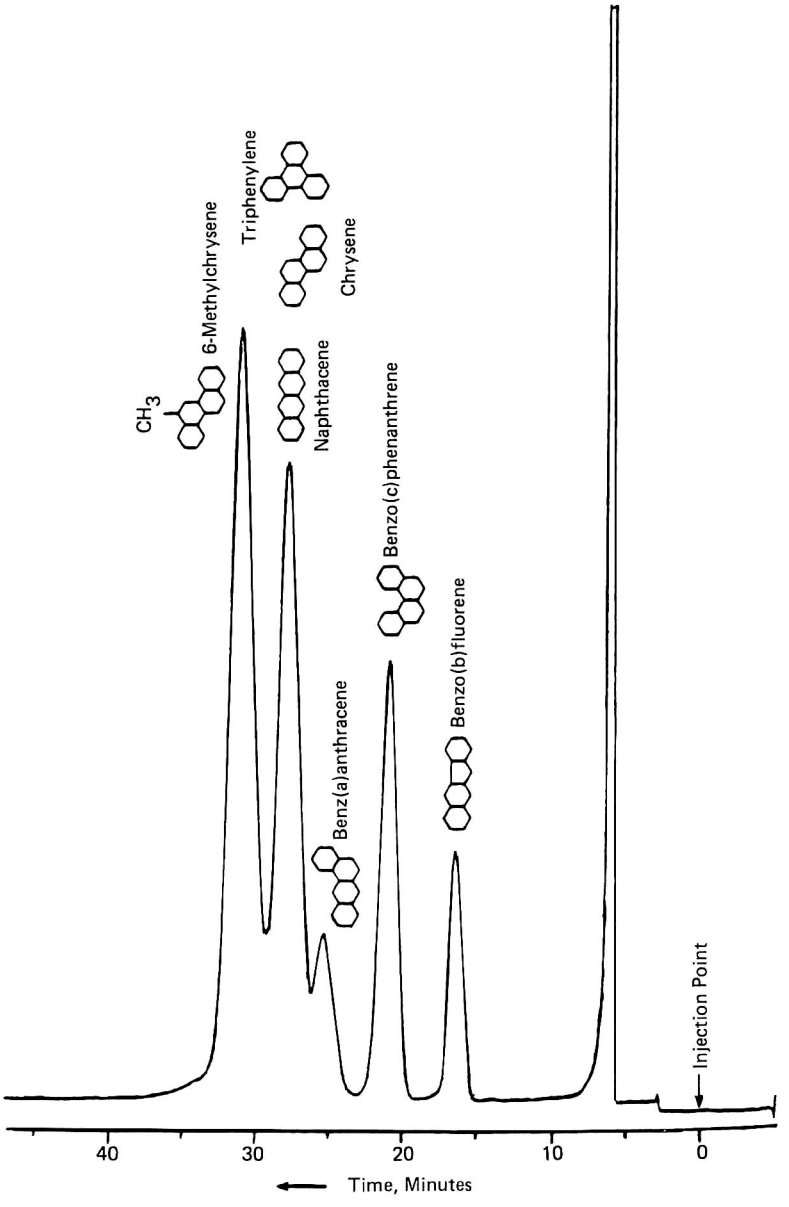

FIGURE 12. Chromatogram of some 4-ring PAH on Vydac Reverse Phase using CO_2 as the mobile phase (ref. 14).

separation of nonpolar polymers (22,45,59). This is analogous to the observation above that 2-propanol gave better selectivity for separating alkylphthalates. For mixed mobile phases, the critical constants of the mixture can be approximated by (65):

$$T_c = X_A T_A + X_B T_B \quad [11]$$

$$P_c = X_A P_A + X_B P_B \quad [12]$$

T_c and P_c are the new critical parameters for the mixed mobile phase, X_A and X_B are the mole fractions of solvents A and B, T_A and T_B are the critical temperatures of the solvents, and P_A and P_B are their critical pressures.

The strong solvating ability of the mobile phase makes the selection of stationary phases one of the biggest problems in supercritical fluid chromatography. Because of this, much work has been done using adsorbents such as alumina, silica, and polystyrene (10,22,66). Size exclusion chromatography has also been used, but poor results were obtained at lower pressures due to adsorption (34). Karayannis, et al. (6) tried a wide number of stationary phases with dichlorodifluoromethane as the mobile phase. The best results were obtained with Carbowax 20M, Versamid 900, Epon 1009, XE-60, and Epon 1001. Polyethylene glycols have been popular as stationary phases because of their insolubility in carbon dioxide and pentane (9,13). Polyethylene has also been used with 2-propanol as the mobile phase (9). The use of bonded non-extractable stationary phases is preferred since it is impossible to dissolve them in the mobile phase. These types of

stationary phases have proven very useful in a wide variety of studies (14,32,40). Bonded stationary phases in SFC are just as important as they are in liquid chromatography.

INSTRUMENTATION

The instrumental requirements of SFC resemble those of high pressure liquid chromatography. This is because both systems make use of high pressures and solvent mobile phases. Characteristics which make the instrumentation for supercritical fluid chromatography different from high pressure liquid chromatography are primarily: (a) the whole column must be under high pressure, not just the first section; (b) supercritical fluid chromatography requires pressure control rather than flow control; and (c) detectors must be designed to either work at high pressures, or provide for a means to handle eluted compounds after the mobile phase pressure is reduced to atmospheric values. The importance of the third point is understood better when one considers the case of a mobile phase which is normally a gas at ambient pressures and temperatures. When this mobile phase is allowed to come to atmospheric pressure, dissolved solutes will precipitate out. This has been a source of problems for mass spectrometer interfaces (67) and flame ionization detectors (51,68,69).

There are many published descriptions and reviews of instrumentation for supercritical fluid chromatography (6,7,9,13,16,

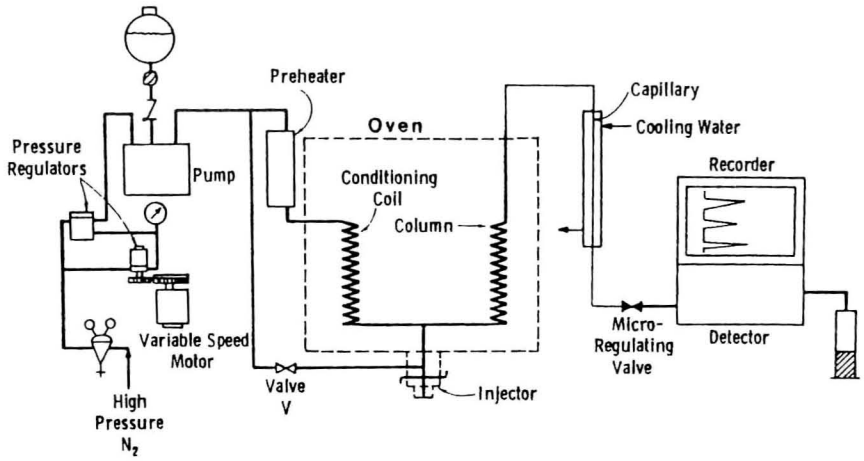

FIGURE 13. Apparatus for Supercritical Fluid Chromatography (ref. 36).

24-27,36,51,60,69,70). A typical schematic of instrumentation used is shown in figure 13 (14).

Solvent delivery systems have differed mainly with respect to the type of mobile phase that they were designed to supply. With mobile phases that are gases at ambient pressures, a gas cylinder has been connected to high pressure reducing valves in order to supply the appropriate mobile phase pressure to the column (6,7). When higher pressures have been needed than that supplied normally by the gas cylinder, two approaches have been used. The cylinder has been heated (7), or the pressure to the column has been raised by means of high pressure pumps (13,24). For mobile phases which are liquids under ambient conditions, high pressure liquid chromatographic pumps can be used after they

are modified for pressure, rather than flow, control (36,60,71). It is very important that the flow output from these pumps be pulseless. Reciprocating piston pumps require pulse damping systems to assure pulseless operation (16), whereas syringe pumps can deliver pulseless flow without damping (27). The pressure control system should also allow for pressure programming in order to increase its usefulness.

Injection systems suitable for high pressure liquid chromatography can often be used. As in liquid chromatography, injection systems using septa have pressure limitations and can cause contamination of the mobile phase (13,27). This has lead to the use of various kinds of high pressure valves as injection devices (9,60). Systems allowing the supercritical fluid mobile phase to act as the injection solvent (9) can be advantageous, but care must be used to ensure that the entire sample is soluble in the mobile phase under the injection conditions (55).

Column and mobile phase temperature control is usually handled by using a constant temperature bath or oven in which both the column and a coil for preheating the mobile phase are located. The importance of having good temperature control of the mobile phase is easily understood in view of the strong effects that the temperature of the mobile phase has on its density and sample solubility as previously discussed. This becomes increasingly important the closer one works to the critical temperature of the mobile phase (26). For large mobile

phase flow rates, the coil used to preheat the mobile phase can become inadequate. Semonian and Rogers studied the ability of preheating coils to bring the mobile phase to the desired temperature under various conditions (40). For n-pentane at flow rates from 10 to 320 ml/hr, a one meter stainless steel tube (0.22 cm i.d.) was found to be adequate.

The most popular detector used has been the ultraviolet detector; various modifications have been made to allow its use for SFC. Capillary columns require on-column detectors to meet their small volume requirements (70). Most high pressure liquid chromatography detectors are not designed to operate under high pressures. In order to use ultraviolet detection systems with mobile phases such as CO_2 and N_2O, extremely high pressure cells are required for detection in the liquid or supercritical fluid state (6,13,22). Systems have also been devised to allow for cooling the mobile phase to the liquid state, followed by detection in low pressure ultraviolet detector cells (9,60). This method allows for any of the liquid chromatographic detectors available to be used.

Flame ionization detectors can also be used, but tend to present some problems because of sample condensation during mobile phase decompression (7,21,69,72). This same problem is present in the interfacing of supercritical fluid chromatographs to mass spectrometers (27,67,73). Other detectors which have

been used are refractive index (74), adsorption (23), scintillation (14), and thermoconductivity detectors (12).

If the mobile phase is a liquid under ambient conditions, fraction collection is easily accomplished by cooling the column effluent and collecting the liquefied mobile phase as one would in liquid chromatography (17,27). In the case where the mobile phase is gaseous at atmospheric pressure, two alternatives have been used. In one case, a specially constructed high pressure sample collection chamber (13,27) was maintained at high pressures with a high pressure nitrogen source so that liquid CO_2 fractions could be collected. By gradually bleeding off the pressure in these chambers, the solvent free fractions could be obtained. The other alternative involved mixing the mobile phase with another higher boiling solvent, such as acetone, at the exit end of the column (16). This in turn was emptied into the fraction collector.

CONCLUDING COMMENTS

SFC has several characteristics which make it useful for the study of wide molecular weight range mixtures and for situations which demand repeated high speed analysis of high molecular weight or thermally labile samples. The development of efficient, easy to use instrumentation will help propagate this analytical method. Detectors still need considerable improvement to make this technique more widely applicable. Another aspect which

needs to be further explored and demonstrated is the determination of the types of samples for which SFC is best suited and can handle. The narrower choice of mobile phases as compared to liquid chromatography puts some restrictions on the sample types which can be conveniently handled. This also points out the need for more research on additional mobile phases, and the mixing or doping of mobile phases with small percentages of modifiers to achieve special effects. The use and further development of insoluble stationary phases will be beneficial for providing better selectivity and allowing mobile phase conditions which would normally wash other stationary phases out of the column. As people become increasingly aware of how SFC can be valuable in solving specific analytical problems, its use will increase.

REFERENCES

1. Hannay, J. B.; Hogarth, J. Proc. Roy. Soc., London 1879, 29, 324-326.

2. Klesper, E.; Corwin, A. H.; Turner, D. A. J. Org. Chem. 1962, 27, 700-701.

3. Karayannis, N. M.; Corwin, A. H. Anal. Biochem. 1968, 26, 34-50.

4. Karayannis, N. M.; Corwin, A. H. J. Chromatogr. 1970, 47, 247-256.

5. Karayannis, N. M.; Corwin, A. H. J. Chromatogr. Sci. 1970, 8, 251-256.

6. Karayannis, N. M.; Corwin, A. H.; Baker, E. W.; Klesper, E.; Walter, J. A. Anal. Chem. 1968, 40, 1736-1739.

7. Sie, S. T.; Van Beersum, W.; Rijnders, G. W. A. Sep. Sci. 1966, 1, 459-590.

8. Sie, S. T.; Rijnders, G. W. A. Sep. Sci. 1967, 2, 699-727.

9. Sie, S. T.; Rijnders, G. W. A. Sep. Sci. 1967, 2, 729-753.

10. Sie, S. T.; Rijnders, G. W. A. Sep. Sci. 1967, 2, 755-777.

11. Sie, S. T.; Rijnders, G. W. A. Analyt. Chim. Acta. 1967, 38, 31-44.

12. Bartmann, D., Ph.D. Dissertation, Universität Bochum, Germany, 1972.

13. Jentoft, R. E.; Gouw, T. H. Anal. Chem. 1972, 44, 681-686.

14. Jentoft, R. E.; Gouw, T. H. Anal. Chem. 1976, 48, 2195-2200.

15. Jentoft, R. E.; Gouw, T. H. J. Polym. Sci. Polym. Lett. Ed. 1969, 7, 811-813.

16. Klesper, E.; Hartmann, W. J. Polym. Sci. Polym. Lett. Ed. 1977, 15, 9-16.

17. Hartmann, W.; Klesper, E. J. Polym. Sci. Polym. Lett. Ed. 1977, 15, 713-719.

18. Sie, S. T.; Bleumer, J. P. A.; Rijnders, G. W. A. "Proc. 7th Int. Symp. Gas Chromatogr. and Its Exploitation," ed. Harbourn, C. L. A.; Institute of Petroleum, London, 1969, p. 235.

19. Gere, D.; Board, R.; McManigill, D. in work presented at the 1982 Pittsburgh Conference on Analytical Chemistry, Atlantic City, New Jersey, March 8-13, 1982.

20. Giddings, J. C.; Myers, M. N.; McLaren, L.; Keller, R. A. Science 1968, 159, 197-199.

21. Myers, M. N.; Giddings, J. C.; in Perry, E. S.; Van Oss, C. J. "Progress in Separation and Purification," vol. 3, Wiley-Interscience, New York, 1970, pp. 133-152.

22. Nieman, J. A.; Rogers, L. B. Sep. Sci. 1975, 10, 517-545.

23. Cashaw, J. L.; Segura, R.; Zlatkis, A. J. Chromatogr. Sci. 1970, 8, 363-369.

24. Van Wasen, U.; Swaid, I.; Schneider, G. M. Angew. Chem. Int. Ed. Engl. 1980, 19, 575-587.

25. Klesper, E. Angew. Chem. Int. Ed. Engl. 1978, 17, 738-746.

26. Gouw, T. H.; Jentoft, R. E. J. Chromatogr. 1972, 68, 303-323.

27. Gouw, T. H.; Jentoft, R. E. Adv. Chromatogr. 1975, 13, 1-40.

28. Gouw, T. H.; Jentoft, R. E. Chromatogr. Sci. (Chromatogr. Pet. Anal.) 1979, 11, 313-327.

29. Czubryt, J. J.; Myers, M. N.; Giddings, J. C. J. Phys. Chem. 1970, 74, 4260-4266.

30. Graham, J. A.; Rogers, L. B. J. Chromatogr. Sci. 1980, 18, 75-84.

31. Novotny, M.; Bertsch, W.; Zlatkis, A. J. Chromatrogr. 1971, 61, 17-28.

32. Gere, D. R.; Board, R.; McManigill, D. Anal. Chem. 1982, 54, 736-740.

33. Stephan, K.; Lucas, K. "Viscosity of Dense Fluids," Plenum Press, New York, 1979.

34. Giddings, J. C.; Bowman, L. M., Jr.; Myers, M. N. Anal. Chem. 1977, 49, 243-249.

35. Hofmann, K.; Halasz, I. J. Chromatogr. 1979, 173, 211-228.

36. Jentoft, R. E.; Gouw, T. H. J. Chromatogr. Sci. 1970, 8, 138-142.

37. Giddings, J. C. "Dynamics of Chromatography," Marcel Dekker, New York, 1965, pp. 223-224.

38. Giddings, J. C. Anal. Chem. 1964, 36, 741-744.

39. Myers, M. N.; Giddings, J. C. Anal. Chem. 1965, 37, 1453-1457.

40. Semonian, B. P.; Rogers, L. B. J. Chromatogr. Sci. 1978, 16, 49-60.

41. Weissman, S. J. Chem. Phys. 1964, 40, 3397-3406.

42. Lielmezs, J. Z. Phys. Chem. Neue Folge 1974, 91, 288-300.

43. Giddings, J. C. "Dynamics of Chromatography," Marcel Dekker, New York, 1965, pp. 233-234.

44. Schneider, G. M. Angew. Chem. Int. Ed. Engl. 1978, 17, 716-727.

45. Conaway, J. E.; Graham, J. A.; Rogers, L. B. J. Chromatogr. Sci. 1978, 16, 102-110.

46. Novotny, M.; Springston, S.R.; Peaden, P.A.; Fjeldsted, J.C.; Lee, M.L. Anal. Chem. 1981, 53, 407A-414A.

47. Springston, S.R.; Novotny, M. Chromatographia 1981, 4, 83-85.

48. Giddings, J. C. "Dynamics of Chromatography," Marcel Dekker, New York, 1965, p. 279.

49. Van Wasen, J.; Schneider, G. M. Chromatographia 1975, 8, 274-276.

50. Hubert, P.; Vitzthum, O. G. Angew. Chem. Int. Ed. Engl. 1978, 17, 710-715.

51. Giddings, J. C.; Myers, M. N.; McLaren, L.; Keller, R. A. Science 1968, 162, 67-73.

52. Stahl, E.; Schilz, W.; Schütz, E.; Willing, E. Angew. Chem. Int. Ed. Engl. 1978, 17, 731-738.

53. Meek, J. L. Anal. Chem. 1980, 52, 1370-1371.

54. Hurtubise, R. J.; Hussain, A.; Silver, H. F. Anal. Chem. 1981, 53, 1993-1997.

55. Bartmann, D.; Schneider, G. M. J. Chromatogr. 1973, 83, 135-145.

56. Lentz, H.; Franck, E. U. Angew. Chem. Int. Ed. Engl. 1978, 17, 728-730.

57. Giddings, J. C.; Myers, M. N.; King, J. W. J. Chromatogr. Sci. 1969, 7, 276-283.

58. Jandera, P.; Colin, H.; Guiochon, G. Anal. Chem. 1982, 54, 435-441.

59. Klesper, E.; Hartmann, W. J. Polym. Sci. Polym. Lett. Ed. 1977, 15, 707-712.

60. Klesper, E.; Hartmann, W. Europ. Polym. J. 1978, 14, 77-88.

61. Bowman, L.M., Jr.; Myers, M.N.; Giddings, J.C. Sep. Sci. Tech. 1982, 17, 271-287.

62. Peaden, P.A.; Lee, M.L., submitted for publication.

63. Giddings, J. C. Sep. Sci. 1966, 1, 73-80.

64. Asche, W. Chromatographia 1978, 11, 411-412.

65. Reed, R. C.; Sherwood, T. K. "The Properties of Gases and Liquids," 2nd ed., McGraw-Hill, New York, 1966.

66. Fuzita, K.; Shimokobe, I.; Nakazima, F. Nippon Kagaku Kaishi 1975, 8, 1348-1351.

67. Randall, L. G.; Wahrhaftig, A. L. Anal. Chem. 1978, 50, 1703-1705.

68. Bartmann, D; Schneider, G. M. Ber. Bunsenges. Phys. Chem. 1969, 73, 917-926.

69. Bartmann, D. Ber. Bunsenges. Phys. Chem. 1972, 76, 336-339.

70. Peaden, P.A.; Fjeldsted, J.C.; Lee, M.L.; Springston, S.R.; Novotny, M. Anal. Chem. 1982, 54, 1090-1093.

71. Van Lenten, F. J.; Rothman, L. D. Anal. Chem. 1976, 48, 1430-1432.

72. Bartmann, D.; Schneider, G. M. Chem.-Ing.-Tech. 1970, 42, 702-704.

73. Gouw, T. H.; Jentoft, R. E.; Gallegos, E. J. "High Pressure Science and Technology," ed. Timmerhaus, K. D.; Barber, M. S.; Plenum Publishing Co., New York, 1979, pp. 583-592.

74. Asche, W., Dissertation, Universität Clausthal-Zellerfeld, 1977.

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USE OF CROWN ETHERS IN LIQUID CHROMATOGRAPHY

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ABSTRACT

The use of crown ethers in liquid chromatography is one of the most attractive applicationsof them in analytical chemistry. Low-molecular-weight crown ethers can be employed as a component of both stationary and mobile phases in liquid chromatography. Insoluble crown ether resins, which can be prepared by condensation polymerization of crown ethers, and chemical modification or coating of crown ether derivatives on organic or inorganic support, have been so far studied extensively as the stationary phases. Some of crown ether-modified silicas have been attempted for the use in high performance liquid chromatography (HPLC). The cation-binding properties of crown ethers are considerably reflected in the chromatographic behavior.

I. INTRODUCTION

Crown ether, which is one of the most sensational compounds, has received considerable attention since the discovery of their complexing abilities with various metal ions. Crown ethers possess respective cation-selectivities, which depend mainly on the following fac-

tors: (i) relative size of ion and crown ether cavity; (ii) type, number, and placement of hetero atoms (binding sites); (iii) conformational flexibility of crown ether ring; (iv) electrical charge of ion; (v) solvent interaction with crown ether. A large number of crown ether analogs have been designed in order to get highly cation-selective crown ethers. Numerous articles have appeared concerning the applications of crown ethers for the past decade. Especially, their application in analytical chemistry, such as extraction analyses, electrochemical analyses, and chromatographic separation, have been, so far, developed extensively. There are a number of books about the progress of crown ether chemistry published recently (1-3), and a very important review paper on the analytical application has been written by Kolthoff (4).

This review paper deals with the use of crown ethers in liquid chromatography, chiefly the application in chromatographic separation of ions. The topics to be covered include the use of low-molecular-weight crown ethers in liquid chromatography and insoluble crown ether resins as the stationary phase. The goal is to stimulate the work about the application of crown ethers in this field, because there are very few examples concerning the practical use of them in liquid chromatography, particularly of crown ether resins as the stationary phase.

II. LOW-MOLECULAR-WEIGHT CROWN ETHERS

Since various useful applications of crown ethers have been developed, many crown ether derivatives have become commercially available. Analytical chemists also came up with great ideas about their application in liquid chromatography. In liquid chromatography, crown ethers can be employed in two different ways; as a component of the stationary and mobile phases.

II-1. Use as a Component of Stationary Phase

The first example for the use of crown ether as the stationary phase is probably the use of dibenzo-18-crown-6 on separation of some rare earth metal ions (5). Separation of Pr^{3+} and Er^{3+} were attempted using a column packed with the crown ether and acetone-hexane (2:1) eluent. The heavier rare earth metal ion, Er^{3+}, is likely to be eluted faster than the lighter one, Pr^{3+}, probably because the former cannot form as stable complex with the crown ether as the latter. In this column chromatography , moderate separation of the rare earth metal ions was observed, although it was not complete. However, the composition of acetone-hexane mixture and the flow rate seem to be rather critical. For example, 1:3 and 2:3 acetone-hexane mixtures cannot afford any separation of the ions. The 1:3 mixture is too nonpolar to elute the ions, whereas the 2:3 mixture is so polar that it elutes the ions from the column at a rate too fast to achieve detectable separation.

Extraction chromatography using crown ethers has been applied in separation of radionucleides of alkali and alkaline earth metals (6,7). The stationary phases were prepared by mixing siliconized kieselguhr with an appropriate crown ether dissolved in chloroform or mesit ylene, followed by evaporation of the excess of the solvent from the slurry. As crown ether extractant dibenzo-18-crown-6, dicyclohexyl-18-crown-6, and benzo-15-crown-5, and a noncyclic crown ether, 1,13-bis(8-quinolinyl)-1,4,7,10,13-pentaoxatridecane were used. The column was eluted with 0.01 M NaSCN aqueous solution at a flow rate of 0.02 - 0.03 ml/min. A typical extraction chromatogram shows good separation of the metal ions, revealing that the chromatographic behavior for these ions reflects the extractability of dibenzo-18-crown-6 chloroform solution (FIG. 1). In the case that mesitylene was utilized instead of chloroform, the elution order , however, changed much, which suggests that the separation of the ions in extraction chromatography depends significantly on the kind of diluent (or solvent). Also, the solvent composition in the mobile phase seems to affect their separation, although a large quantity of organic solvent cannot be employed because of the miscibility with the diluents.

Another stationary phase containing low-molecular-weight crown ether is the precipitate formed between phospho-

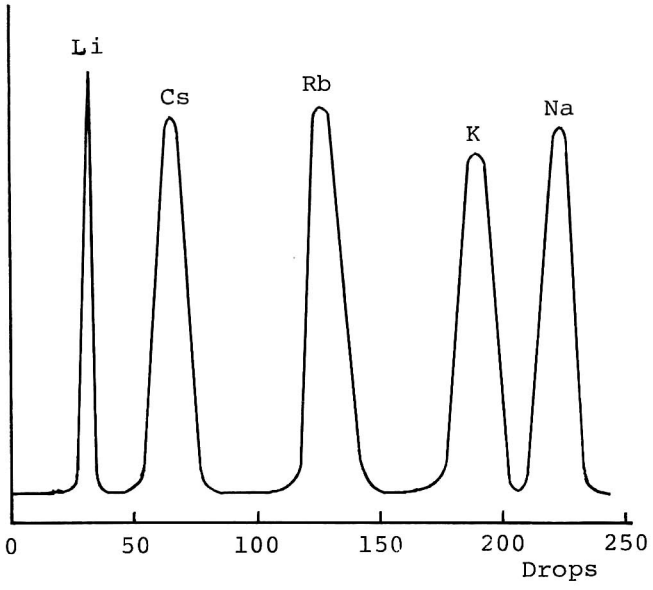


FIG. 1 Separation of alkali metals by extraction chromatography using dibenzo-18-crown-6 diluent: $CHCl_3$, eluent: 0.01 M, pH 7. (Ref. 7)

molybdic acid and crown ethers like benzo-15-crown-5, dibenzo-18-crown-6, dibenzo-24-crown-8, or cryptand [2.2.2] (8). The precipitate was easily prepared by shaking a solution of the crown ether in methylene chloride and a solution of the acid in 2 M aqueous nitric acid. The ion-exchange behavior of the sorbent was checked by measuring distribution coefficients of alkali and alkaline earth metal ions, whichwere found to be strongly dependent on the cation-binding ability of the crown ether. The sorption behavior for metal ions can be explained in the terms

of a simple ion-exchange mechanism with H_3O^+ in the crown ether cavity. Column chromatography using the precipitates afforded successful separation of Na^+ and K^+.

II-2. Use as a Component of Mobile Phase

Low-molecular-weight crown ethers can be added to the mobile phase in liquid chromatography so as to improve chromatographic separation. Effects of crown ether on ion-exchange behavior of alkali metal ions were investigated using Aminex A7 resin (sulfonated polystyrene cation-exchanger) and 18-crown-6 or cyclohexyl-18-crown-6 (9). The distribution coefficients of alkali metal ions between solutions containing HCl or HNO_3 - crown ether - 80%(v/v) methanol and the cation-exchanger were studied as a function of the methanol concentration, acidity, and crown ether concentration, and then the data were compared with those calculated theoretically from the formation constants of the crown ether complexes. Predictive modelling equations, derived from the distribution coefficients of alkali metal ions in solutions containing dicyclohexyl-18-crown-6, proved to be useful for devising separation of metal ions by ion-exchange chromatography, by which sodium isotopes, ^{22}Na and ^{24}Na, could be separated.

It is well known that crown ether complexes with ammonium ions besides alkali and alkaline earth metal ions. Taking advantage of this fact, attempts were made to improve separation efficiency in reversed-phase chromatog-

raphy of aromatic amines, amides, and amino acids, introducing crown ethers into the mobile phase (10). The pH of mobile phase and the type of the resulting ammonium ion influences, remarkably, the complexation of amino compounds with crown ethers, on which the chromatographic profile is dependent. The separation of the amino compounds is also influenced by crown ether concentration, as illustrated in FIG. 2. That is to say, amino compounds

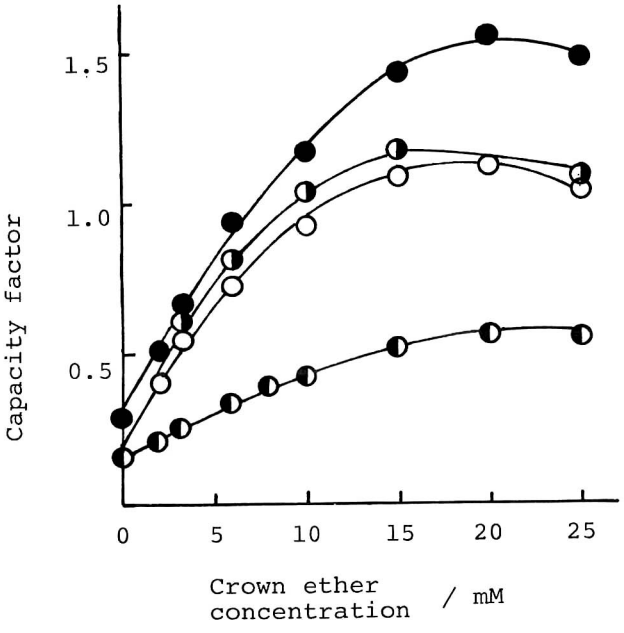


FIG. 2 Effect of dicyclohexyl-18-crown-8 concentration in mobile phase upon capacity factor of amino acids at pH 3.0 phenylglycine (○), phenylalanine (◑), tyrosine (◐), tryptophan (●) (Ref. 10)

of which ammonium species can form stable complexes with crown ethers in the mobile phase tend to be retained on the stationary phase by hydrophobic interaction. From this point of view, the role of crown ether in the mobile phase of liquid chromatography is similar to that of large organic ions in so-called ion-pair chromatography. However, crown ethers are hydrophobic non-electrolytes which can interact specifically with some organic cations unlike the organic salts for ion-pair chromatography. In reversed-phase chromatography of the amide derivatives, addition of crown ether to the mobile phase did not cause any change in the chromatogram, which observation could be anticipated readily from the fact that they can interact with crown ethers only by weak hydrogen bonding. In a similar reversed-phase chromatography of biogenic amines using 18-crown-8 in the mobile phase, selective group separation of primary and secondary amines was carried out, which is based on the selective formation of primary amines with the crown ether (11). In order to elucidate the effect of crown ethers on normal-phase adsorption chromatography of aromatic sulfonates, to the organic mobile phase was again added 15-crown-5, 18-crown-6, or dicyclohexyl-18-crown-6 (12). Sulfonic acid derivatives, which are very ionic, generally interact strongly with the polar stationary phases. Crown ethers can complex with the countercation of the sulfonate, K^+ or Na^+, resulting in

increased solubility of the sulfonates in the organic mobile phase, therefore, marked decrease of the capacity factor of the sulfonates in liquid chromatography. This use of crown ethers seems to successfully improve the separation of various sulfonic acids by normal-phase liquid chromatography.

II-3. Miscellaneous Use in Liquid Chromatography

Besides the above-mentioned examples, there are several interesting ways to use low-molecular-weight crown ethers in liquid chromatography. Chromatographic behavior of dibenzo-18-crown-6 complexes with mercury chloride, bromide, and iodide was studied in reversed-phase chromatography (13). Typical chromatograms of the crown ether - mercury halide systems are depicted in FIG. 3, showing good separation between the complexes and the crown ether itself. In the case that the sample was prepared by extraction of an aqueous solution of mercury halide mixture with the crown ether in dichloromethane (FIG. 3C), a crown ether complex with mixed halide, HgClBr, was also detected. The extraction - chromatographic technique was tested for the practical determination of mercury chloride in aqueous samples. A straight line was obtained in the calibration plots over mercury concentration range of 5 - 100 ppm. and the relative standard deviation in the mean value of peak area was about 4 % for 5 ppm.

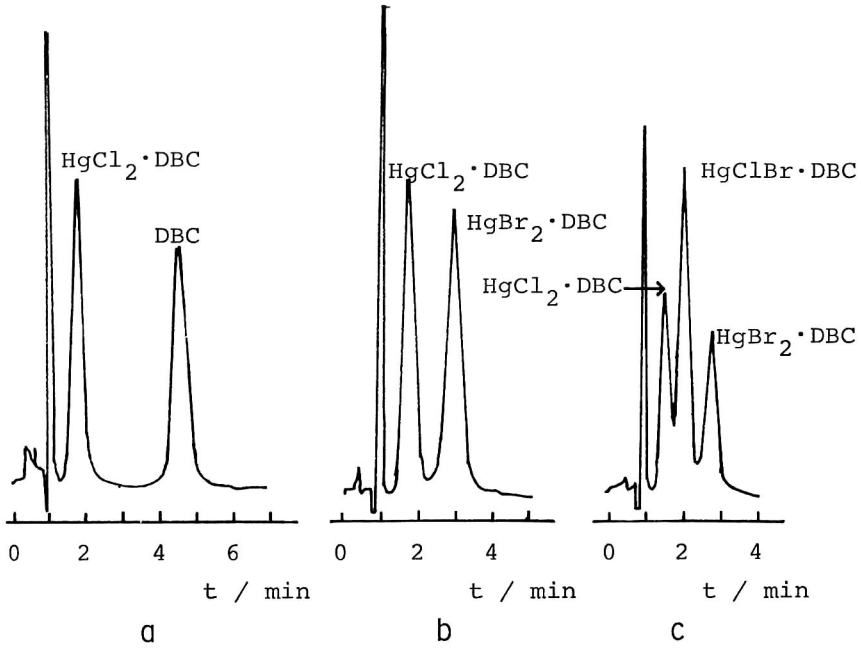


FIG. 3 Separation of dibenzo-18-crown-6(DBC) - Hg^{2+} complexes by reversed-phase chromatography. On chromatography C, the sample was obtained by extraction of aqueous mixture of Cl^- and Br^- with Hg^{2+} and the crown ether. (Ref. 13)

It is possible to measure stability constants of crown ether - metal complexes by means of reversed-phase chromatography (14). Using the physicochemical basis of solute retention in the stationary phase, the stability constants of alkali metal complexes of dibenzo-18-crown-6 and dibenzo-24-crown-8 were evaluated, which were in good agreement with the literature data. This chromatographic method may be very convenient for the determination of stability

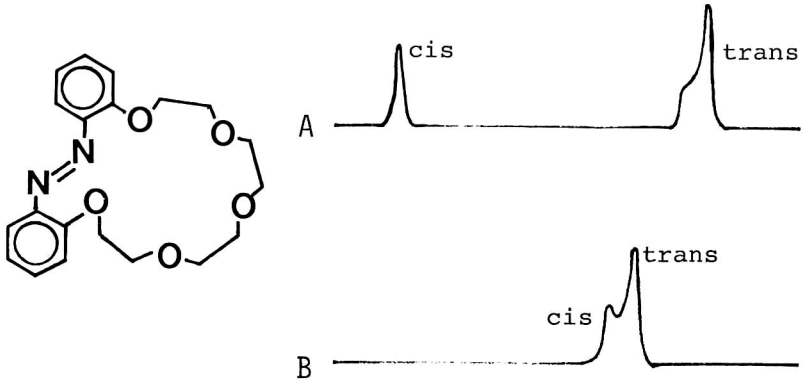


FIG. 4 Ion-dipole association chromatography of photo-isomerized azocrown ether on ion-exchanger gel (Na^+ type) (A) and polystyrene gel (B).
eluent: MeOH (Ref. 15)

constants, compared with the conventional methods like pH and calorimetric titrations.

A new type of liquid chromatography for crown ethers is "ion-dipole association chromatography" (15). This chromatography of crown ether derivatives was run using sulfonate-type cation-exchanger loaded with a specific metal ion as the stationary phase and methanol or THF as the mobile phase. The difference n the ion-dipole interaction with a loaded metal ion between crown ether derivatives brings about their separation on the stationary phase. Obviously, the chromatographic behavior of crown ethers is remarkably dependent on the kind of loaded metal ion, because they generally have different cation-binding

selectivity. A typical example for this type of chromatography, separation of azocrown ether isomers, is given in FIG. 4. Photo-irradiation causes isomerization of the azocrown ether from the trans- to the cis-form, which in turn results in change of the crown ether cavity size, therefore, that of the cation-binding selectivity of crown ether. Thus, the crown ether isomers could be separated easily in this chromatography. This type of liquid chromatography is probably applicable to the separation of other crown ether derivatives.

III. INSOLUBLE CROWN ETHER RESINS

Low-molecular-weight crown ethers can be easily purchased, so they are often applied for the liquid chromatography as mentioned above. There are, however, some unresolved problems in using the crown ethers in liquid chromatography. If crown ethers, which are still expensive, are utilized as a component of mobile phase, a large quantity of crown ether is eluted out. Even if they are used as a component of stationary phase, some loss of crown ethers, and then deterioration of the stationary phase, are inevitable; also, it is generally accepted that soluble crown ethers, notably water-soluble ones, are highly toxic. A way to eliminate these problems is immobilization of crown ethers on organic or inorganic supports. Therefore, insoluble crown ether resins or poly-

meric crown ethers have been developed as the stationary phase in liquid chromatography.

III-1. Condensation-type Poly(crown ether)s and Crown Ethers Immobilized on Organic Support

Blasius and his coworkers have reported extensive investigation of polymer resins bearing crown ethers and cryptands for chromatographic application (16-25). Various types of poly(crown ether)s and polycryptands have been synthesized, typical ones of which are shown in FIG. 5. Phenol-formaldehyde resins containing crown ethers were obtained by condensation polymerization of formaldehyde with dibenzocrown ethers in formic acid, or with monobenzocrown ethers in a mixture of formic and sulfuric acids, using crosslinking agents as phenol, resorcinol, and xylol. Also, some crown ethers were immobilized on crosslinked polystyrene by reaction of hydroxymethylbenzocrown ethers with the chloromethylated resin. Moreover, crosslinked poly(crown ether)s were synthesized by copolymerization of vinylbenzocrown ethers with divinylbenzene. Cryptands were employed instead of crown ethers in some of these systems. These crown ether resins were characterized in detail by means of pyrolysis - gas chromatography and - mass spectroscopy (25). The cation-binding abilities of the crown ether resins were examined by batch extraction of alkali and alkaline earth metal salts in methanol, in order to obtain some prelim-

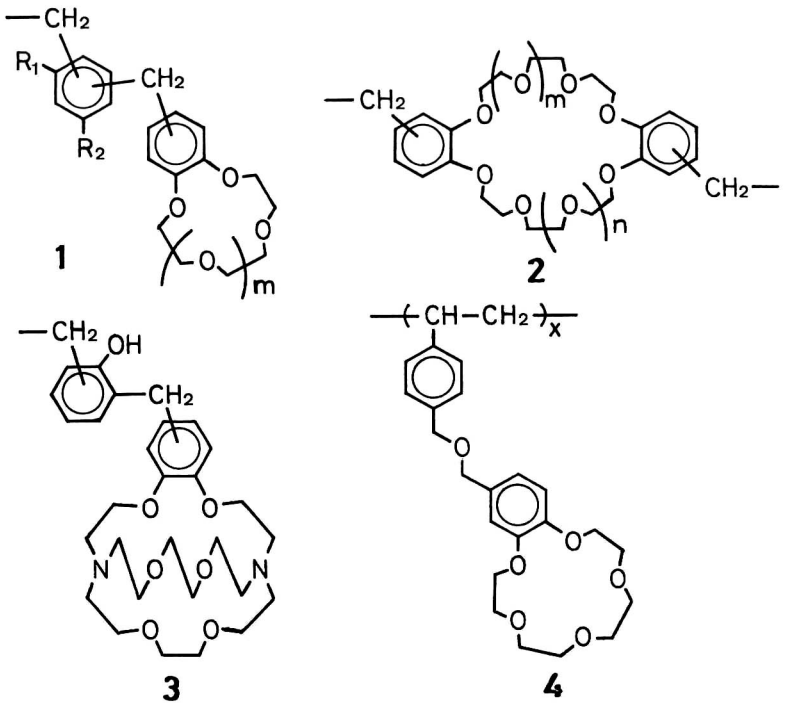


FIG. 5 Typical condensation-type crown ether resins and crown ether immobilized on crosslinked polystyrene. (Ref. 20)

inary information about the chromatographic behavior of the resins. Conceivably, the cation-binding ability (the partition coeffient) is dependent on the kind of metal ion, its counterion, and solvent. On the whole, the resins containing benzo-15-crown-5 or dibenzo-18-crown-6 bind K^+ efficiently in the group of alkali metal ions. The dibenzo-24-crown-8 resins prefer Cs^+, while Rb^+ seems to

be favored by the dibenzo-21-crown-7 resins. Li^+ does not seem to be bound very strongly by these crown ether resins. For alkaline earth metal ions, Ba^{2+} is favored in any of these resins. Although the anion dependence of cation-binding ability for the crown ether resins varies slightly from one resin to another, it follows, approximately, the polarizability sequence of the anions. For example, the order of the anion dependence for the dibenzo-18-crown-6 resin is $SCN^->I^->Br^->Cl^->F^->OH^-$ in methanol. When methanol is replaced by water, the cation-binding ability decreases drastically. Actually, these crown ether resins were applied as the stationary phase of liquid chromatography. FIG. 6 demonstrates a typical chromatogram, in which thiocyanates of Li^+, Na^+, and K^+ are separated excellently by dibenzo-21-crown-7 resin 2 (m=1,n=0). Chromatographic separations of other cations such as alkaline earth metal, transition metal, rare earth metal, and alkylammonium ions were also attempted using the crown ether resins. Moreover, separation and enrichment of some radioisotopes have been reported. It is of much interest that, unlike the conventional cation-exchangers, these crown ether resins are capable of separating anions besides the cations due to the marked anion dependence of the cation-binding ability for the neutral ion-exchangers. FIG. 7 depicts a chromatogram of a mixture of potassium halides and thiocyanate on a column of crown ether resin

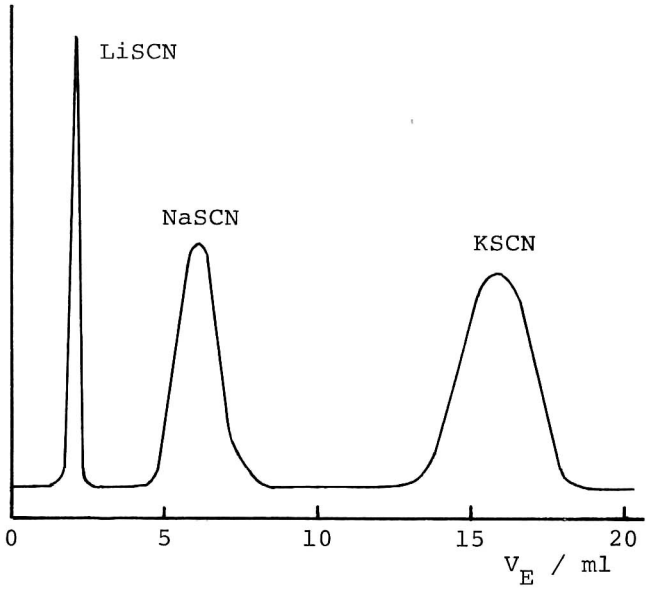


FIG. 6 Chromatographic separation of alkali metal thiocyanates on a column of crown ether resin 2 (m=1,n=0). LiSCN: 0.065 mg; NaSCN: 0.48 mg; KSCN: 1.77 mg, eluent: H_2O. (Ref 20)

2 (m=1,n=0). In this connection, interconversion of mixtures of two salts is feasible. For instance, a mixture of NH_4SCN and RbCl can be interconverted to a mixture of NH_4Cl and RbSCN by liquid chromatography using benzo-15-crown-5 resin 1 (R_1=H,R_2=OH,m=2). Another interesting application of the crown ether resins is chromatographic separation of neutral organic compounds. FIG. 8 reveals a chromatogram of furan, thiophene, and pyrrole on a column of dibenzo-30-crown-10 resin 2 (m=n=2). It is most likely that sulfur- and nitrogen-containing solutes are bound more

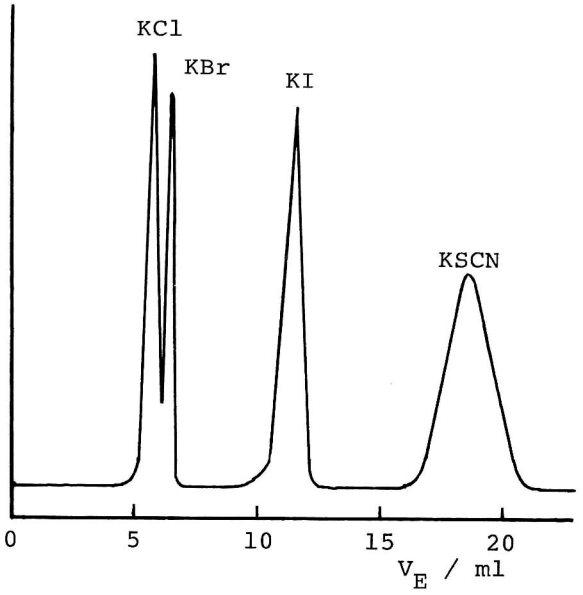


FIG. 7 Chromatographic separation of anions on a column of crown ether resin 2 (m=1,n=0). KCl: 0.42 mg; KBr: 0.60 mg; KI: 1.60 mg; KSCN: 1.77 mg, eluent: H_2O. (Ref. 20)

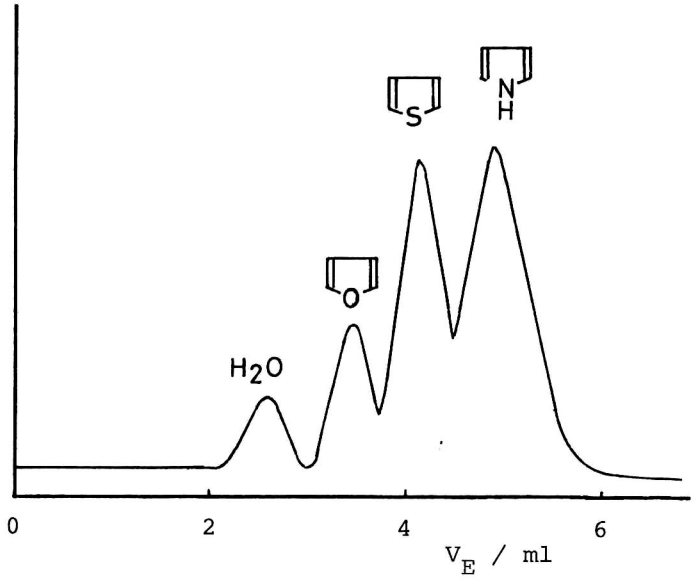
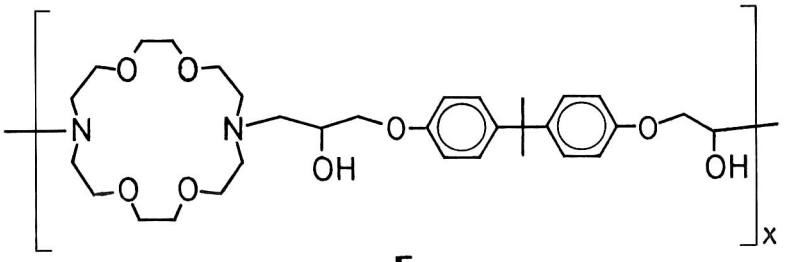


FIG. 8 Chromatographic separation of neutral organic compounds on a column of crown ether resin 2 (m=n=2). furan: 0.2 mg; thiophene: 0.2 mg; pyrrole: 0.2 mg, eluent: MeOH. (Ref. 21)

strongly than oxygen-containing ones on the crown ether resin, although it has not been explained what the binding force is between the organic compounds and the crown ether resins. Chromatographic separation of urea - thiourea mixture is also possible on the crown ether resins in a similar way. In still another application, hydration water of salts can be determined by liquid chromatography using the crown ether resins. When $CaCl_2 \cdot xH_2O$ was eluted with methanol on a column of dibenzo-21-crown-7 resin 2 (m=1,n=0), $CaCl_2$ and the hydration water were separated completely. The linear calibration plot afforded the



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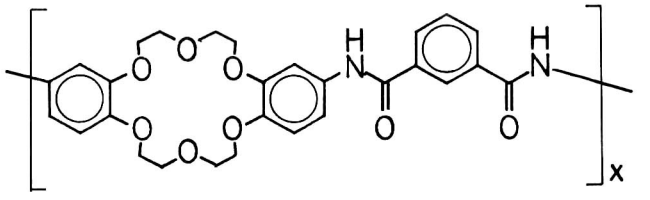

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FIG. 9 Epoxy-type and polyamide-type crown ether resins. (Ref. 27 and 30)

determination of water in the salt. A cryptand resin of this type is commercially available now, and separation of calcium isotopes ($^{48}Ca/^{40}Ca$) could be realized using a column packed with the resin (26).

Gramain and Frere synthesized condensation-type poly-(diazacrown ether)s 5 and polycryptands, which were immobilized on chloromethylated polystyrene resin, and then applied the resins for chromatographic separation of alkali and alkaline earth metal ions (27). Other examples of crown ether resins containing various supports (except silica gel) for the stationary phase of liquid chromatography are diazacrown ether - immobilized acrylamide gel (28), dibenzocrown ether - immobilized cellulose (29), polyamide-type benzocrown ether 6 (30), and poly(glycidyl methacrylate)-type crown ether resins coated on Chromosorb (31).

III-2. Crown Ethers Immobilized on Silica Gel

The above-mentioned crown ether resins, which consist of condensation-type poly(crown ether)s, crown ether - modified polystyrene, and crosslinked poly(vinylcrown ether)s, may not be suited for HPLC where high pressure is required. Inorganic materials containing hard cores like silica gel seem to be preferable to the packing support of liquid chromatography.

The synthesis and chromatographic behavior of poly-(crown ether) - modified silicas have been reported by the

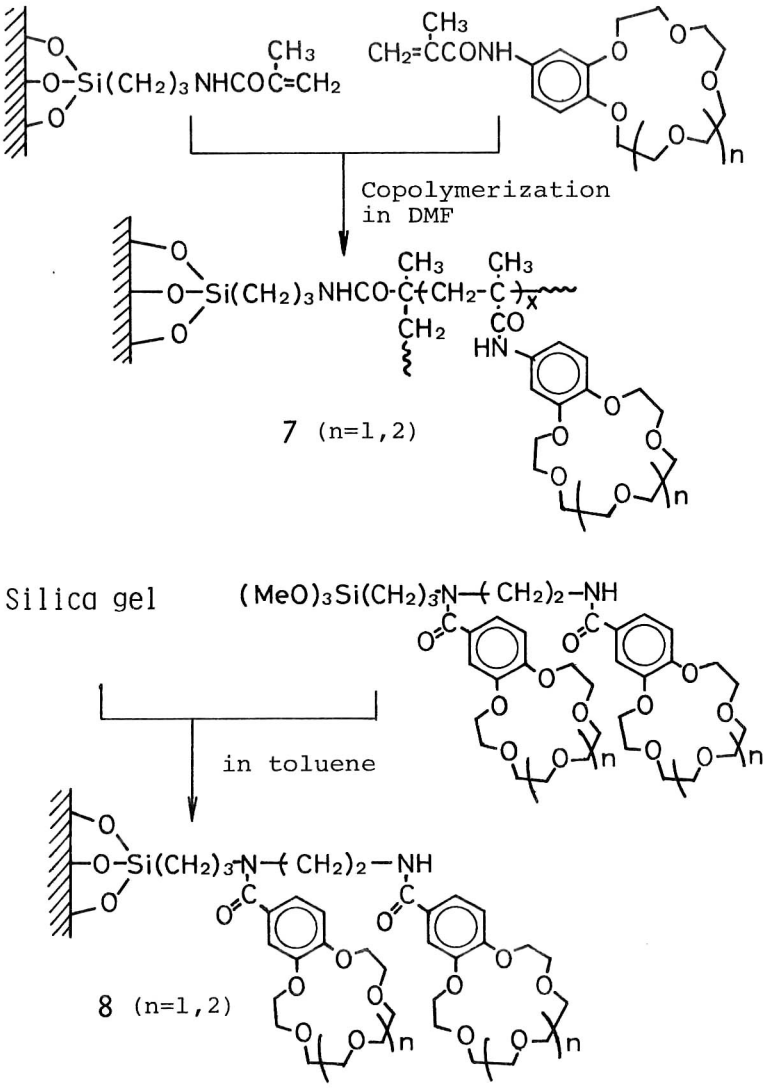

FIG. 10 Structure and preparation of silicas modified by poly- and bis(benzocrown ether)s.

authors (32). The structure and preparation of the crown ether silicas are shown in FIG. 10. Poly(crown ether) - modified silicas 7 were prepared by incorporating vinyl groups on silica gel (10μ, for HPLC), followed by radical copolymerization of the vinyl-modified silica with an appropriate crown ether monomer. The HPLC of ions was coducted using columns which were packed with the crown ether silicas by a conventional slurry packing method. The poly(crown ether) silicas bind alkali metal salts, even the halides, so strongly that methanol cannot elute out K^+ and Rb^+ salts from the columns. Therefore, water, containing a small quantity of methanol, is generally a good eluent for the chromatography of alkali metal salts using these crown ether silicas. Typical chromatographic separations of alkali metal halides by poly(benzo-15-crown-5)- and poly(benzo-18-crown-6) - modified silicas are depicted in FIGS. 11 and 12, respectively (32,33). The retention sequence of alkali metal chlorides on 7 (n=1) is $Li^+ < Na^+ << Cs^+ < Rb^+ < K^+$, and that of 7 (n=2) $Li^+ < Na^+ < Rb^+ < K^+ < Cs^+$. Poly(crown ether)s, which carry crown ether moieties in the side chain, are known to possess attractive cation-binding properties compared to their corresponding monomeric analogs (34,35). That is to say, they are capable of forming sandwich-type 2:1 (crown ether ring/metal ion) complexes with particular metal ions effectively by cooperative action of two adjacent crown ether rings in

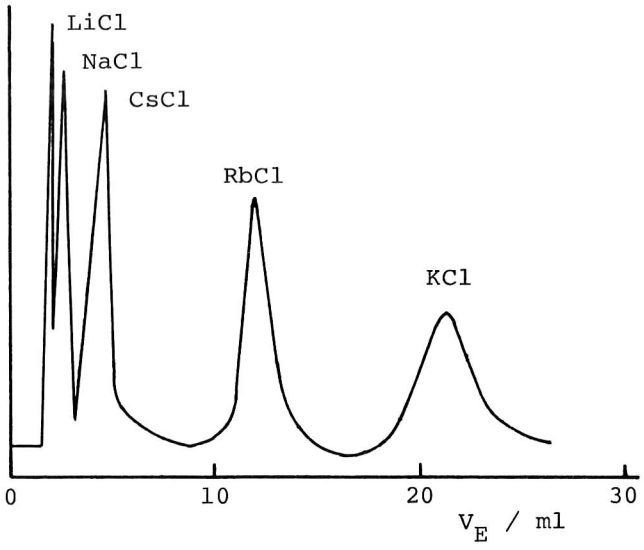

FIG. 11 Separation of alkali metal chlorides on a column of poly(crown ether)-modified silica 7 (n=1) by elution with H_2O-MeOH (90/10). LiCl: 6.4 μg; NaCl: 9.4 μg; KCl: 17.2 μg; RbCl: 17.2 μg; CsCl: 26.9 μg.

the polymer chain, which often accounts for their excellent cation-binding selectivity. For example, poly-(benzo-15-crown-5) and poly(benzo-18-crown-6) exhibit pronounced selectivities for K^+ and Cs^+, respectively. The cation-binding properties of poly(crown ether)s seem to be reflected in the chromatographic behavior of these poly(crown ether) - modified silicas. The poly(crown ether) silicas were also tested for their usefulness in liquid chromatography of alkaline earth metal ions (FIG. 13) (33). Moreover, chromatographic separation of var-

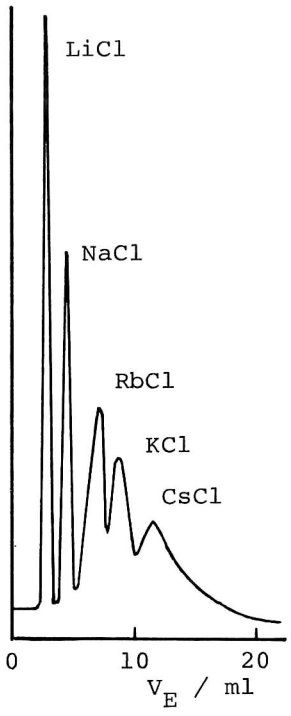

FIG. 12 Separation of alkali metal chlorides on a column of poly(crown ether)-modified silica 7 (n=2) by elution with H_2O-MeOH (90/10). LiCl: 0.80 mg; NaCl: 0.12 mg; KCl: 0.15 mg; RbCl: 0.24 mg; CsCl: 0.34 mg.

ious anions is feasible with the poly(crown ether) silicas, by analogy with Blasius' crown ether resins. Bis(crown ether)s, dimeric model compounds of the poly(crown ether)s, also exhibit similar cation-binding properties to them (35), and the bis(crown ether) - modified silicas are almost the same in chromatographic behavior for the alkali and alkaline metal ions as the corresponding poly(crown

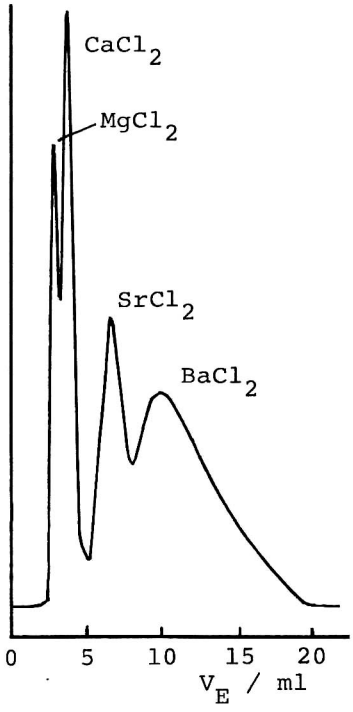

FIG. 13 Separation of alkaline earth metal chlorides on a column of poly(crown ether)-modified silica 7 (n=2) by elution with H_2O-MeOH (90/10). $MgCl_2$: 0.10 mg; $CaCl_2$: 0.15 mg; $SrCl_2$: 0.27 mg; $BaCl_2$: 0.63 mg.

ether) silicas (33). It was found that poly- and bis-(12-crown-4) derivatives have Na^+-selectivity in binding alkali metal ions (36,37), so silicas modified by the 12-crown-4 derivatives may be expected to show different chromatographic behavior from those of the silicas modified by the 15-crown-5 and 18-crown-6 derivatives. Further, poly(thiacrown ether)-modified silicas, in which some of

oxygen atoms are replaced by sulfur atoms in the crown ether ring of poly(crown ether) silica 7 (n=1,2), are promising candidates of the stationary phase for chromatographic separation of heavy metals , such as Ag, Hg, Cd, and other transition metals (38).

A few additional ways have been reported to immobilize crown ethers on silica gel (24,39). Benzo-15-crown-5 [neither poly- nor bis(crown ether) derivative] was also immobilized on silica gel by covalent bonding (24). In liquid chromatography of alkali metal chlorides using the benzo-15-crown-5 - modified silica, elution with pure methanol allowed good separation among Li^+, Na^+ Cs^+, and K^+ (or Rb^+). The cation-binding ability of the benzo-15-crown-5 silica is, however, not so good as that of the foregoing poly(benzo-15-crown-5) silica. In addition, the separation between K^+ and Rb^+ is incomplete on the monomeric crown ether silica unlike the polymeric crown ether silica, although the former seems to resemble the latter in the retention sequence of alkali metal ions. Another approach for immobilization of crown ethers on silica gel was attempted by Igawa et al. (39). In that case, silica gel for HPLC was coated by polyamide-type crown ether resin 6. In liquid chromatography of alkali metal salts using the crown ether-coated silica, the metal ions were hardly separated, whereas good separation between various anions was observed.

In other words, unlike the foregoing crown ether resins, this one permits only separation of anions, although the reason has not been mentioned. A column of this crown ether resin was tested for its usefulness in chromatographic analysis of anions in sea water.

III-3. Optical Resolution by Chiral Crown Ether Resins

Some chiral crown ethers can form complementary complexes with ammonium salts of chiral amines and amino acids (or the esters) (40). The feasibility of resolving their racemic mixtures was demonstrated, using resins incorporationg chiral crown ethers, by Cram and coworkers (41,42). Bis(dinaphtho-22-crown-6) derivatives were immobilized on silica gel or crosslinked polystyrene. Excellent chromatographic separation of phenylglycine perchlorate or methyl phenylalaninate hydrochloride was attained with (R,R)-bis(dinaphtho-22-crown-6) resins, as illustrated in FIG. 14. Some attempts were also made to resolve racemic mixtures of threonine, aspartic acid, histidine, tryptophan, phenylalanine, and valine by liquid chromatography using phenol-formaldehyde resin of dibenzo-18-crown-6 2 (m=n=0), which does not contain any chirality in the chemical structure, as shown in 2 (m=n=0) of FIG. 5 (43). Slight resolution of the racemates was observed in the chromatography, which might suggest that some chirality was produced in the crown ether resin during preparation.

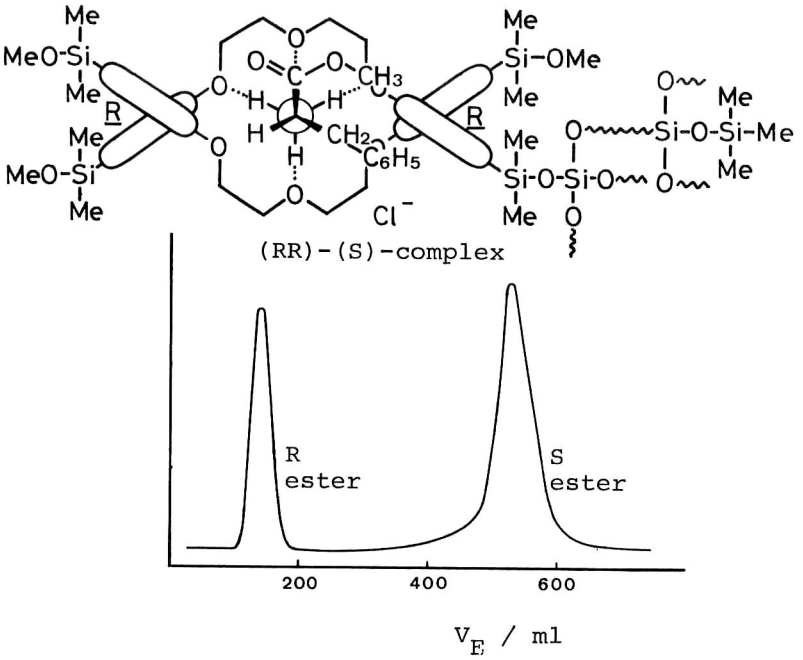

FIG. 14 Chromatographic optical resolution of methyl phenylalaninate hydrochloride salt by (R,R)-bis(dinaphtho-22-crown-6) - bound silica. (Ref. 41)

III-4. Noncyclic Crown Ether Resins

Noncyclic polyethers like poly(oxyethylene) sometimes act as if they were their macrocyclic analogs, and exhibit similar cation-binding abilities to crown ether derivatives. Therefore, they are often referred to as noncyclic crown ethers. The synthesis and chromatographic behavior of resins containing noncyclic crown ethers have been reported by Fujita et al. (44). Poly(oxyethylene)

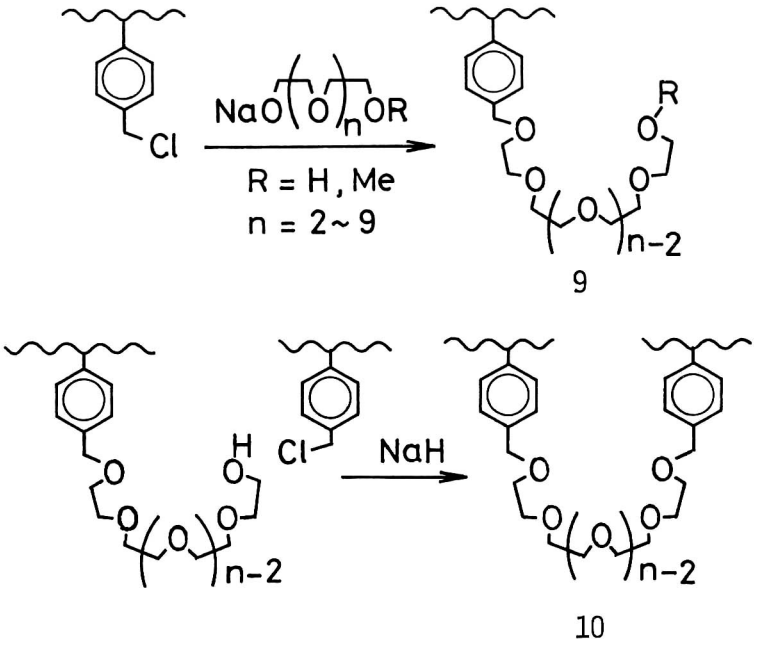

FIG. 15 Noncyclic poly(oxyethylene) bound to crosslinked polystyrene (noncyclic crown ether resins) (Ref. 44 and 45)

with various chain lengths were immobilized on crosslinked polystyrene, as depicted in FIG. 15. The cation-binding ability of the resins is likely to decrease with decreasing chain length of poly(oxyethylene) bound to the support. The noncyclic crown ether resins cannot interact with alkali metal ions so strongly as the "cyclic" crown ether resins. Nevertheless, good separation of thiocyanates of Li^+, Na^+, and K^+ was attained in liquid chromatography using a column of resin 9 (n=10) and water-

acetone (10/90) eluent. Polymeric pseudocrown ethers, synthesized and coined by Warshawsky et al., which are essentially the same as resin 10 in FIG. 15, could also be a substitute for the crown ether resins as the stationary phase of liquid chromatography (45). Simon et al. have immobilized 3,6-dioxaoctanedioic acid diamide derivatives on crosslinked polystyrene or NH_2-modified silica gel for HPLC (46). The resins were applied to chromatographic separation of alkali and alkaline earth metal ions. Only poor separation of the chloride salts was observed in liquid chromatography on the noncyclic crown ether resins with elution of water or water-alcohol mixtures. However, successful separation of the iodide salts was achieved on the resins, using a technique of solvent gradient of water and alcohol.

IV. CONCLUSION

This work is a review on application of crown ethers in liquid chromatography. The works published so far indicate that crown ether is one of the most excellent tools for separation chemistry using liquid chromatography. The chromatography using crown ethers is useful for the separations of not only metal ions but also amino compounds such as amino acid derivatives. Also, it seems feasible to separate some neutral organic compounds on crown ether resins, taking advantage of the fact that many crown ethers also form molecular complexes with them.

In order to attain highly efficient separations and analyses of ions and neutral compounds, extensive investigations concerning the application of crown ethers in liquid chromatography will continue in the future. It is, however, recommended that insoluble crown ether resins are utilized for studying the separation chemistry of crown ether in liquid chromatography, because the recovery of soluble crown ethers will be a significant problem.

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REFERENCES

(1) Synthetic Macrocyclic Compounds, Izatt, R. M. and Christensen, J. J. Ed., Academic Press, New york, 1978.

(2) Progress in Macrocyclic Chemistry, Vol. 1 and 2, Izatt, R. M. and Christensen, J. J. Ed., Wiley New yok, 1979 and 1981.

(3) Topics in Current Chemistry, Vol. 98, Vogtle, F. Eds., Spriger-Verlag, Berlin, 1981.

(4) Kolthoff. I. M., Anal. Chem., 51, 1R, 1979.

(5) King, R. B., Heckley , P. R., J. Am. Chem. Soc., 96, 3118, 1974.

(6) Smulek, W., Lada, W. A., Radiochem. Radioanal. Lett., 30, 199, 1977 and 34, 41, 1978.

(7) Smulek, W., Lada, W. A., J. Radioanal. Chem., 50, 169, 1979.

(8) Fernado, L. A., Miles, M. L., Bowen, L. H., Anal. Chem., 52, 1115, 1980.

(9) Delphin, W. H., Horwitz, E. P., Anal. Chem., 50, 843, 1978.

(10) Nakagawa, T., Mizunuma, H., Shibukawa, A., Uno, T., J. Chromatogr., 211, 1, 1981.

(11) Wiechmann, M., J. Chromatogr., 235, 129, 1982.

(12) Brugman, W. J. T., Kraak, J. C., J. Chromatogr., 205, 170, 1981.

(13) Mangia, A., Parolari, G., Anal. Chim. Acta, 92, 111, 1977.

(14) Horvath, C., Melander, W., Nahum, A., J. Chromatogr., 186, 371, 1979.

(15) Aoki, S., Shiga, M., Tazaki, M., Nakamura, H., Takagi, M., Ueno, K., Chem. Lett., 1981, 1583.

(16) Blasius, E., Adrian, W., Janzen, K.-P., Klautke, G., J. Chromatogr., 96, 89, 1974.

(17) Blasius, E., Janzen, K.-P., Chemi.-Ing.-Tech., 47, 594, 1975.

(18) Blasius, E., Maurer, P. G., J. Chromatogr., 125, 511, 1976.

(19) Blasius, E., Maurer, P. G., Makromol. Chem., 178, 649, 1977.

(20) Blasius, E., Janzen, K.-P., Adrian, W., Klautke, G., Lorscheider, R., Maurer, P. G., Nguyen, V. B., Nguyen-Tien, G., Scholten G., Stockemer, J., Fresenius' Z. Anal. Chem., 284, 337, 1977.

(21) Blasius, E., Janzen, K.-P., Luxemburger, H., Nguyen, V. B., Klotz, H., Stockemer, J., J. Chromatogr., 167, 307, 1978.

(22) Blasius, E., Janzen, K.-P., Keller, M., Lander, H., Nguyen-Tien, T., Scholten, G., Talanta 27, 107, 1980.

(23) Blasius, E., Janzen, K.-P., Adrian, W., Klein, W., Klotz, H., Luxemburger, H., Mernke, E., Nguyen, V. B., Nguyen-Tien, T., Rausch, R., Stockemer, J., Toussaint, A., Talanta, 27, 127, 1980.

(24) Blasius, E., Janzen, K.-P., Klein, W., Klotz, H., Nguyen, V. B., Nguyen-Tien, T., Pfeiffer, R., Scholten G., Simon, H., Stockemer, H., Toussaint, A., J. Chromatogr., 201, 147, 1980.

(25) Blasius, E., Lander, H., Fresenius' Z. Anal. Chem., 303 , 177 and 272, 1980.

(26) Heumann, K. G., Schiefer, H.-P., Angew. Chem., Int. Ed. Engl., 19, 406, 1980.

(27) Gramain, P., Frere, Y., Ind. Eng. Chem., Prod. Res. Dev., 20, 524, 1980.

(28) Kutchukov, P., Ricard, A., Quivoron, C., Eur. Polym. J., 16, 753, 1980.

(29) Djamali, M. G., Burba, P., Lieser, K. H., Angew. Makromol. Chem., 92, 145, 1980.

(30) Igawa, M., Ito, I., Tanaka, M., Bunseki Kagaku, 29, 580, 1980.

(31) Yagi, K., Sanchez, M. C., Makromol. Chem., Rapid Commun., 2, 311, 1981.

(32) Kimura, K., Nakajima, M., Shono, T., Anal. Lett., A9, 741, 1980.

(33) Nakajima, M., Kimura, K., Shono, T., to be published.

(34) Kopolow, S., Hogen Esch, T. E., Smid, J., Macromolecules, 6, 133, 1973.

(35) Kimura, K., Maeda, T., Shono, T., Talanta, 26, 945, 1979.

(36) Maeda, T., Ouchi, M., Kimura, K., Shono, T., Chem. Lett., 1981, 1573.

(37) Shono, T., Okahara, M., Ikeda, I., Kimura, K., Tamura, H., J. Electroanal. Chem., 132, 99, 1982.

(38) Oue, M., Ishigaki, A., Matsui, Y., Maeda, T., Kimura, K., Shono, T., Chem. Lett., 1982, 275.

(39) Igawa, M., Saito, K., Tsukamoto, J., Tanaka, M., Anal. Chem., 53, 1942, 1981.

(40) Cram, D. J., Helgeson, R. C., Sousa, L. R., Timko, J. M., Newcomb, M., Moreau, P., De Jong, F.,

Gokel, G. W., Hoffman, D. H., Domeier, L. A., Peacock, S. C., Madan, K., Kaplan, L., Pure Appl. Chem., 41, 327, 1975.

(41) Sousa, L. R., Sogah, G. D. Y., Hoffman, D. H., Cram, D. J., J. Am. Chem. Soc., 100, 4569, 1978

(42) Sogah, G. D. Y., Cram, D. J., J. Am. Chem. Soc., 101, 3055, 1979.

(43) Blasius, E., Janzen, K.-P., Klautke, G., Fresenius' Z. Anal Chem., 277, 374, 1975.

(44) Fujita, H., Yanagida, S., Okahara, M., Anal. Chem., 52, 869, 1980.

(45) Warshawsky, A., Kalir, R., Deshe, A., Berkovitz, H., Patchornik, A., J. Am. Chem. Soc., 101, 4249, 1979.

(46) Grossmann, P., Simon, W., J. Chromatogr., 235, 351, 1982.

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RECENT DEVELOPMENTS IN LC/MS INTERCONNECTION

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ABSTRACT

Recent innovations in LC/MS interconnection and desirable operating characteristics of an LC/MS interface are discussed. Relative strengths and weaknesses are examined for the three commercially available LC/MS interfaces and for a number of experimental designs. Possible areas for future research are suggested.

INTRODUCTION

The analysis of organic compounds by mass spectrometry provides the capability of determining elemental composition, molecular weight and molecular structure. Several techniques have recently extended analytical mass spectrometry for the analysis of compounds once considered intractable for reasons of volatility or stability (1). These include desorption chemical ionization (2), secondary ionization (3-4), fast atom bombardment (5-6), field desorption (7), laser desorption (8), electrohydrodynamic ionization (9), and Cf-252 plasma desorption (10).

Combination of liquid chromatography with some of the newer ionization techniques makes it possible to separate and identify almost any organic compound. All except field desorption can be

directly coupled to an LC/MS; and by collecting fractions, it is possible to apply LC/MS to field desorption. Use of pyrolysis (11) with liquid chromatography permits mass spectral analysis of compounds with even less volatility. Coupling MS/MS (12-13) with liquid chromatography further aids in structure elucidation and provides additional separation for complex mixtures.

There are numerous ways to interconnect liquid chromatographs and mass spectrometers. Recent reviews of liquid chromatography/mass spectrometry (14-15) have discussed LC/MS coupling from a historical standpoint whereas this paper stresses some of the more recent developments in LC/MS interconnection. Direct liquid introduction, continuous sample preconcentration, and mechanical transfer are discussed first because they form the basis for commercially available LC/MS interfaces. More esoteric methods of LC/MS interconnection are also examined.

TABLE 1

Operating Characteristics Desired from an LC/MS Interface

LC Operation

- No restriction on solvents
- Gradient elution
- Buffers: volatile and nonvolatile
- Ion-pair reagents
- Flow up to 2.0 ml/min.

MS Operation

- Maintain vacuum
- Ionization method: both EI and CI, FD desirable
- Both positive and negative ion mode
- Select reagent gas for CI
- Unimpaired sensitivity, hence noise and background must be minimized with little interference from solvent impurities

Interface Operation

- High sample enrichment
- High transfer of sample to MS
- No peak broadening
- Vaporize low volatility solvents

DESIRABLE FEATURES OF LC/MS INTERFACES

Desirable operating characteristics of an LC/MS interface are summarized in Table 1 (15). In fact, none of the current LC/MS interfaces meet all of these requirements. LC/MS interconnection problems include poor material transfer and degradation of labile compounds.

COMMERCIALLY AVAILABLE LC/MS INTERFACES

Directly Coupled LC/MS

Directly coupled LC/MS can be accomplished by modification of a solids probe inlet to fit any chemical ionization source. Micro LC columns with a solvent flow rate of up to 10 µl/min. have been directly coupled to CI mass spectrometers (14,16).

In early systems, the LC effluent was passed to the ion source using a capillary tube. The capillary tube interface is easy and economical to fabricate but low volatility solutes tend to clog the tube. This difficulty is avoided by replacing the capillary tube with a pinhole orifice positioned directly adjacent to the ion source (17).

This method requires interruption of routine GC/MS only for the period of the LC/MS experiment, since optimum conditions for separating components of a mixture can be established off line.

Typical sensitivities are 50-500 ng injected onto the LC column for a complete spectrum and 2-20 ng under selected ion monitoring conditions (18). Although limited to CI ionization, negative CI can be used to obtain complementary information on many samples. Peak broadening is minimal (19). Good stability, precision, and sensitivity have been demonstrated for thermally labile and nonvolatile compounds (20).

Almost any solvent can be used if it acts as a good reagent for CI and gives few interfering peaks in the mass spectrum. Since

polar solvents readily yield reagent ions, the method matches well the requirements of reversed-phase LC. The choice of mobile phase for LC separation must exclude only highly chlorinated and caustic materials (21). Acetate and formate buffers have been successfully used with the technique (18). Since the solvent acts as the CI reagent, sensitivity for each component may change as a function of the solvent composition when gradients are used.

Flow diverting in the absence of peaks of interest keeps the source cleaner when running biological samples (22). Efficient volatilization of the solvent and reduction of ion source background is accomplished by maintaining the ion source temperature at 250°C (23).

Applications of directly coupled LC/MS include acids (13), alkaloids (24-25), amides (18,26-28), aromatic hydrocarbons (18,21,25,29), drugs (17-23,25-27,30-34), esters (21,35-36), flavors (14,29), nitro compounds (37), nucleosides and nucleotides (18,27), peptides (32,38), pesticides (14,18,23,25,27,33,39), quinones (21,40), steroids (17-18,27,41-42), sugars (24), and vitamins (21).

Continuous Sample Preconcentration

Continuous sample preconcentration is accomplished by allowing the LC effluent to flow down an electrically heated tapered wire in a stream of warm nitrogen (43). The current to the wire is controlled by a feedback loop from a volume-sensing photocell. The concentrated efflent flows through a very small needle valve which regulates the flow into the mass spectrometer. The valve is constructed such that liquid is sprayed into the ion source of the mass spectrometer.

In normal phase operation, virtually the total sample reaches the mass spectrometer without excess solvent. Approximately 95%

of the solvent is evaporated providing a signal enhancement of about 20-fold and sensitivity in the low nanogram range (43,44). With reversed phase solvents such as water, a modest split is required (44). Typical acceptable flows to the concentrator for some solvents are: n-pentane 3 ml/min; 2,2,4-trimethyl pentane 2.8 ml/min; methanol 1.7 ml/min; 50% methanol/water 0.7 ml/min. The maximum flows obtained correlate inversely with the heats of vaporization, but volatility, viscosity, and surface tension also are important (43).

Almost any LC solvent can be used. The LC solvent may serve as the CI reagent gas. Polar, mixed solvent, and linear gradient capability are feasible. Use of a tandem source CI/EI mass spectrometer allows both CI and EI spectra of the same LC fraction with extremely high sensitivity (44). This feature is of great value for structural elucidation of organic compounds by mass spectrometry.

Relatively sharp sample peaks indicate transfer of the solute to the mass spectrometer without substantial peak broadening. The interface retains sample separation even for narrow LC peaks with little or no tailing. The quantitative reproducibility is excellent, indicating that the interface is free of memory effects.

This interface can be readily incorporated into any mass spectrometer. Since there are no moving parts, it is simple in function and easy to operate. The interface shows less tendency to clog than direct liquid introduction systems since a mechanism is provided to unplug the orifice without breaking vacuum. Fewer problems are encountered with contamination than with belt interfaces. Reduced pyrolysis and degradation of sensitive samples are observed. Neither fast pumping speeds nor cryogenic pumps are needed for the vacuum system.

Applications of continuous sample preconcentration LC/MS include amines (43,45), aromatics (43,45), esters (44), ketones (44), and phenols (43).

Mechanical Transport

Solvent separators in which effluent from the LC is introduced into the mass spectrometer by a continuously moving transport system are currently popular. Scott's initial work with a thin wire limited uptake of LC effluent to around 10 µl/min. McFadden (47) extended the flow range by using a moving belt of Kapton polyamide or stainless steel in place of the wire. Stainless steel belts have higher solvent capacity and are not damaged by vaporizer temperatures above 300°C, but Kapton belts are more commonly used because they permit vaporization of labile samples at lower temperatures and have smoother mechanical operation (48).

A moving belt interface permits operation in EI and CI modes without restricting conventional LC or MS except that the background spectrum is of higher intensity (49). Sample to solvent enrichment is in the 10^5 range and yields are 30-40% (50). The detection limit is often below 1 ng with a dynamic range of 10^4 (49). The effluent capacities noted for some common solvent types with moving belts are nonpolar solvents 1.5 - 2 cc/minute; intermediate solvents 0.8 - 1.2 cc/minute; polar solvents 0.5 - 0.8 cc/minute; water and water solutions 0.1 - 0.3 cc/minute (50). A splitter allows higher solvent flows. Microbore LC offers another effective solution to problems requiring polar solvents (51).

There is considerable evidence that it is possible to produce mass spectra from thermally unstable and relatively involatile samples if they are vaporized very quickly and close to the electron beam in the ion source. This "in-beam" vaporization is easily accomplished with the moving belt. Aerosol deposition (52)

increases the volatility range of samples that can be handled by the moving belt method.

Reversed phase liquid chromatography, even with nonvolatile buffers, is possible using segmented flow continuous liquid-liquid extraction to transfer solutes from polar or strongly ionic solvents into an immiscible volatile organic phase. The mixture is then admitted to the mass spectrometer via a moving belt (53).

The moving belt can be used exclusively as an automatic sampling probe. Alternatively it can be run separately and later introduced to the MS at a fast rate to give sharp, sensitive peaks or a slow rate for analysis of selected portions by MS/MS. Ribbon storage is also used with conventional flash heating/electron impact ionization to remove increasingly less volatile components in successive ribbon passes through the ion source (11). Use of a moving ribbon interface with SIMS, FAB (12,54-55) or laser desorption (56) provides subnanogram sensitivity for quite nonvolatile organic compounds.

Applications of mechanical transfer LC/MS include alfatoxins (50), alkaloids (57-59), amides (49,60), aromatics (50,61-62), biochemicals (63), drugs (46,49-50,57,59-60,64-71), esters (50,72), flavors (57,73), halogenated compounds (61,74-75), liquid crystals (76-77), natural products (46,57), nitro compounds (60), nucleosides and nucleotides (59,68,78), organometallics (68), peptides (46,68), pesticides (50,57,66,79-84), phenols (60), polymers (49), steroids (46,49-50,60,66), sugars (59,68), sulfur compounds (60), and vitamins (46,61,66).

Applications of mechanical transfer LC/MS combined with continuous liquid/liquid extraction include acids (53,85), alcohols (53,85), amines (53,85), drugs (86), ketones (53,85), pesticides (53,85,87), and sulfur compounds (86).

Applications of mechanical transfer LC/MS combined with secondary ion mass spectrometry include amino acids (12,88-91), and drugs (88).

EXPERIMENTAL LC/MS INTERFACES

Explosive Evaporation

Among the most promising noncommercial LC/MS interfaces today are the explosive evaporation methods which rapidly vaporize the liquid jet as it emerges from the LC with a laser (92-93), oxyhydrogen flames (94), or ultrasonically (95). Molecular beam techniques efficiently transport and ionize the sample with minimal contact with solid surfaces.

Flow rates of up to 1 ml/min for a number of common solvents such as methanol, acetonitrile, hexane, and chloroform, and approximately 0.5 ml/min of water may be used (92). At least 50% of the sample can be transferred to the ion source of the mass spectrometer along with, at most, 5% of the solvent vapor (94). Low microgram quantities of sample injected into the LC can be detected (92). The system is applicable to all of the separation techniques presently employed in modern LC including reversed phase. Ion exchange using a phosphate buffer builds up deposits inside the vacuum but acetate buffer presents no difficulties (92).

An interesting sidelight was the discovery that ions are produced spontaneously in the interface. No ionizing source such as an electron beam or strong electric field is required (96). Underivatized pentapeptide and dinucleotide spectra are very similar to those obtained by field desorption (97).

Applications of the explosive evaporation LC/MS interface include acids (98), amines (98-100), amino acids (94,101-103), aromatics (92,95,98-100,104), drugs (103), esters (98-100), halogenated compounds (99-100,105), nucleic acid bases (92,94,106-109), nucleosides and nucleotides (92,94,97,99,101-104,106-109), peptides

(94,97,101-103), pesticides (92,98,100), phenols (98-100), and phosphorus compounds (92).

Atmospheric Pressure Ionization

The use of atmospheric pressure ionization (API) provides a novel solution to the design of the LC/MS systems. The entire effluent stream may be vaporized and directed through the source. Since a pressure change is not required, the mechanical and physical problems of transferring the solute from the LC column to the source are not great. The ionization is solvent mediated, but there is much choice in the way in which ionization can be effected (110).

The API source restricts the method to solvents of low proton or electron affinity. Severe interferences can occur from solvent impurities. Such a restriction has limited the general applicability of the system, and it has not yet been widely applied in LC/MS (14).

Applications of atmospheric pressure ionization LC/MS interface include amines (43,45), aromatics (43,45,110), drugs (111), and phenols (43).

Jet Separator

On-line coupling of a micro liquid chromatograph and mass spectrometer through a jet separator of the type commonly used for GC/MS has been reported (112). Significant sample enrichment is not achieved, ionization is restricted to CI mode and a large diffusion pump is necessary to obtain a free expanding jet (14).

Applications of jet separator LC/MS include amines (113-115), amino acids (115-116), aromatics (112), drugs (113-114,116), esters (112,116), pesticides (115), polymers (116), and steroids (115).

Membrane

A silicon rubber membrane molecular separator has been used for the coupling of a mass spectrometer to a liquid chromatograph. The use

of the membrane device limits the solvents to readily volatile, polar compounds (117). High enrichment of sample to solvent is not achieved, and significant peak broadening is experienced in the interface system. The lack of versatility of the interface with respect to solvent selection has discouraged further development (14).

PROBABLE FUTURE TRENDS

A round table discussion (118) emphasized the advantages of LC/MS for samples that cannot be separated by gas chromatography. LC/MS coupling techniques directed toward analysis of nonvolatile or thermally labile compounds will be stressed in the future. A combination of interfaces seems likely, such as continuous sample preconcentration with either direct liquid introduction or the moving belt. The moving belt may be combined with some ionization method involving a reactive plasma. Methods which eliminate the solvent as the sample is ionized, such as electrohydrodynamic ionization, laser desorption, Cf-252 plasma desorption, fast atom bombardment, secondary ion mass spectrometry, and thermospray procedures may receive more attention in coming years. LC column technology may simplify interfacing by providing columns that require nothing more polar than water to elute polar compounds. Whatever direction LC/MS interfacing takes, it is likely to be a prolific field of research due to the advantages of mass spectrometry as a detector for liquid chromatography.

REFERENCES

(1) Daves, G.D., Jr.: Acc. Chem. Res., 12, 359, 1979.

(2) Cotter, R.J.: Anal. Chem., 52, 1589A, 1980.

(3) Colton, R.J.: J. Vac. Sci. Technol., 18, 737, 1981.

(4) Day, R.J., Unger, S.E., and Cooks, R.G.: Anal. Chem. 52, 557A, 1980.

(5) Taylor, L.C.E.: Ind. Res./Dev., 23, 124, 1981.

(6) McNeal, C.J.: Anal. Chem., 54, 43A, 1982.

(7) Beckey, H.D.: Principles of Field Ionization and Field Desorption Mass Spectrometry, Pergamon, New York, 1977.

(8) Kistemaker, P.G., Lens, M.M.J., Van Der Peyl, C.J.Q., and Boerboom, A.J.H.: Advances in Mass Spectrometry, Vol. 8A, Quayle, A., ed., Heyden, London, 1980, p. 928.

(9) Stimpson, B.P., Simons, D.S., Evans, C.A., Jr.: J. Phys. Chem., 82, 660, 1978.

(10) NcNeal, C.J., MacFarlane, R.D.: J. Am. Chem. Soc., 103, 1607, 1981.

(11) Smith, R.D., Johnson, A.L.: Anal. Chem., 53, 1120, 1981.

(12) Smith, R.D., Burger, J.E., and Johnson, A.L.: Anal. Chem., 53, 1603, 1981.

(13) Voyksner, R.D., Hass, J.R., and Bursey, M.M.: To Be Published.

(14) Arpino, P.J., and Guiochon, G.: Anal. Chem., 51, 682A, 1979.

(15) McFadden, W.H.: J. Chromatogr. Sci., 18, 97, 1980.

(16) Takeuchi, T., Ishii, D., Saito, A., and Ohki, T.: J. High Res. Chromatogr./Column Chromatogr., 5, 91, 1982.

(17) Henion, J.D., and Wachs, T.: Anal. Chem., 53, 1963, 1981.

(18) Melera, A.: Advances in Mass Spectrometry, Vol. 8B, Quayle, A., ed., Heyden, London, 1980, p. 1597.

(19) Henion, J.D.: J. Chromatogr. Sci., 18, 101, 1980.

(20) Henion, J.D.: Advances in Mass Spectrometry, Vol. 8B, Quayle, A., ed., Heyden, London, 1980, p. 1241.

(21) Henion, J.D.: 24th Annual Conf. on Mass Spectrom. and Allied Topics, San Diego, 1976, p. 414.

(22) Kenyon, C.N., Melera, A., and Erni, F.: J. Chromatogr. Sci., 18, 103, 1980.

(23) Henion, J.D., Anal. Chem., 50, 1687, 1980.

(24) Kenyon, C.N.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 608.

(25) Ind. Res./Dev., 22, C1, 1980.

(26) Beggs, D.P., and Melera, A.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 724.

(27) Melera, A.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 363.

(28) Serum, J.W., and Melera, A.: 26th Annual Conf. on Mass Spectrom. and Allied Topics, St. Louis, 1978, p. 655.

(29) Arpino, P.J., and Krien, P.: 26th Annual Conf. on Mass Spectrom. and Allied Topics, St. Louis, 1978, p. 426.

(30) Arpino, P.J., Guiochon, G., Devant, G., and Krien, P.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 366.

(31) Arpino, P.J., and Krien, P.: J. Chromatogr. Sci., 18, 104, 1980.

(32) Henion, J.D., and Maylin, G.A.: 32nd Pittsburgh Conf. on Anal. Chem. and Appl. Spectrosc., Atlantic City, 1981, p. 723.

(33) Henion, J.D.: 25th Annual Conf. on Mass Spectrom. and Allied Topics, Washington, 1977, p. 377.

(34) Henion, J.D.: Advances in Mass Spectrometry, Vol. 7B, Daly, N.R., ed., Heyden, London, 1976, p. 865.

(35) Price, P.C., McDonie, R.A., and Wellons, S.L.: 28th Annual Conference on Mass Spectrom. and Allied Topics, New York, 1980, p. 610.

(36) McLafferty, F.W., Knutti, R., Venkataraghavan, R., Arpino, P.J., and Dawkins, B.G., Anal. Chem., 47, 1503, 1975.

(37) Voyksner, R.D., Tondeur, Y., Parker, C.E., Henion, J.D., and Yinon, J.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 297.

(38) Dawkins, B.G., McLafferty, F.W., and Venkataraghaven, R.: 24th Annual Conf. on Mass Spectrom. and Allied Topics, San Diego, 1976, p. 462.

(39) Arpino, P.J., Colin, H., and Guiochon, G.: 25th Annual Conf. on Mass Spectrom. and Allied Topics, Washington, 1977, p. 189.

(40) Imabayashi, S., Nakamura, T., Sawa, Y., Hasegawa, J., Sakaguchi, K., Fujita, T., Mori, Y., and Kawabe, K.: Anal. Chem., 51, 534, 1979.

(41) Arpino, P., Dawkins, B., and McLafferty, F.W.: 22nd Annual Conf. on Mass Spectrom. and Allied Topics, Philadelphia, 1974, p. 314.

(42) Sugnaux, F.R., Gunatilaka, A.A.L., and Djerassi, C.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 467.

(43) Christensen, R.G., Hertz, H.S., Meiselman, S., and White, V, E.: Anal. Chem., 53, 171, 1981.

(44) Lo, H.H., and Siegel, M.W.: 30th Annual Conf. on Mass Spectrom. and Allied Topics, Honolulu, 1982.

(45) Christensen, R.G., Hertz, H.S., Meiselman, S., and White V, E.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 620.

(46) Scott, R.P.W., Scott, C.G., Munroe, M., and Hess, J., Jr.: J. Chromatogr., 99, 395, 1974.

(47) McFadden, W.H., Schwartz, H.L., and Evans, S.: J. Chromatogr., 122, 389, 1976.

(48) McFadden, W.H.: Finnigan Corp. Technical Report TR8022.

(49) Millington, D.S., Yorke, D.A., and Burns, P.: Advances in Mass Spectrometry, Vol. 8B, Quayle, A., ed., Heyden, London, 1980, p. 1819.

(50) Liquid Chromatograph/Mass Spectrometer Interface for Finnigan GC/MS Systems, Finnigan Corp.

(51) Games, D.E., Lant, M.S., Westwood, S.A., and Woodhall, B.J.:

29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 476.

(52) Smith, R.D., and Johnson, A.L.: Anal. Chem., 53, 739, 1981.

(53) Karger, B.L., Kirby, D.P., Vouros, P., Foltz, R.L., and Hidy, B.: Anal. Chem., 51, 2324, 1979.

(54) Smith, R.D.: 29th Annual Conf. on Mass Spectrometry and Allied Topics, Minneapolis, 1981, p. 165.

(55) Smith, R.D., and Johnson, A.L.: 29th Annual Conf. on Mass Spectrometry and Allied Topics, Minneapolis, 1981, p. 482.

(56) Hunt, D.F., Bone, W.M., and Shabanowitz, J.: 28th Annual Conf. on Mass Spectrometry and Allied Topics, New York, 1980, p. 620.

(57) Eckers, C., Games, D.E., Lewis, E., Rao, K.R.N., Rossiter, N., and Weerasinghe, N.C.A.: Advances in Mass Spectrometry, Vol. 8B, Quayle, A., ed., Heyden, London, 1980, p. 1396.

(58) Games, D.E., Eckers, C., Swann, B.P., and Mallen, D.N.B.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 484.

(59) Games, D.E., Games, M.L., Eckers, C., Kuhnz, W., and Lewis, E.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 318.

(60) Thruston, A.D., Jr., and McGuire, J.M.: 28th Annual Conf. on Mass Spectrometry and Allied Topics, New York, 1980, p. 614.

(61) Dymerski, P.P.: 28th Annual Conf. on Mass Spectrometry and Allied Topics, New York, 1980, p. 624.

(62) Hilker, D., Dymerski, P.P., and Champlin, P.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 625.

(63) McFadden, W.H., Bradford, D.C., Eglinton, G., Hailbrahim, S.K., and Nicolaides, N.: J. Chromatogr. Sci., 17, 518, 1979.

(64) Kelley, P.E.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 276.

(65) Roy, T.A., DeRoos, F.L., Hidy, B.J., and Howard, C.C.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 615.

(66) Scott, R.P.W.: Natl. Bur. Stds. Spec. Publ. 519, Trace Organic Analysis, 1979, p. 637.

(67) Games, D.E., Lewis, E., Haskins, N.J., and Waddell, K.A.: Advances in Mass Spectrometry, Vol. 8B, Quayle, A., ed., Heyden, London, 1980, p. 1233.

(68) Games, D.E., Games, M.L., Lewis, E., Rossiter, M., and

Weerasinghe, N.C.: 26th Annual Conf. on Mass Spectrom. and Allied Topics, St. Louis, 1978, p. 656.

(69) Games, D.E., Gower, J.L., Lee, M.G., Lewis, I.A.S., Pugh, M.E., and Rossiter, M.: Methodol. Surv. Biochem., 7, 185, 1978.

(70) Kelley, P.E., Skinner, R.F., and Phillips, C.R.: Finnigan Corp. Application Report No. 9, 1979.

(71) Tway, P.C., and Caldwell, W.B.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 278.

(72) Privett, O.S., and Erdahl, W.L.: Chem. Phys. Lipids, 21, 361, 1978.

(73) Games, D.E., Gower, J.L., and Rossiter, M.: 25th Annual Conf. on Mass Spectrom. and Allied Topics, Washington, 1977, p. 360.

(74) Stalling, D.L., Petty, J.D., Dubay, G.R., and Smith, R.A.: J. Chromatogr. Sci., 18, 107, 1980.

(75) Dymerski, P., Kennedy, M., and Kaminsky, L.: Nat. Bur. Stds. Spec. Publ. 519, Trace Organic Analysis, 1979, p. 685.

(76) Martin, T.I., and Haas, W.E.: Anal. Chem., 53, 593A, 1981.

(77) Martin, T.I.: J. Chromatogr. Sci., 18, 104, 1980.

(78) Brunnee, N.C., Delgmann, L., Dielmann, G., Meyer, W., and Thorenz, P.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 280.

(79) Wright, L.H., and Oswald, E.O.: 26th Annual Conf. on Mass Spectrom. and Allied Topics, St. Louis, 1978, p. 47.

(80) Games, D.E., Weerasinghe, N.C.A., and Westwood, S.A.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 316.

(81) Games, D.E., and Weerasinghe, N.C.A.: J. Chromatogr. Sci., 18, 106, 1980.

(82) Skinner, R.F., Thomas, Q., Giles, J., and Crosby, D.G.: J. Chromatogr. Sci., 18, 108, 1980.

(83) Skinner, R.F., Thomas, Q., Giles, J., and Crosby, D.G.: Finnigan Corp. Application Report No. 8, 1979.

(84) Wright, L.H.: J. Chromatogr. Sci., 20, 1, 1982.

(85) Karger, B.L., Kirby, D.P., and Vouros, P.: J. Chromatogr. Sci., 18, 111, 1980.

(86) Kirby, D.P., Karger, B.L., Vouros, P., Petersen, B., and Hidy, B.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 314.

(87) Karger, B.L., Kirby, D.P., Vouros, P., Hidy, B., and

Foltz, R.L.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 616.

(88) Benninghoven, A., Eicke, A., Junack, M., Sichtermann, W., Krizek, J., and Peters, H.: 32nd Pittsburgh Conf. on Anal. Chem. and Applied Spectrosc., Atlantic City, 1981, p. 859.

(89) Benninghoven, A., and Peters, H.: 32nd Pittsburgh Conf. on Anal. Chem. and Applied Spectrosc., Atlantic City, 1981, p. 861.

(90) Smith, R.D.: 32nd Pittsburgh Conf. on Anal. Chem. and Applied Spectrosc., Atlantic City, 1981, p. 613.

(91) Smith, R.D., and Burger, J.E.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 310.

(92) Blakley, C.R., McAdams, M.J., and Vestal, M.L.: J. Chromatogr., 158, 261, 1978.

(93) Lory, E.R., and McLafferty, F.W.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 644.

(94) Blakley, C.R., Carmody, J.J., and Vestal, M.L.: Anal. Chem., 52, 1636, 1980.

(95) Udseth, H.R., Orth, R.G., and Futrell, J.H.: 26th Annual Conf. on Mass Spectrom. and Allied Topics, St. Louis, 1978, p. 659.

(96) Carmody, J.J., Blakley, C.R., and Vestal, M.L.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 156.

(97) Blakley, C.R., Carmody, J.J., and Vestal, M.L.: J. Am. Chem. Soc., 102, 5931, 1980.

(98) McAdams, M.J., Blakley, C.R., and Vestal, M.L.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 548.

(99) Blakley, C.R., McAdams, M.J., and Vestal, M.L.: Advances in Mass Spectrometry, Vol. 8B, Quayle, A., ed., Heyden, London, 1980, p. 1616.

(100) McAdams, M.J., and Vestal, M.L.: J. Chromatogr. Sci., 18, 110, 1980.

(101) Blakley, C.R., and Vestal, M.L.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 275.

(102) Blakley, C.R., Carmody, J.J., and Vestal, M.L.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 312.

(103) Blakley, C.R., Carmody, J.J., and Vestal, M.L.: 28th Annual Conf. on Mass Spectrom. and Allied Topics, New York, 1980, p. 320.

(104) Vestal, M.L.: Natl. Bur. Stds. Spec. Publ. 519, Trace Organic Analysis, 1979, p. 647.

(105) Blakley, C.R., and Vestal, M.L.: 25th Annual Conf. on Mass Spectrom. and Allied Topics, Washington, 1977, p. 718.

(106) Edmonds, C.G., Jenkins, E.E., McCloskey, J.A., Blakley, C.R., Vestal, M.L., De, N.C., Farber, A., Dutta, S.P., and Chheda, G.B.: 29th Annual Conf. on Mass Spectrom. and Allied Topics, Minneapolis, 1981, p. 469.

(107) McAdams, M.J., Blakley, C.R., and Vestal, M.L.: 26th Annual Conf. on Mass Spectrom. and Allied Topics, St. Louis, 1978, p. 418.

(108) Blakley, C.R., McAdams, M.J., and Vestal, M.L.: 27th Annual Conf. on Mass Spectrom. and Allied Topics, Seattle, 1979, p. 622.

(109) McAdams, M.J., Blakley, C.R., and Vestal, M.L.: 25th Annual Conf. on Mass Spectrom. and Allied Topics, Washington, 1977, p. 376.

(110) Carroll, D.I., Dzidic, I., Stillwell, R.N., Haegele, K.D., and Horning, E.C.: Anal. Chem., 47, 2369, 1975.

(111) Thomson, B.A., Henion, J.D., and Dawson, P.H.: 33rd Pittsburgh Conf. on Anal. Chem. and Applied Spectrosc., Atlantic City, 1982, p. 198.

(112) Takeuchi, T., Hirata, Y., and Okumura, Y., Anal. Chem., 50, 659, 1978.

(113) Tsuge, S., Hirata, Y., and Takeuchi, T.: Anal. Chem., 51, 166, 1979.

(114) Tsuge, S., Yoshida, Y., and Takeuchi, T.: Chem., Biomed. Environ. Instrum., 10, 405, 1980.

(115) Yoshida, Y., Yoshida, H., Tsuge, S., Takeuchi, T., and Mochizuki, K.: J. High Res. Chromatogr./Col. Chromatogr., 3, 16, 1980.

(116) Hirata, Y., Takeuchi, T., Tsuge, S., and Yoshida, Y.: Org. Mass Spectrom., 14, 126, 1979.

(117) Jones, P.R., and Yang, S.K.: Anal. Chem., 47, 1000, 1975.

(118) Talrose, V.L.: Advances in Mass Spectrometry, Vol. 7B, Daly, N.R., ed., Heyden, London, 1976, p. 949.

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DETERMINATION OF ANTICONVULSANTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In this paper the determination of anticonvulsants by high performance liquid chromatography is reviewed. The paper is not an all-inclusive review but an author's selection of those publications dealing with the determination of common-used anticonvulsants in serum. Attention is given to detection, selection of the chromatographic system, sample pretreatment and to the quantitative aspects.

Introduction

The primary use of anticonvulsant drugs is in the prevention and the control of epileptic seizures [1]. In most cases the treatment is symptomatic and frequently whole life. Since a feeling of inferiority and selfconsciousness often causes a withdrawl from society, this disease constitutes a major public health problem. Clinical studies have shown a strong correlation between serum anticonvulsant concentration and seizure control [2]. Improving patient compliance as well as adjusting the prescribed dosage based on serum anticonvulsant levels, enhancement of seizure control has been acquired.

With respect to pharmacological properties of anticonvulsants it can be mentioned that besides quantitative and fast resorption after oral dose and small sedative or hypnotic activity, attention must be paid in particular to acceptable tolerance, prolonged therapeutic action and lack of drug addiction[3]. Since rarely a single preparation meets all these requirements, several active compounds are prescribed to approach the ideal case.
Physical conditions favourable for seizure prevention are dehydration, systematic acidosis and freedom from stress[1]. For this reason adjuncts to anticonvulsants therapy include drugs such as tranquilizers and diuretic agents with concommittant acidifying activity.
When determining the concentration of anticonvulsants in biological samples, one must be aware that co-administered drugs and metabolites of whatever origin might interfere seriously.
A variety of analytical methods[2] has been applied for the determination of anticonvulsants in serum, such as spectrophotometry, thin layer-, gas - and liquid chromatography, enzyme multiplied immuno technique (EMIT) and radio immuno assay (RIA).
In the present paper high performance liquid chromatography (HPLC), as analytical technique for the determination of anticonvulsants, will be reviewed. When analyzing drugs in serum by HPLC, one can distinguish three optimization steps: detection, separation and sample treatment. All these steps are linked together and have to be tuned to each other to meet the requirements of optimal quantitation. Therefore, the state of art of these three optimization steps will be discussed separately.

DETECTION OF ANTICONVULSANTS

Accurate quantitative analysis of trace amounts of solutes in complex matrices by chromatography requires the registration of the solute of interest resolved from other substances present in the sample at a large signal to noise ratio.

The relation between the resolution R_{ji} of two successively eluting compounds i,j and the chromatographic variables is given by equation 1 .

$$R_{ji} = \frac{t_{Rj} - t_{Ri}}{\sigma_{ti}} = (r_{ji}-1)\left(\frac{k_i'}{k_i' + 1}\right)(L/H)^{\frac{1}{2}} \qquad (1)$$

inwhich: t_{Rj}, t_{Ri} = retention time of j and i ($t_{Rj} > t_{Ri}$)

σ_{ti} = standard deviation of peak i

r_{ji} = selectivity factor (= k_j'/k_i')

k_i' = capacity factor of i

L = column length

H_i = theoretical plate height of i

The maximal concentration of a solute i (height of the peak-maximum) in the mobile phase at the column end ($c_{i,m}^{max}$) is given by the following relation:

$$c_{i,m}^{max} = \frac{V_{inj} \cdot c_{i,sample}}{\varepsilon_m \cdot A \cdot (1 + k_i')(2\pi)^{\frac{1}{2}}(L.H)^{\frac{1}{2}}} \qquad (2)$$

inwhich: V_{inj} = injection volume

$c_{i,sample}$ = concentration of i in the sample injected

ε_m = interstitial porosity of the column

A = cross sectional area of the column

With respect to detectability $c_{i,m}^{max}$ should be as large as possible According to equation 2 this can be realized by choosing $V_i.c_{i,sam}$ as large as possible and the column diameter,capacity factor , column length and theoretical plate height as small as possible.

However, these requirements contradict those put on the resolution and are in conflict with some practical limitations. For instance in order to realize the desired resolution a certain number of theoretical plates N (N=L/H) or, at a given theoretical plate height, a certain column length is required. Also very small k_i' values cannot be easy realized in practice, as usually a disturbance occurs in the first part of the chromatogram, particularly at sensitive detector settings, and therefore k_i should have a certain minimum value in order to allow for undisturbed detection. Reduction of the column diameter is also limited in practice due to the relative increase of the contribution of the injection and detection systems to the overall peakbroadening which affects the resolution unfavourably. The same unfavourable effect occurs when the injection volume (V_{inj}) is increased above a certain limit. Moreover, injection of too large quantities of the sample mixture can overload the column and, hence, may result in loss of resolution. Because of the above mentioned reasons, in practice the choice of the column dimensions and chromatographic conditions will be based on a compromise between resolution and detection.

UV detection is by far the most applied detection principle in HPLC and its suitability to monitor the anticonvulsant concentration levels in serum can be estimated from equation 2 and table 1. Equation 2 can be rewritten in terms of injected amount of solute Q_i and the void volume of the column V_o :

$$\frac{Q_i}{(1 + k_i')} = \frac{c_{i,m}^{max} \cdot V_o \cdot (2\pi)^{\frac{1}{2}} \cdot M_i}{(N)^{\frac{1}{2}}} \qquad (3)$$

where: M_i = the molecular weight of solute i

By means of equation 3 the minimal amount of solute i that must be injected into a selected HPLC system to obtain a peakheight that exceeds the noise of the detector by a desired factor can be calculated providing the molecular absorption coefficient ε_o of the solute is known. The relation between V_o , ε_o and Q_i , at constant

TABLE 1

PARTIAL LISTING OF ANTICONVULSANTS

GENERAL NAME	TRADE NAME	ABBREVIATION	THERAPEUTIC LEVEL	TOXIC LEVEL
phenobarbital	luminal	PB	20 - 40 ug/ml	>50 ug/ml
mephobarbital	mebaral	MPB	-	-
phenytoin	dilantin	PNT	10 - 20 ug/ml	>25 ug/ml
mephenytoin	mesantin	MPNT	5 - 16 ug/ml	>20 ug/ml
trimethadione	tridione	TMO	20 ug/ml	-
paramethadione	paradione	PMO	>700 ug/ml	>1500 ug/ml
ethosuximide	zarontin	ESM	40 - 100 ug/ml	> 150 ug/ml
diazepam	valium	DZP	.2 - .5 ug/ml	> 1 ug/ml
clonazepam	clonopin	CLZP	30 - 60 ng/ml	>80 ng/ml
carbamazepine	tegretol	CBZ	6 - 10 ug/ml	>15 ug/ml
phenacemide	phenurone	PAC	-	-
acetazolamide	diamox	ALM	-	-
primidone	mysoline	PRM	8 - 12 ug/ml	>15 ug/ml
valproic acid		VP	.4 - 1.2 ug/ml	-
Sulthiame		SU	.4 - 15 ug/ml	-

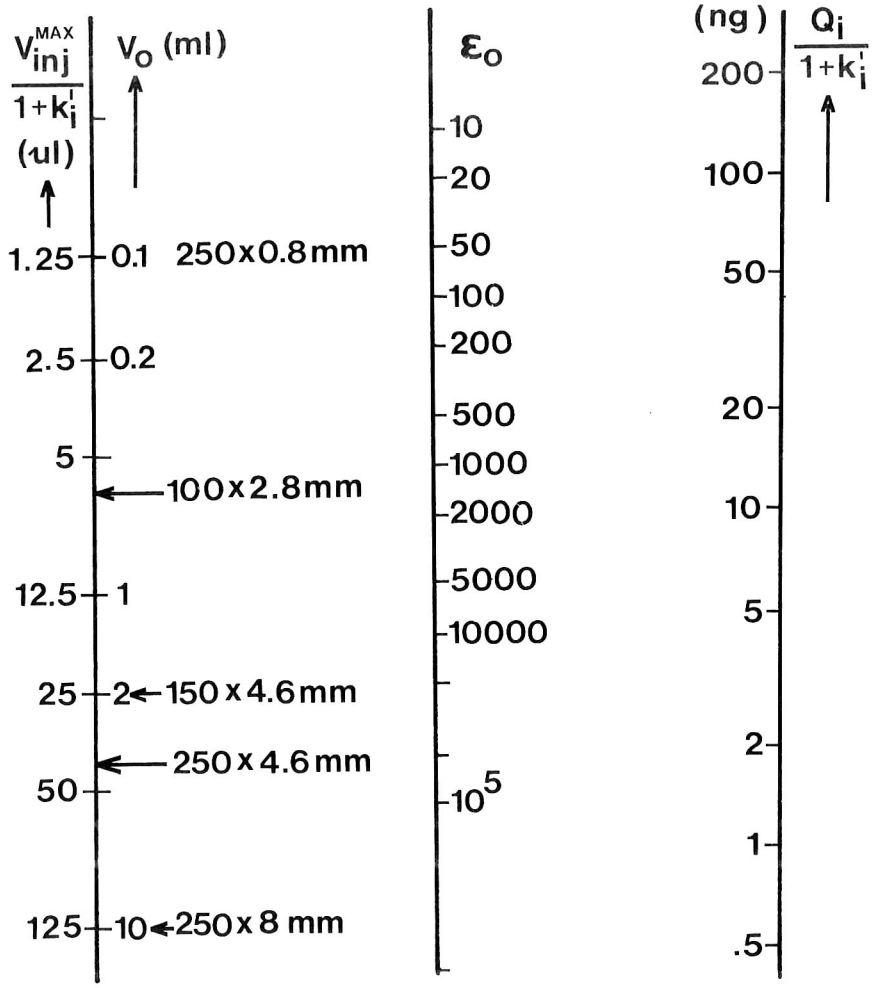

Fig.1 Nomogram representing the relation between the column void volume (V_o), the molecular extinction coefficient (ε_o) of a solute and the minimal amount of solute (Q_i) which must be injected to obtain a signal to noise ratio of 50 for a column with 6400 theoretical plates and a UV detector with a noise level of 10^{-4} AU.

TABLE 2

MOLECULAR EXTINCTION COEFFICIENT OF ANTICONVULSANTS

	WAVELENGTH (nm)	MOL.EXTINCTION COEFF.
phenytoin	258	740
ethotoin	259	225
trimethadion	226	1220
phensuximide	257	280
methsuximide	256	370
ethosuximide	248	200
carbamazepine	255	6500
phenacemide	257	285
primidone	258	220
phenobarbital	240	1470[1]
''	240	10000[2]

1) at pH = 2
2) at pH = 10

N,can be represented in a nomogram as given in Fig.1. The plate number (6400) , the noise level of the UV detector (1.10^{-4}) , the signal to noise ratio (50) and the molecular weight (200) used to compose this nomogram are representative for a common practical HPLC system with UV detection. When further assuming that 1 ml of serum is available for the analysis of anticonvulsants or another drug ,then the minimum amount of drug which must be present in 1 ml serum for accurate determination with the selected HPLC system can be estimated from this chromatogram,using the molar absorption coefficients as given in Table 2. When comparing the Q_i values,estimated with a maximal allowable k_i' of about 10,with the therapeutic levels of anticonvulsants as given in Table 1,the following conclusions can be drawn:

- on basis of the expected therapeutic serum concentration levels most common anticonvulsants can be accurately analyzed when monitoring at their UV molecular absorption maximum.
- in most cases an injection,equivalent to 0.01 - 0.1 ml of serum will be sufficient to detect the drugs with a signal to noise ratio of about 50.
- for the analysis of MSM,CLZP and DZP an injection,equivalent to 1 - 2 ml of serum will be necessary to obtain accurate results.
- the therapeutic levels of several anticonvulsants can be accurately measured with a simple 254 nm wavelength detector.
- the use of a variable wavelength UV detector has some advantages compared to the fixed wavelength detector. For a number of drugs the absorptivity at 254 nm is too small and measurement at their UV maximum is required. Further,a 2-40 fold increase of the sensitivity can be obtained if detection at 195 - 200 is applied. Under these circumstances one measures the absorptivity of the single chromophoric groups as present in the molecules of anticonvulsants. At this low UV wavelength all anticonvulsants can be detected with good sensitivity[4,5]. However,one must be aware that also the co-extracted interfering substances might show increased UV absorptivity. A drawback of monitoring at 195-200 nm is the fact that many common-used solvents for liquid chromatography cannot be used because of their strong UV absorption in the low UV range.
- UV detection fails in detecting valproic acid,as this compound show very little UV absorptivity. However, this drug can be sensitively detected at 280 nm,after pre-column derivatization with a bromomethylarylketone[6,7].

Another advantage of the use of a variable wavelength detector is the possibility to monitor the sample at different wavelengths. Comparison of the ratio of the absorptivity (via the peakheights) at the selected wavelengths with the ratio measured with the pure compounds,might give valuable information whether the registered peaks can be attributed to the drugs of interest or are interfered

by coextracted UV-active compounds eluting with the same retention as the drug[8-10].

It is convenient when the applied detection system can be used over a large concentration range. In this respect, the common-used UV detectors show a linear behaviour up to an extinction of about 1. This range is large enough to cover the expected concentrations of the anticonvulsants in serum[11]. The maximal allowable injected amount can be estimated from Fig.1 by calculation of the estimated minimal injected amount with a factor of 100-200. However, it must be noticed that no column overloading may occur .

SELECTION OF THE CHROMATOGRAPHIC PHASE SYSTEM

Of the separation modes available for HPLC , normal phase and reversed phase adsorption systems have shown to be the most suitable for the analysis of anticonvulsants.

In normal phase chromatography the retention is caused by the distribution of the solute between an organic mobile phase and the surface of a solid bearing active polar sites. The degree of retention depends on the type and number of functional groups in the solute molecule, the composition of the mobile phase and on the type of adsorbent. The distribution process can be very well described by a competition between solute and mobile phase molecules to be adsorbed on the active sites[12]. This means that the solute retention can be varied by changing the mobile phase composition. Silica gel is by far the most suitable adsorbent for normal phase liquid chromatography. The mobile phase is usually composed of a non polar or medium polar organic solvent (alkanes, chlorinated alkanes) and a small amount of a polar solvent (alcohols, tetrahydrofuran, dioxan, ethylacetate). In some cases the mobile phase is buffered with acetic acid or ammonia.

Until now normal phase liquid chromatography has found only limited application for the analysis of anticonvulsants[13-17], despite it s unique potential. This is partly caused by the fact that the commonly used mobile phases show considerable UV absorption

in the low UV range which limits the accurate detection of a number of anticonvulsants as was mentioned before. Also, the large retention of co-extracted polar serum constituents is mentioned to be a drawback, as this may lead to undesired long analysis times or even severe column contamination. However, this is not yet properly confirmed by investigations and is further not a specific problem for normal phase itself. A significant part of these problems might be solved by proper selection of the clean up procedure.

The applicability of normal phase liquid chromatography for the determination of carbamazepine and some other commonly used anticonvulsants in serum has been nicely demonstrated by Westenberg [14]. These authors used a mixture of dichloromethane and tetrahydrofuran as mobile phase. The retention of the anticonvulsants can be varied over a wide range by the tetrahydrofuran content as is shown in Fig.2. The complete analysis route is very simple and involves serum extraction with dichloromethane and injection of the organic phase directly into the HPLC system. Fig.3 shows a chromatogram of a serum extract obtained by this method. The described analysis method lends itself to automatic sample clean up coupled to HPLC.[18]

A drawback of normal phase chromatography is the fact that relative polar metabolites of the drugs will be more retained than the parent drug. In those cases where small metabolite concentrations have to be measured this might lead to detection problems. Bugge[15] demonstrated the use of normal phase for the determination of diazepam and it's main metabolite in blood, using a mixture of heptane/iso-propanol/methanol (40/10/1) as the mobile phase. This mobile phase is rather UV transparent, which allows detection at 232 nm.

Others preferred to use more complicated mobile phases consisting of propanol/diethylether/hexane/ammonia [17] or chloroform/dioxane /isopropanol/acetic acid [13] for the separation of phenobarbital, phenytoin and carbamazepine. However, such complicated mobile phases don't seem to be necessary when comparing those as used in Ref. 14,15.

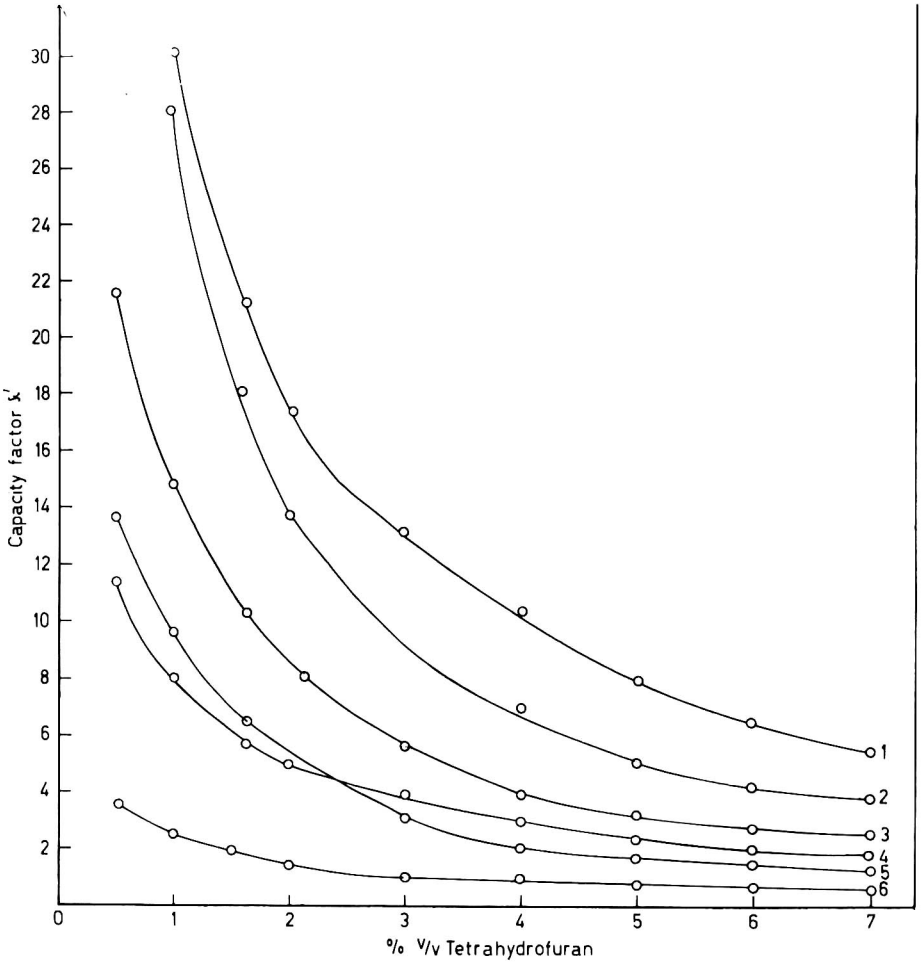

Fig.2 Effect of the percentage of tetrahydrofuran in dichloromethane on the capacity factor (k_i') of some anticonvulsants. 1 = carbamazepine ; 2 = nitrazepam ; 3 = phenytoine ; 3 = ethosuximide ; 5 = phenobarbital ; 6 = methylphenobarbital. Stationary phase : Lichrosorb SI 100. (taken from ref. 14)

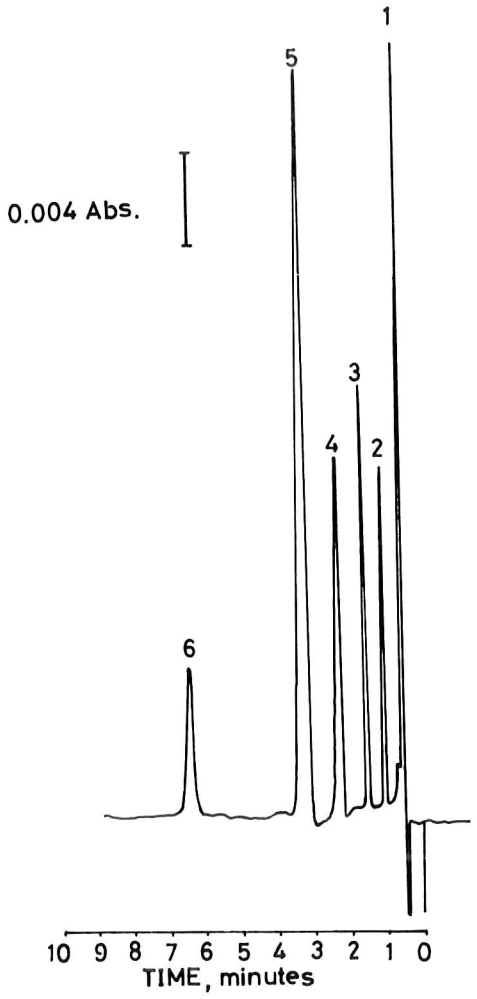

Fig.3 Chromatogram of a plasma extract. 1 = solvent front
2 = phenobarbital ; 3 = phenytoin ; 4 = nitrazepam ; 5 = carbamazepine ; 6 = carbamazepine 10,11- epoxide.
Stationary phase : Lichrosorb SI 100 250x2.1 mm
Mobile phase : dichloromethane + 5% tetrahydrofuran
(taken from ref. 14)

For the analysis of anticonvulsants ,the normal phase mode is completely overruled by the reversed phase mode. However,in our opinion the potential of normal phase chromatography on silica gel has not yet been fully developed and is most probably underestimated.

Reversed phase liquid chromatography has been applied in about 95% of all described HPLC methods for the analysis of anticonvulsants in literature[4,5,7,9,10,19 - 41]. In this separation mode the column packing consists of an alkyl-modified silica gel. An alkyl-chain (usually C_8,C_{18}) is chemically bonded to the silica which makes the surface non polar[42]. In contrast to normal phase systems, where the retention increases with increasing polarity of the solute (type and number of functional groups),in reversed phase systems the retention increases with increasing size of the hydrophobic moiety in the solute molecule. Although the distribution process in reversed phase systems is not yet well elucidated,the system can be described satifactorily as a reversed phase adsorption system. Just as is the case in normal phase systems,the retention of solutes can be changed by varying the mobile phase composition. Water is, in nearly all cases, one of the mobile phase constituents. Organic solvents are added in order to regulate the retention. These organic, less polar, solvents are often indicated as modifiers because they decrease the retention.

Acetonitrile and aliphatic alcohols have proved to be favourable organic modifiers. An additional advantage of these solvents is their good UV transparency which allows detection down to 195-200 nm.

The degree of retention in reversed phase systems,at fixed mobile phase composition,is also dependent on the type of packing (length of the alkyl chain,surface area etc.). As ionic species are hardly retained on reversed phase packings,the retention of ionizable substances can sometimes also be regulated by proper adjustment of the pH of the mobile phase by means of buffers. However,it must be noticed that,in practice,the upper limit of pH is 7-8,as the silica structure is broken down in alkaline medium[42]. The pH of the mo-

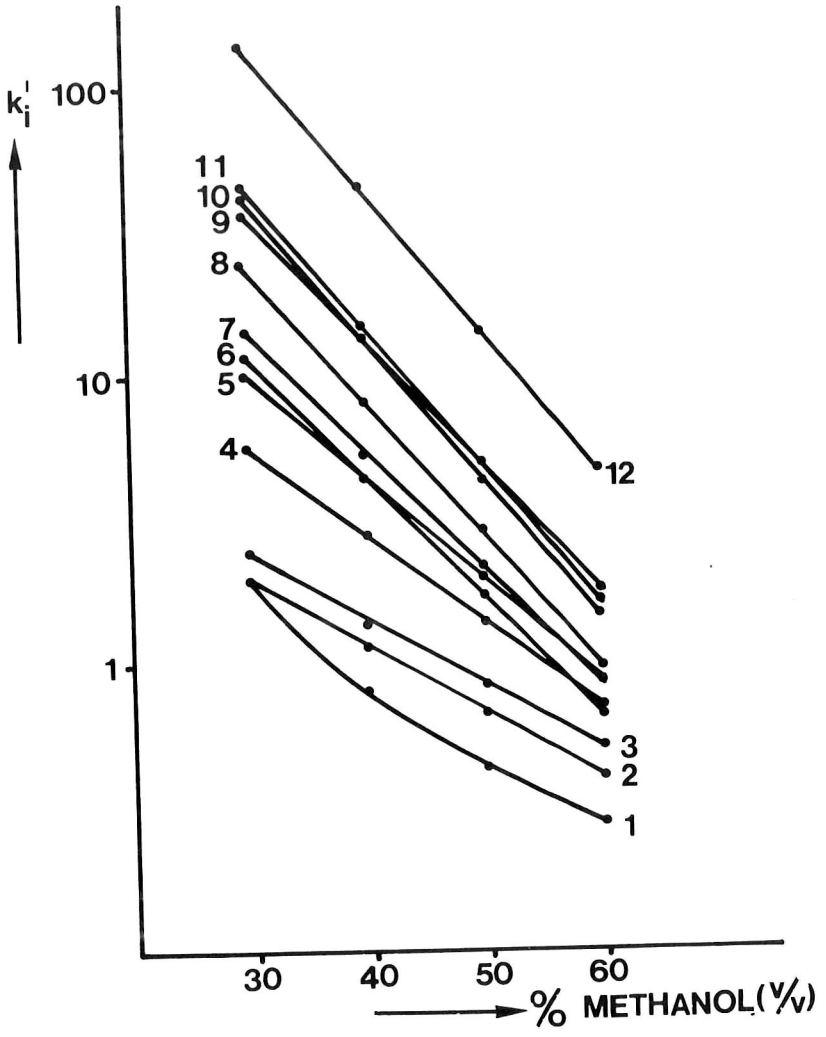

Fig.4 Effect of the percentage of methanol in water on the capacity factor of some anticonvulsants. 1 = sulthiam ; 2 = phenylethyl malon diamide ; 3 = ethyl-methyl suximide ; 4 = primidon ; 5 = carbamazepine 10,11 - epoxide ; 6 = phenobarbital ; 7 = phenytoin ; 8 = 5-(4-hydroxyphenyl)-5-phenyl-hydantoin ; 9 = carbamazepine ; 10 = gluthemide ; 11 = diphenyl hydantoin ; 12 = diazepam.
Stationary phase : Lichrosorb RP-8

bile phase might also significantly diminish the background caused by co-extracted interfering ionizable substances, as they can be shifted into the front peak.

For the analysis of anticonvulsants, acetonitrile and methanol have been most frequently applied. In many cases, the mobile phase is buffered with phosphate at pH 3-7. The reported modifier concentrations ranges between 15-50% (v/v) for acetonitrile and 25-55% (v/v) for methanol. This relatively large concentration range depends on the type and number of anticonvulsants to be separated and on the type of commercially available reversed phase packings. The effect of the modifier concentration on the retention of anticonvulsants on a C_8-modified silica gel is shown in Fig.4. As can be seen from this figure, the order of retention of some solutes differs significantly with different methanol contents. The separation of all anticonvulsants with one mobile phase composition is difficult. However, in practice, only a few combinations of anticonvulsants are used together, which simplifies the selection of the mobile phase composition. The commonly used anticonvulsants can be easily separated on reversed phase systems as is shown in Fig.5.

For the analysis of possibly formed metabolites, reversed phase systems offer advantages compared to normal phase, as the metabolites are more polar and, therefore, elute in front of the parent drug. This favours their detection when present at low levels. As in normal phase systems, one must be aware of column contamination by irreversible adsorption of strongly retained serum constituents like lipids. Often, a column washing with pure acetonitrile, dimethylsulfoxide or tetrahydrofuran after a number of analyses is required to maintain the separation efficiency of the column[16,20,22]. Despite these treatments, usually a limited number of analyses (2000-5000) can be realized with one column because the reversed phase packing slowly decomposes in the presence of polar solvents[42]. In this respect, the normal phase systems behave more favourably.

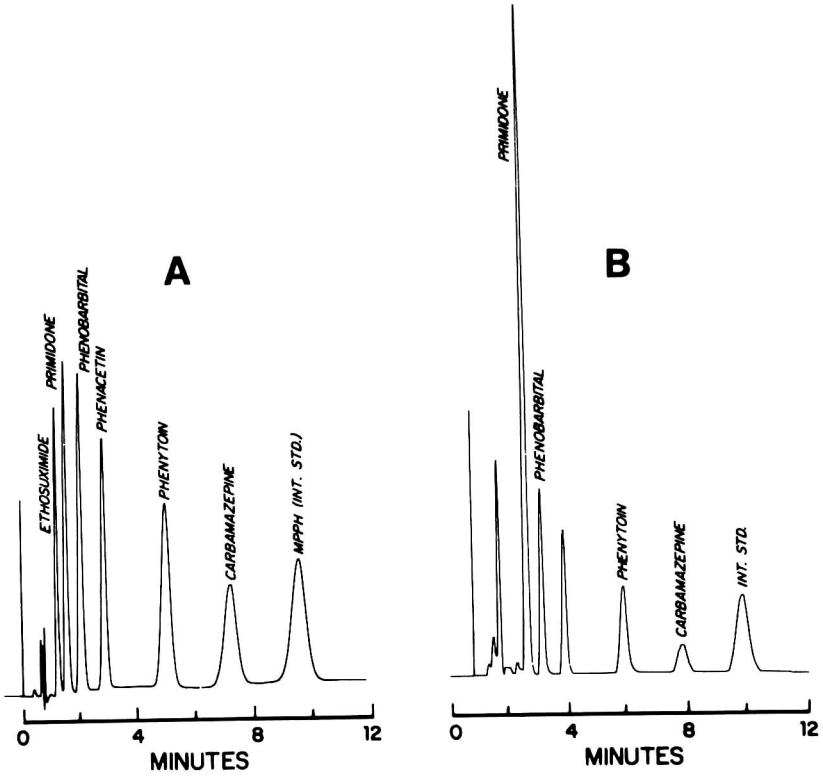

Fig.5 A), Chromatogram of a test mixture of anticonvulsants.
B), Chromatogram of an extract of a spiked serum.
Stationary phase : ODS-Sil-X-I 250 x 2.6 mm
Mobile phase : water + 15% (v/v) of acetonitrile
(taken from ref. 29)

Table 3 gives an overview of the mobile phase compositions and type of reversed phase packings applied for the separation and analysis of anticonvulsants and some of their metabolites in serum. Summarizing the selection of the chromatographic system it can be stated that the separation efficiency of normal and reversed phase systems is sufficient for the simultaneous separation of the common-

TABLE 3

APPLIED CHROMATOGRAPHIC CONDITIONS FOR THE SEPARATION OF ANTICONVULSANTS BY RPHPLC

PACKING [1]	COLUMN(mm)	% ORG.MODIFIER	pH	SAMPLE(ml)	ANTICONVULSANTS	REFERENCES
lichrosorb RP2	100x2.8	40% MeOH	-	1.0	barbiturates	24
Fast LC-8	150x4.6	36%MeOH+2%Acn	7.06	0.15	PM,PB,PNT,CBZ,ESM,CBZO	39
ODS-SIL X-1	250x2.6	15% Acn	-	0.05	ESM,PM,PB,PNT,CBZ,TMO,PMO	29
Zorbax C8	250x4.6	35% Acn	5.6	0.2-2.0	PB,PNT,CBZ,PM,CBZO,CLCP,NZP	33
Lichrosorb RP8	150x3.0	8% BuOH	3.0	1.0	ESM,PM,PB,CBZ,PNT,CBZO,SU,PEM	40
Partisil ODS	250x4.5	35% Acn	4.6	2.0	OZP,DDZP,DZP	35
Bondapack C18	300x4.0	65% Acn	-	2.0	DZP,DDZP	26
Lichrosorb RP8	100x4.6	45%MeOH+5%Acn	-	0.1	DZP,DDZP,OZP	36
Lichrosorb RP8	100x5.5	30% Acn	5.8	0.5	PM,ESM,PB,CPZ,PNT,CBZO,SU	34
Bondapack C18	300x4.0	50% Acn	8.0	0.025	PB,PMO,ESM,PNT,CBZ	20
Bondapack C18	300x4.0	19% Acn	4.4	0.2	PB,PNT,PMO,ESM,CBZ	22,48
Bondapack C18	300x4.0	55% MeOH	-	1.0	CBZ,CBZO	25
Spherisorb ODS	250x4.6	27% Acn	2.7	0.2	PB,PNT,CBZ,PMO	32
Bondapack C18	300x4.0	55% MeOH	-	1.0	PB,PNT,CBZ,HPPH	38
Bondapack C18	300x4.0	17%Acn+28%MeOH	7.0	0.4	ESM,PMO,PB,DMSM,PNT,CBZ	41
Lichrosorb RP§	125x4.0	16.4% Acn	5.3	0.2-0.5	ESM,PR,PB,PT,CBZ,CBZO,PEMA	46

1) for suppliers see ref. 50

ly used anticonvulsants. The possibly formed metabolites are usually completely separated from the parent drug. However, if more anticonvulsants are co-administered,one must be aware that the metabolites might interfere. The same might occur when other drugs are co-administered as was extensively investigated by several authors[4,5,19,32,39,41].
In a few papers,ion-exchange[43,44] and ion-pair chromatography[5] has been applied as separation mode. However,there seems to be very few reasons to apply these less efficient separation modes for the determination of anticonvulsants.

SAMPLE PRE TREATMENT

The pre-treatment of samples prior to injection into the HPLC system will be necessary in most cases for the following reasons:

- The separation power of the chromatographic system is limited. To match the separation power of the selected chromatographic system,the large number of interfering compounds present in body fluids usually has to be decreased by selective pre-fractionation of the sample components.
- Due to the presence of many compounds in body fluids,widely differing in structure and thus in retention behaviour,there might be a chance that the column is slowly detoriated with strongly adsorbed compounds after injection of a number of samples. To maintain the separation efficiency these substances have to be removed before the sample is injected.
- In certain cases the concentration of the drugs of interest in the sample is too small to be detected directly in the sample, accounting for the limit put on the injection volume (see fig.1). In these situations pre-concentration is necessary.
- Sample components which might precipitate in the chromatographic system (proteins) must be removed before injection of the sample as otherwise serious and often irreparable blockage of the injection valve and column occurs.

The extent of sample pre-treatment for the analysis of drugs in body fluids ranges between just deproteinization and complicated procedures involving all four steps as mentioned before.

There are two ways to realize the requirements put on the sample composition suitable for injection into an HPLC system. The sample is treated in such way that either the interfering substances are removed from the sample, leaving the solutes of interest in the original sample fluid, or the solutes are selectively withdrawn from the sample leaving the interfering substances in the original sample fluid. In practice, however, often both ways have to be applied successively in order to obtain clean samples.

Solvent and solid extraction are the separation methods of choice to realize the clean up steps as mentioned above. Although both separation methods have been applied for sample pre-treatment of anticonvulsants in serum, solvent extraction is by far most frequently used.

Most chromatographic systems deteriorate in performance when the sample solution differs much in solvent composition from the mobile phase. In many pretreatment procedures, one ends up with a solution of analytes in a solvent which is not compatable with the chromatography. Evaporation to (near) dryness and redissolution in the mobile phase is then inevitable. When ethosuximide is present in the sample, the evaporation temperature must be kept below $35^{o}C$. For reversed phase systems it is possible to inject the analytes if finally present in an aqueous solution.

Of course, losses of analyte due to the clean up manipulations with the sample (less than 100% recovery) have to be avoided as much as possible. However, this is often difficult in practice and one has to accept a certain percentage of loss. As long as the whole procedure is reproducible this does not invalidate the value of the analysis. Even in some cases, the acceptance of a somewhat lower recovery might be profitable with respect to co-extraction of interfering substances. If the sample clean up procedure may have an impredictable volumetric manipulation with phases and aliquots, the use of an internal standard is indispensable.

SOLVENT EXTRACTION: Various organic solvents of different polarity have been applied as extractant for the isolation of anticonvulsant drugs from serum and urine. Meijer e.a.[45] studied the distribution behaviour of a number of anticonvulsants in water-organic solvent mixtures as function of the pH of the aqueous phase (see Table 4). From this table it can be seen that the effect of the pH of the aqueous phase on the partition coefficient is insignificant at pH below 7.4 . As can be expected, the partition coefficient of the drugs with acidic character (phenobarbital and phenytoin) becomes very small when the aqueous phase is strongly alkaline. The influence of pH on the distribution of drugs can be used to separate acidic from neutral or basic drugs as was demonstrated by Eichelbaum [46] who determined carbamazepine by HPLC. Phenytoin and phenobarbital interfered with the analysis, but these drugs could be removed from the organic extract by shaking the organic phase with an alkaline aqueous solution bywhich the acidic drugs are completely extracted into the aqueous phase. Also,significant differences in partition coefficients are found with various extraction solvents. Acetone,in the presence of a large amount of ammoniumsulfate in the aqueous phase,seems to be the solvent which gives the largest partition coefficients. However,it must be noticed that still a considerable amount of water, which originally belongs to the biological sample,remains dissolved in the acetone phase bywhich a significant number of water soluble substances can be co-extracted. The amount of water in acetone and thus the co-extraction of water soluble substances can be significantly decreased by repeated salting out techniques as was demonstrated recently[10].

According to Table 4 ,ethylacetate seems to be a more or less universal extraction solvent for the common anticonvulsants. However, it is advantageous to keep the polarity of the extraction solvent not toohigh in order to minimize the co-extraction of interfering substances. This is,in particular,the case when applying normal phase chromatography where the retention increases with increasing

TABLE 4

PARTITION COEFFICIENTS OF SOME ANTICONVULSANTS AS FUNCTION OF pH AND TYPE OF SOLVENT[1]

		CHLOROFORM	DICHLOROETHANE	DIETHYLETER	ETHYLACETATE	ACETONE[2]	TOLUENE	HEXANE
phenobarbital	pH = 3.4	4.2	5.3	16.4	57	-	0.54	0.006
	pH = 7.4	2.3	2.9	17.4	52	760	0.55	0.005
	pH =12.2	0.004	0.001	0.003	-	-	0.0006	0.0004
phenytoin	pH = 3.4	28.8	28.7	40.8	197	-	4.1	0.008
	pH = 7.4	25.4	26.0	30.3	178	2600	2.6	0.004
	pH =12.2	0.007	0.02	0.009	-	-	0.001	0.0001
carbamazepine	pH = 3.4	97	90	3.4	28	-	7.6	0.1
	pH = 7.4	91	79	2.4	21	746	5.2	0.06
	pH =12.2	91	70	2.5	-	-	4.6	0.05
primidone	pH = 3.4	0.7	0.6	0.5	4	-	0.06	0.0006
	pH = 7.4	0.6	0.5	0.4	3.3	400	0.05	0.0002
	pH =12.2	0.5	0.4	0.3	-	-	0.03	0.0006
ethosuximide	pH = 4.0	9						

1) taken from reference 45 2) measured between water saturated with ammonium sulphate

polarity of the solute and thus might cause long analysis times or even severe column contamination.

Toluene seems to be a less suitable extraction solvent,while hexane does not extract the anticonvulsants at all. This shows that hexane can be used to remove possibly interfering lipids from serum as was recommended by some authors[36] to prolong the life time of reversed phase columns.

Until now,chloroform[16,21,25,29,44],dichloromethane[13,14,32,34,46],diethylether[26,27,38],sometimes containing a small amount of propanol,are the most frequently used extraction solvents. For the more polar drugs and the metabolites of the liphophilic drugs, ethylacetate[5,31,37,43],chloroform/propanol mixtures[39],acetone and acetonitrile[10,36] have been successfully applied.

In most reports the organic phase/sample ratio ranges between 5 - 10. In some applications repeated extractions are recommended. The reported absolute recoveries of anticonvulsants vary between 70 - 100%.

The solvent extraction can also be performed on a so-called extraction column[17,47]. Such columns are filled with large sized diatomite earth with a large pore volume. The aqueous sample is applied to a dry column which adsorbs the aqueous phase as a stationary liquid phase on the porous support. The column is then eluted with an organic solvent not miscible with water (usually one of the solvents used in table 4). The lipophilic drugs are extracted from the aqueous phase (the sample) into the organic eluent. An advantage of this mode of solvent extraction is an emulsion free organic extract which is sometimes not the case with conventional solvent extractions.

The solvent extraction,deproteinization,evaporation and redissolution lends itself to automation as was shown by Snyder [39]. In this report a complete automated sample clean up unit,suitable for anticonvulsants,was directly coupled to HPLC (see Fig.6). The drugs are on line extracted from the serum sample. After phase separation the organic solvent flows onto a continuously moving te-

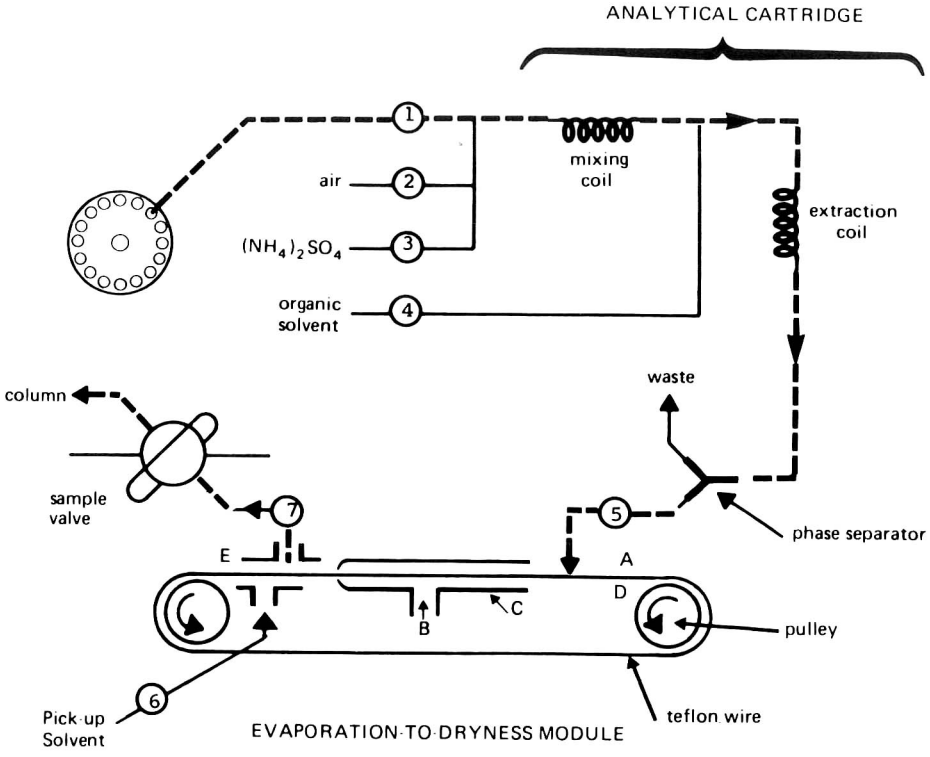

Fig.6 Flow diagram of sample pretreatment module for Fully Automated Sample Treatment - Liquid Chromatography (FAST-LC System) -----sample pathway ; ——— other liquid streams. (taken from ref. 39)

flon wire and is evaporated by hot air. After this,the Teflon wire is washed with a pick-up solvent of composition suitable for injection into the HPLC system. The pick-up solvent is pumped through the sample loop of the injection valve of the HPLC system. Fig.7 shows a chromatogram of a human serum obtained by this FAST-LC system.

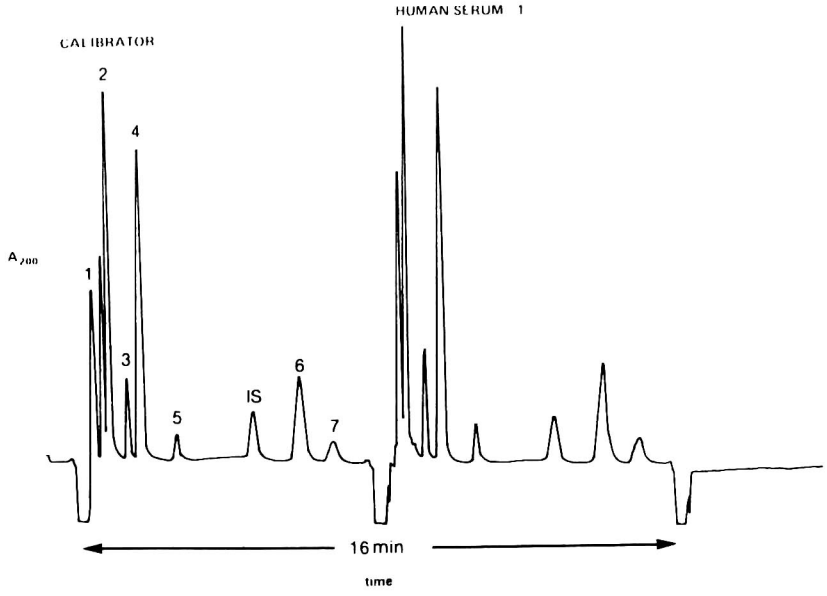

Fig.7 Chromatograms provided by FAST-LC assay for anticonvulsants and metabolites. (see fig.6)
1 = phenyl ethyl malonamide ; 2 = ethosuximide ; 3 = primidone ; 4 = phenobarbital ; 5 = carbamazepine 10,11-epoxide ; 6 = phenytoin ; 7 = carbamazepine ; IS = hexobarbital. (taken from ref. 39)

DEPROTEINIZATION: Direct injection of deproteinized serum has been applied by a number of authors[20,22,28,35,40,41]. The serum samples are deproteinized with acetonitrile[20,22,28,35], acetone[41] or perchloric acid[40], centrifuged and 25 - 100 ul of the supernatant is injected in a reversed phase system. This method is only applicable when the drug serum concentrations are relatively large. A drawback is the fact that almost all serum constituents are injected onto the column, which might give problems with the background and column contamination. For instance, when using per-

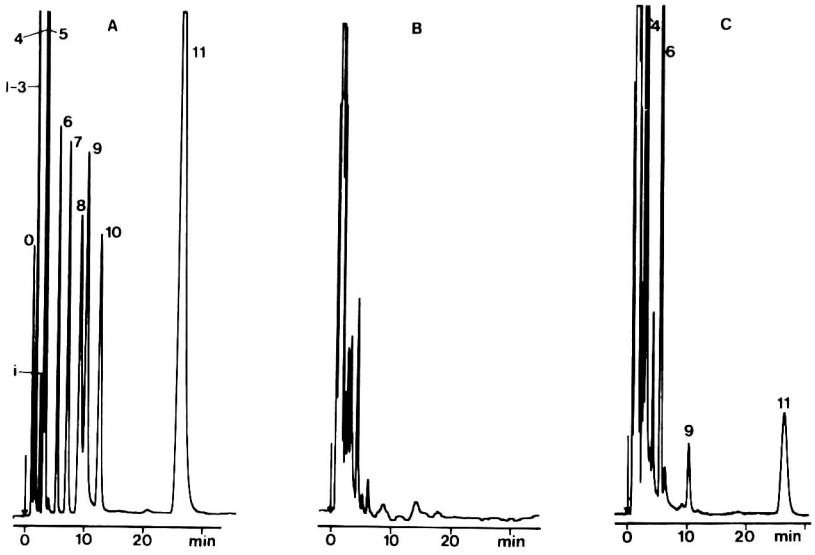

Fig.8 Chromatogram of a test mixture of anticonvulsant (A) , of a deproteinized blank serum (B) and of a deproteinized serum of a patient receiving daily primidone,carbamazepine and diphenylhydantoine (C). (the numbers correspond to those in fig.4)

The serum samples were deproteinized with perchloric acid ,centrifuged and 100 ul of the supernatant injected. (taken from ref. 40)

chloric acid as deproteinizating agent,a large disturbance occurs in the beginning of the chromatogram as is shown in Fig.8. This disturbance prevents the analysis of the more polar drugs and metabolites. When using acetonitrile as deproteinizating agent a significantly better background is obtained as is shown in Fig.9. All the described methods,where direct injection of deproteinized serum is applied,suffer from serious column contamination after a limited number of injections. To maintain the separation effi-

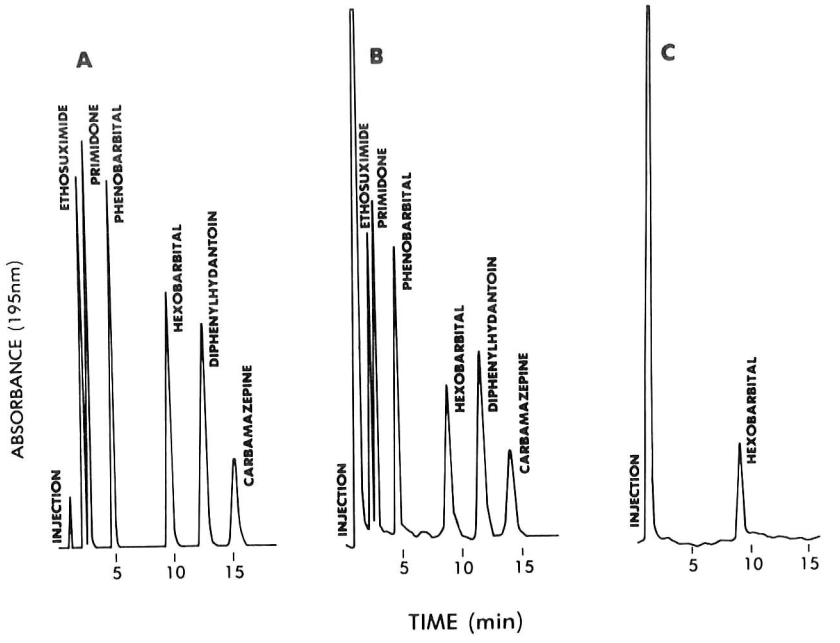

Fig.9 Chromatogram of a test mixture of anticonvulsants (A) ; of a spiked serum (B) and of a blank serum spiked with the internal standard (C).
The serum samples were deproteinized with acetonitrile,centrifuged and 20 ul of the supernatant injected. (taken from ref. 22)

ciency,the column has to be washed regularly with pure organic solvents as recommended in Ref.20,22. Another way to protect the analytical column is the installation of a small disposable precolumn[36] which can be replaced after a number of injections.
SOLID PHASE EXTRACTION: Another way to simplify the matrix of the sample is by adsorbing the solutes at an adsorbent. This way of sample clean up has been applied with polystyrene resins (XAD)[33], charcoal[4,19] and alkylmodified silicas[48]. The serum sample is applied to a small column filled with the solid material onwhich

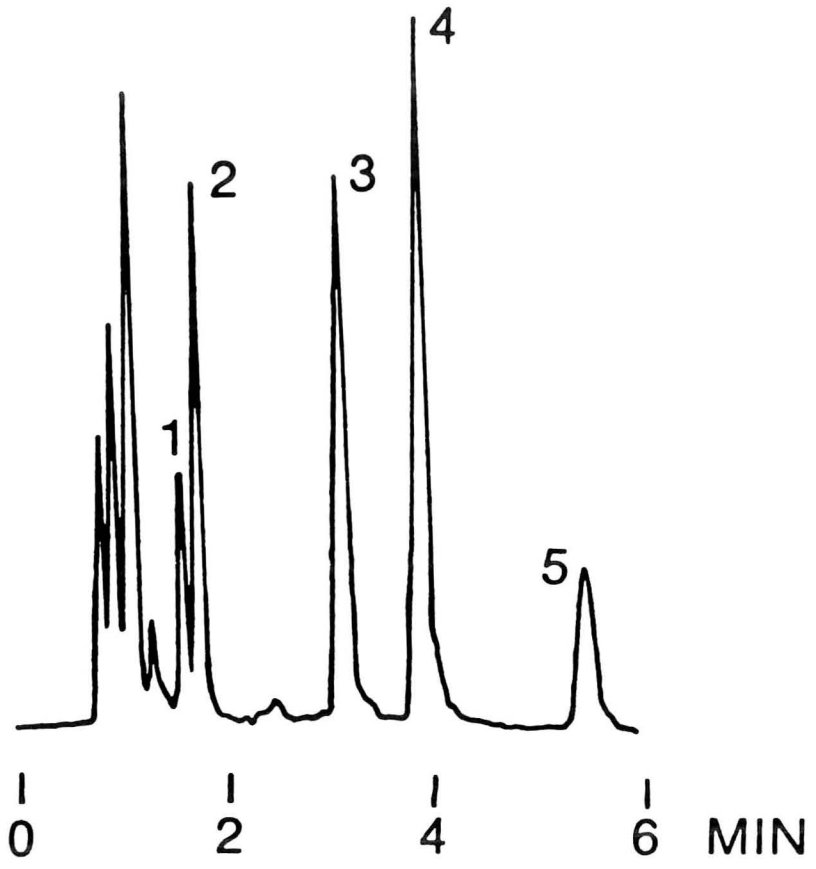

Fig.10 Chromatogram of a solid phase extract of a patient serum. 1 = phenylethylmalonamide ; 2 = primidone ; 3 = phenobarbital ; 5 = alphenal ; 6 = phenytoin. (taken from ref. 33)

the drugs of interest are strongly adsorbed. The column is then washed with a solvent (usually water) to elute weakly retained serum constituents. Than the drugs are eluted from the column with an organic solvent. The organic solvent is collected and evaporated and the residue dissolved in the eluent. The recoveries with solid

extraction techniques ranges between 60 - 100%. The extracts obtained with solid extraction are rather clean as is shown in Fig.10 The described clean-up procedures have also been successfully applied to urine[20,31,37] and saliva [24] samples. However, as the urine samples contain considerably larger concentrations of interfering substances, the clean-up is more laborious than for serum samples.

QUANTITATIVE ASPECTS

The precision of HPLC for the determination of anticonvulsants in serum is determined by the accuracy of the various steps in the analysis route (e.g. sample manipulations, chromatography, detection). With respect to chromatography, the constancy of the flow-rate and the retention characteristics are the main sources which might affect the precision. Modern liquid chromatographs and column packings enable reproduction of retention data with a coefficient of variation less than 1-2%. The influence of the detection system on the precision becomes significant when measuring at a small signal to noise ratio or out of the linear dynamic range. For the determination of anticonvulsants at therapeutic levels, this is hardly the case, as the solutes can easily be detected at a signal-to-noise ratio of 50 - 100 (see also Fig.1). Under these conditions, the reproducibility of the peakheight or peak area is usually 1 - 2%. The chromatographic and detection system are minor error sources compared with those introduced by the clean-up manipulations. The often unpredictable phenomena occuring during the sample preparation require, in most cases, the use of an internal standard to obtain acceptable precision. In these procedures a compound with similar physical and chemical properties as the solutes, is added to to the original sample and processed through the complete analysis route. The serum drug concentrations are determined by comparison of the ratio of the peak-heights or peak areas of the solute and internal standard with a calibration curve composed from ratios obtained by analysis of

drug free serum samples spiked with known amounts of pure solute and internal standard. The precision of the linear calibration curve can be characterized by a regression coefficient or preferably by a standard deviation of error[49]. Unfortunately, values of regression analysis of calibration curves of anticonvulsants are seldomly reported[13,28]. Despite this lack of information, data about the precision of the determination of anticonvulsants by HPLC, using internal standardization, have been published. For therapeutic levels the reported coefficients of variation for within-day analyses ranges between 2 - 6%[13,17,19,20-22,31-33,39 7,14,28,29,38,41] and for day to day between 6 - 9%[5,16,20,27,31,39] From the reproducibility of the recovery, which have been reported to range between 2 - 6% [4,13,14,19,31,33,35,39,40,44] it can be concluded that the coefficients of variation ultimately achieved can be greatly ascribed to the irreproducibility of the isolation method. This pleads in favour of completely automated sample treatment systems as applied in Ref.39.
When analyzing samples with lower concentration levels, the coefficients of variation increases considerably and are of the order of 10 - 15% at levels < 0.1 ug/ml[24,38,39].

CONCLUSIONS

From the many reports which have appeared in lit erature, it can be concluded that HPLC, combined with UV detection, is very suitable for the analysis of anticonvulsants in serum. The separation efficiency of the available phase systems enables the simultaneous analysis of a large number of anticonvulsants and some of their metabolites. In contrast to gas chromatography, the sample pretreatment for HPLC is simple. Sample pretreatment by means of solvent extraction lends itself to automation, coupled to HPLC. In this respect automated on line solid extraction coupled to HPLC might have advantages compared to solvent extraction and is worth investigating.

The anticonvulsant concentrations ,determined by HPLC,correlate well with those obtained by means of other techniques like EMIT and GC [5,13,17,19,20-21,25,31,32,38,39].

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REFERENCES

1) Textbook of Organic Medicinal and Pharmaceutical Chemistry, C.O.Wilson,O.Gisvold and R.F.Doerge (eds.), J.B.Lippincott Comp.,Philadelphia (1976)
2) Antiepileptic drugs: Quantitative analysis and Interpretation, C.E,Pippenger,J.Kiffin Penry,Henn Kutt (eds.) , Raven Press, New York (1978)
3) Arzneimittelchemie I , E.Schröder,C.Rufer,R.Schmiecken (eds.), George Thieme Verlag, Stuttgart (1976)
4) R.F.Adams in Advances in Chromatography, J.C.Giddings (ed.), 15 (1977) p131.
5) J.A.Christofides and D.E.Fry, Clin.Chem. 26 (1980) 499
6) G.J.Schmidt and W.Slavin, Chromatogr. Newsletter, 6(1978) 22
7) R.Alric,M.Cociglio,J.P.Blayac and R.Puech, J.of Chromatogr. 224 (1981) 289
8) S.J.Soldin and J.J.Hill, Clin.Chem. 23 (1977) 2352
9) V.A.Raisys,P.N.Friel,P.R.Graaff,K.E.Opheim,A.J.Wilensky, J.of Chromatogr. 183 (1980) 441
10) M.Riedman,B.Rambeck and J.W.A.Meijer, Hewlett-Packard Application note 232-19 (1981)
11) H.Poppe in Instrumentation for HPLC,J.F.K.Huber (de.), J.of Chromatogr.Library, vol 13,cpt.7
12) L.R.Snyder, Principles of Adsorption Chromatography, Marcel Dekker , New York (1968)
13) S.H.Atwell,V.A.Green and W.G.Haney, J.Pharm.Sci.,64 (1975) 806
14) H.G.M.Westenberg and R.A.de Zeeuw, J.of Chromatogr.118 (1976) 217

15) A.Bugge, J. of Chromatogr., 128 (1976) 111
16) P.M.Kabra,G.Gotelli,R.Stanfill and L.J.Marton, Clin.Chem. 22 (1976) 824
17) P.J.Helmsing,J.van der Woude,O.M.van Eupen, Clin.Chim.Acta, 89 (1978) 301
18) M.Bonnafé , Swiss Pharm. 1(10) (1979) 21
19) R.F.Adams and F.L.VanderMark, Clin.Chem. 22 (1976) 25
20) S.J.Soldin and J.G.Hill, Clin.Chem. 22 (1976) 856
21) P.M.Kabra and L.J.Marton, Clin.Chem., 22 (1976) 1070
22) P.M.Kabra , B.E.Stafford and L.J.Marton, Clin. Chem. 23 (1977) 1284
23) E.Grassi,G.L.Passetti and A.Trebbi, J.of Chromatogr. 144 (1977) 132
24) U.R.Tjaden,J.C.Kraak and J.F.K.Huber, J.of Chromatogr. 143 (1977) 183
25) J.W. Mihaly,J.A.Philips,W.J.Louis and F.J.Vajda, Clin. Chem., 23 (1977) 2283
26) R.R.Brodie,L.F.Chasseaud and T.Taylor,J.of Chromatogr. 150 (1978) 361
27) P.M.Kabra,G.L.Stevens and L.J.Marton, J.of Chromatogr. 150 (1978) 355
28) J.E.Slonek,G.W.Pengand and W.L.Chion, J.Pharm.Sci. 67 (1978) 1462
29) R.F.Adams,G.J.Schmidt and F.L.VanderMark, J.of Chromatogr. 145 (1978) 275
30) A.Castro,J.Ibanez,J.L.DiCesare,R.F.Adams and H.Malkus,Clin. Chem., 24 (1978) 710
31) R.W.Dykeman and D.J.Ecobichon, J.of Chromatogr. 162 (1979) 104
32) M.Pesh-Iman,D.W.Fretthold,I.Sunshine,S.Kumar,S.Terrentine and C.E.Willis, Therap.Drug Monitoring, 1 (1979) 289
33) R.C.Williams and J.L.Viola, J.of Chromatogr. 185 (1979) 505
34) D.J.Berry,L.A.Clarke and G.E.Valkins, J.of Chromatogr. 171 (1979) 363
35) A.Astier and M.Maury, J.of Chromatogr. 164 (1979) 235

36) T.B.Vree,A.M.Baars.Y.A.Hesker and E.van der Klein, J.of Chromatogr. 162 (1979) 605

37) R.J.Perchalski,J.Bruni,B.J.Wilder and L.J.Willmore, 163 (1979) 187

38) R.J.Sawchuk and L.L.Cartier, Clin.Chem. 26 (1980) 835

39) J.W.Dolan,Sj.van der Wal,S.J.Bannister and L.R.Snyder, Clin. Chem. 26 (1980) 871

40) J.C.Kraak,F.Smedes and J.W.A.Meijer, Chromatographia, 13,11 (1980) 673

41) G.K.Szabo and T.R.Browne, Clin.Chem. 28 (1982) 100

42) K.K.Unger, Porous Silica, J.Chromatogr. Library, vol 16, Elsevier, Amsterdam (1979)

43) B.Moore,G.Nickless,C.Hallett and A.G.Howard, J.of Chromatogr. 137 (1977) 215

44) S.Kitazawa and T.Komuro, Clin.Chim.Acta, 73 (1976) 31

45) D.M.Goedhart,O.M.J.Driessen and J.W.A.Meijer, Arzneimittel Forschung/Drug research 28 (11) (1978) 19

46) Determination of Antiepileptic drugs by HPLC: sample preparation with Extralut, Merck (Darmstadt) . Application note IFCC congress 1981 (Vienna)

47) M.Eichelbaum and L.Bertilson,J.of Chromatogr. 103 (1975) 135

48) R.C.George, Clin.Chem. 27 (1981) 198

49) Statistics, M.R.Spiegel, 1^{e} edition, McGraw-Hill ,New York (1972)

50) Lichrosorb - Merck,Darmstadt (West Germany)
Partisil - Reeve Angel , Clifton,N.J (USA)
FAST LC - Technicon, Tarrytown,NY (USA)
Zorbax - Dupont,Wilmington,Del, (USA)
Bondapack - Waters Ass. ,Milford,Mass. (USA)
ODS-SIL-X - Perkin Elmer,Norwalk,Conn. (USA)
Sherisorb - Alltech,Los Altos,CA (USA)

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BILE PIGMENTS

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ABSTRACT

The application of high-performance liquid chromatography (HPLC) to the analysis of bile pigments is reviewed with particular references to the characterization and determination of bilirubin and its conjugates in body fluids. A comparison of phase systems for the separation of bile pigments is given.

INTRODUCTION

Bile pigments are linear tetrapyrroles formed in the cells of the reticulo-endothelial system by enzymic oxidation of the haem of haem protein with loss of the protein moiety, fission of the haem IX ring mainly at the α-bridge and removal of the iron atom (Fig.1). The first bile pigment formed, biliverdin IXα , is rapidly reduced in the tissue to bilirubin IXα . Bilirubin is excreted in the bile mainly as diglucuronide after conjugation in the liver. In the gut bilirubin is hydrogenated by intestinal flora either before or after deconjugation to a mixture of urobilinogens which are oxidised to stercobilin, half-stercobilin and urobilin. Stercobilin is stable while half-stercobilin and urobilin may be oxidised to half-stercoviolin and mesobiliviolin respectively. Further oxidation of mesobiliviolin to mesobiliverdin or glaucobilin may also occur (Fig.2).

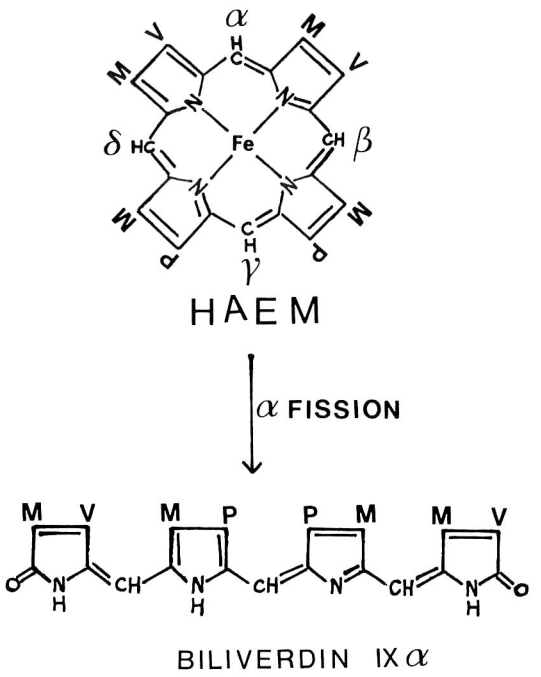

FIGURE 1. Formation of biliverdin from haem.

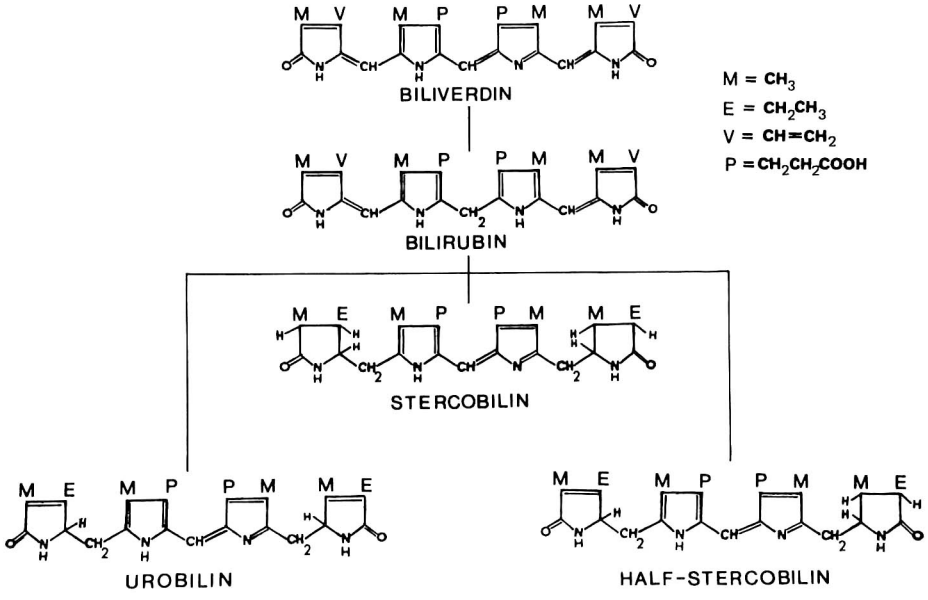

FIGURE 2. Structures of bile pigments.

The bile pigments are classified primarily according to the extent of conjugation ranging from the colourless urobilinogens with no conjugation to the orange-red "urobilins", violet biliviolins and the green or blue biliverdins. The most important bile pigment being the yellow bilirubin IXα .

SEPARATION OF SYNTHETIC BILE PIGMENTS

There is need for a wide range of pure model bile pigments for comparative study in chemistry and biochemistry with the increasing number of new compunds being isolated from natural sources. High-performance liquid chromatography (HPLC) with its superior resolving power and sensitive detectors is increasingly being used for the analytical and preparative separation of bile pigments in place of the widely used thin-layer chromatography (t.l.c.).

Separation of biliverdin IXα, IXβ, IXγ, and IXδ dimethyl esters

The oxidative cleavage of haem either enzymically or chemically to biliverdin IXα is accompanied by the formation of the IXβ , IXγ and IXδ isomers as minor products (Fig.1). These isomers which may be important biologically have been separated by adsorption and reversed-phase chromatography. A reverrsed-phase system (1) with methanol-water (78:22) as mobile phase on a C18 column was shown to be superior to resolution obtained on a silica column eluted with toluene-acetone-pyridine (90:10:1). The bile pigment dimethyl esters, however, are usually better separated by adsorption chromatography. This was demonstrated by the improved separation of the isomers on silica with dichloromethane-methanol-water (90:0.9:0.1) as the eluent (2).

Separation of IIIα ,IXα and XIIIα isomers of verdins and violins

Bile pigments containing a central methylene bridge, i.e. rubins and urobilinogens, undergo acid-catalysed rearrangement so that compounds with the natural IXα order of side-chains give symmetrical IIIα and XIIIα isomers (3,4). This reaction has been used to prepare a large number of verdins and violins of the IIIα , IXα and XIIIα series. The separation of these compounds as dimethyl esters has been successfully performed on a μ-Porasil column with isooctane-methyl acetate in various proportion

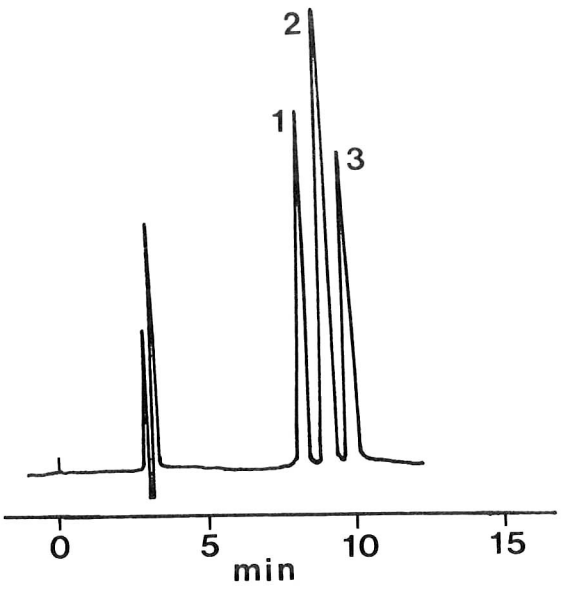

FIGURE 3. Separation of IIIα(1), IXα(2) and XIIIα(3) isomers of biliverdin dimethyl esters.
Column,μ-Porasil; mobile phase,heptane-methyl acetate (2:1); detection,370nm.

as the mobile phase (5). Apart from being able to separate the bile pigments into their major classes the system is also capable of separating the IIIα , IXα and XIIIα isomers (Fig.3) of each compound. The capacity ratio (k') of these compounds increases with decreasing conjugation, and the elution order of the isomers is IIIα < IXα < XIIIα.

Separation of i-urobilin dimethyl esters

The separation of i-urobilin dimethyl ester on silica (μ-Porasil) is particularly interesting. A benzene-ethanol (29:1) solvent mixture containing trace amount of diethylamine not only separated the IIIα, IXα and XIIIα isomers but also resolved each isomer into pairs of diastereoisomers (6,7). The order of elution of the isomers reverses that of the verdins and violins and the RR,SS forms eluted before the RS,SR forms. The presence of diethylamine is essential for the separation.

Separation of bilirubin IIIα, IXα and XIIIα isomers

Bilirubin IIIα and XIIIα isomers can be prepared by acid-catalysed rearrangement of bilirubin IXα (3,4). Commercially available bilirubin also contains variable quantity of IIIα and XIIIα isomers as impurities.

A 20-minute gradient elution on a silica column (25cm x 4.6mm) with acetic acid in chloroform (0.2% to 0.999%) as the gradient mixture has been described for the separation of bilirubin isomers (8). Apart from requiring gradient elution separation of the IXα and XIIIα isomers was incomplete due to peak tailing.

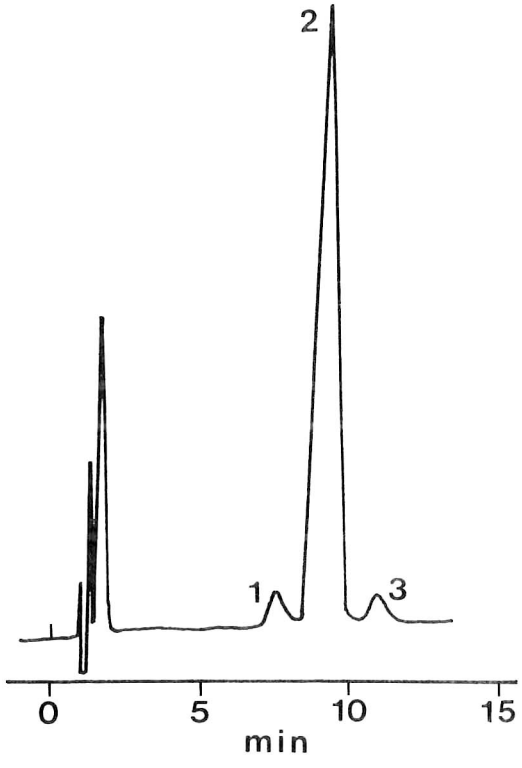

FIGURE 4. Separation of bilirubin XIIIα(1), IXα(2) and IIIα(3) isomers.
Column,SAS-Hypersil;mobile phase,acetonitrile-dimethyl sulphoxide-water(32:32:36);detection,450nm.

An exceedingly long (120 minutes) gradient elution ion-pairing system was employed by Onishi et al (9) using acetonitrile (10% to 40%) in 0.01M phosphate buffer containing 0.1% tetrabutylammonium hydroxide as the mobile phase. A 25cm x 4.6mm Shimadzu PCH column was used as the stationary phase.

Reversed-phase chromatography appeared to give the best results in terms of speed and resolution. Lim et al (10) achieved complete separation of the isomers in less than 15 minutes by isocratic elution on a 10cm x 5mm SAS-Hypersil (C1) or ODS-Hypersil (C18) column. A ternary solvent mixture of acetonitrile- dimethyl sulphoxide-water (typically 30:30:40) was used (Fig.4). The presence of dimethyl sulphoxide improved the solubility of the bile pigments as well as altered the selectivity of the column leading to superior resolution.

CHARACTERIZATION OF BILE PIGMENTS IN BILE

The standard procedure for the characterization of bilirubin and its conjugates in biological fluids is by various modifications of the diazo reaction followed by analysis of the azopigments formed. Little (11) analysed bilirubin p-iodophenyl azopigments from dog bile by reversed-phase ion-pair chromatography on a μ-Bondapak C18 column using tetrabutylammonium ion as the counter ion. The nine peaks separated were not characterised.

Onishi et al separated the bile pigments in dog bile as ethyl anthranilate azopigments (12). A reversed-phase gradient elution system on a Shimadzu PCH column eluted with acetonitrile (20% to 60% in 80 minutes) in 0.1M acetate buffer also separated the azopigments into nine peaks. These were identified as the exo- and endo-vinyl isomers of ethyl anthranilate azodipyrrole (designated $\alpha 0$), azodipyrrole-β-D-monoxyloside ($\alpha 2$), azodipyrrole-β-D-monoglucoside ($\alpha 3$) and azodipyrrole-β-D-monoglucuronide (δ). The last peak was not characterised. The peaks obtained by Little (11) were probably of similar nature.

Conjugated and unconjugated bilirubin in bile can also be separated directly. Lim (13) demonstrated the presence of mono- and di-glucuronide conjugates in rat bile by separation on a μ-Bondapak carbohydrate column. The stability and reproducibility of the column, however,were unsatisfactory. In an attempt to overcome these problems a liquid-liquid partition system with in situ loading of a silica column with tetraethylenepentamine and

eluted with acetonitrile–water containing 0.05% tetraethylenepentamine was developed for the separation of the bile pigments (10). The separation of unconjugated bilirubin was satisfactory but its application to the separation of conjugated bilirubin has not been described. The column while stable required long equilibration for reproducible retention and a small change in solvent composition could led to a large variation of the k' values. A better approach to the direct separation of conjugated and unconjugated bilirubin in bile is by reversed-phase ion-pair chromatography. Jansen and Tangerman (14) and Jansen (15) separated the bile pigments on a C18 column with acetonitrile–0.01M tris HCl buffer (40:60) containing tetrabutylammonium sulphate as the mobile phase. Conjugated and unconjugated bilirubins in the bile of human, monkey, dog, pig and rat have been analysed by this method.

Onishi et al (16) seperated bilirubin, bilirubin diglucuronide and two isomeric bilirubin monoglucuronides in human bile using two C18 columns in series and a 100-minute gradient elution with acetonitrile in 0.1M acetate buffer containing sodium pentanesulphonate as the gradient mixture. The resolution achieved by this system was better than others, however, the much longer retention time plus the need for gradieny elution made it a less praticable method.

ESTIMATION OF URIDINE DIPHOSPHOGLUCURONATE GLUCURONOSYL TRANSFERASE (UDP-GLUCURONYL TRANSFERASE) ACTIVITY

The separation of conjugated and unconjugated bilirubin has been applied by Onishi et al (16) to estimate the activity of the enzyme UDP-glucuronyl transferase.This enzyme, concentrated in the liver microsomes, is essential for the conjugation and biliary excretion of bilirubin. The sensitivity of the HPLC method was over a 100 times that of the t.l.c. densitometric or spectrophotometric method. It is therefore possible to determine very low activity of the enzyme as in liver biopsy specimens of neonates.

Chowdhury et al (17) also developed an assay of human liver UDP-glucuronyl transferase and bilirubin monoglucuronide dismutase based on HPLC separation of bilirubin mono- and di-glucuronide by reversed-phase ion-pair chromatography. The method is readily adaptable to radioassay using labelled bilirubin or UDP-glucuronic acid making it very sensitive and requiring only small specimens.

DETERMINATION OF BILIRUBIN IN PLASMA OR SERUM

The determination of bilirubin in plasma or serum is mainly concerned with the diagnosis and control of treatment of diseases associated with jaundice. The diazo reaction methods currently used in clinical laboratories are probably adequate for the estimation of total bilirubin. Their use for the determination of conjugated and unconjugated bilirubin based on the direct and indirect diazo reactions, however, is very unreliable. The direct spectrophotometric method employed in many paediatric departments measures only total bilirubin and is unable to differentiate bilirubin from photobilirubins (see below).

HPLC is an ideal technique for the simultaneous determination of conjugated and unconjugated bilirubin in plasma. However, while unconjugated bilirubin can be recovered easily from plasma the quantitative extraction of conjugated bilirubin is difficult although their extraction and HPLC separation has been reported (15). Blanckaert et al (18) overcome the extraction problem by converting bilirubin mono- and di-conjugates into mono- and di-methyl ester respectively by alkaline methanolysis. Unconjugated bilirubin was unaffected by the reaction. This elegant and simple procedure is an important advancement in the application of HPLC to the analysis of conjugated and unconjugated bilirubin in plasma. The pigments were then separated by gradient elution on a silica column with chloroform-acetic acid-methanol as the gradient mixture.

To make Blanckaert's method more acceptable for routine applications with possible automation the isocratic reversed-phase system developed by Lim et al (10) for unconjugated bilirubin in serum has been modified (19) for the separation of bilirubin, bilirubin monomethyl ester and bilirubin dimethyl ester (Fig.5). The method does not allow identification of the nature of conjugating groups but is nevertheless useful for routine diagnostic purposes.

In pathological sera containing high concentration of conjugated bilirubin Lauff et al (20) separated four bilirubin species. These were identified as unconjugated bilirubin, bilirubin monoglucuronide, bilirubin diglucuronide and bilirubin tightly bound to albumin. The last of these, the δ fraction, was though to be bilirubin covalently bonded to albumin. It was negligible in serum with only unconjugated bilirubin or with little conjugated bilirubin. The artifactual formation of the δ fraction during the manipulation of sera containing high concentrations of conjugated bilirubin cannot be ruled out. The

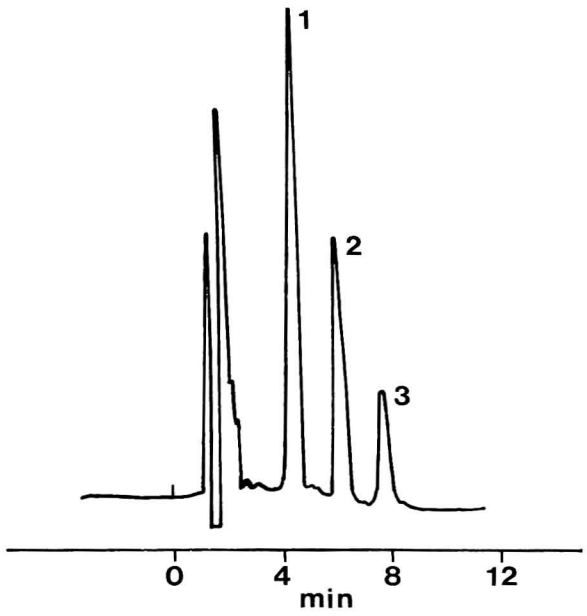

FIGURE 5. Separation of bilirubin monomethyl ester(1), bilirubin(2), and bilirubin dimethyl ester(3) extracted from serum of a patient after alkaline-methanolysis.
Column,SAS-Hypersil;mobile phase,acetonitrile-dimethyl sulphoxide-water(32.5:32.5:35);detection,430nm.

transesterification of such a serum usually leaves a slightly yellow precipitate, presumely containing the δ fraction. In these cases the alkaline methanolysis-HPLC procedure will certainly underestimate the total bilirubin content. The clinical significance of the δ fraction awaits futher investigation.

Bilirubin undergoes photochemical rearrangement when irradiated with UV light giving a mixture of geometric isomers, the photobilirubins (21-25). The presence of photobilirubin in serum of neonates undergoing phototherapy has been demonstrated by Onishi et al (24,25). The separation of bilirubin from photobilirubins is important clinically in monitoring the effectiveness of phototherapy since the spectrophotometric absorption of these compounds are similar and some photobilirubins are diazo positive. The direct spectrophotometric and the diazo methods will therefore give falsely high results for unconjugated bilirubin.

ANALYSIS OF BILIRUBIN IN AMNIOTIC FLUID

The analysis of bilirubin in amniotic fluid was a valuable method of detecting and preventing haemolytic disease in the newborn.

The alkaline methanolysis-HPLC procedure for plasma bilirubin (18) has been applied to the determination of bilirubin in human amniotic fluid (26). Unconjugated bilirubin was detected in the amniotic fluid from normal and rhesus-sensitized pregnancies. Conjugated bilirubin was only found occasionally in amniotic fluid from blood-group-incompatible pregnancies proving the antenatal conjugation of bilirubin by fetuses with excessive bilirubin production.

STUDY OF FAECAL BILE PIGMENTS

In haemolytic diseases the estimation of the daily excretion of faecal bile pigments has been used as an index of the severity of haemolysis. However, due to the complexity and instability of some of the "urobilins", faecal bile pigments are now rarely estimated.

The HPLC analysis of faecal urobilinoids on Hypersil eluted with acetonitrile-water-tetraethylenepentamine (85:15:0.05) showed that they were mixtures of i-stercobilin, half-stercobilin and d-or i-urobilin in various proportions (27). Each of these compounds has been isolated and converted into their dimethyl esters for further separation of the diastereoisomers by adsorption chromatography on Hypersil with heptane-methyl acetate-methanol containing 1% diethylamine (75:25:2) as eluent. Stercobilin dimethyl ester gave a single peak confirming previous observation (28) that it was entirely in the SS forms. Half- stercobilin and urobilin dimethyl esters were resolved into pairs of diasteroisomers, the RR,SS and the RS,SR forms. A mixture of "urobilin" dimethyl esters extracted from faeces will therefore separated into five peaks (Fig.6). The IIIα, IXα and XIIIα isomers of these compounds were not resolved by this system.

It has been shown that synthetic i-urobilin dimethyl ester was separated into pairs of IIIα, IXα and XIIIα diastereoisomers when μ-Porasil eluted with benzene-ethanol-diethylamine was used (6,7); natural stercobilin was separated into the SS forms of IIIα, IXα and XIIIα isomers. The superior resolution of this system

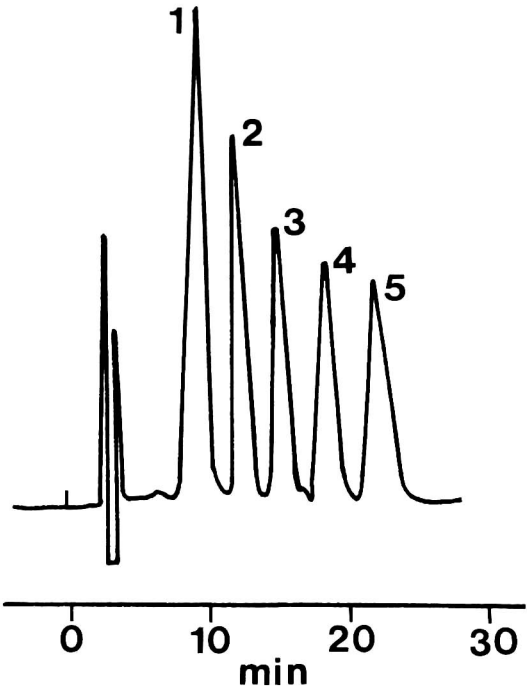

FIGURE 6. Separation of dimethyl esters of (SS)-stercobilin(1), (RR,SS)-half-stercobilin(2), (RR,SS)-urobilin(3), (RS,SR)-half-stercobilin(4), and (RS,SR)-urobilin(5). Column,Hypersil;mobile phase,heptane-methyl acetate-methanol containing 1% diethylamine(75:25:2);detection,490nm.

was unfortunately disadvantageous in actual applications as in samples containing a mixture of "urobilins" the increased number of peaks resulted in overlapping of some of the isomer peaks of stercobilin, half- stercobilin and urobilin. This severely limited its usefulness as a method for faecal bile pigment estimation because the molar absorptivitiy of stercobilin, half-stercobilin and urobilin are different. A complete separation is therefore essential. The molar absorptivities of the IIIα, IXα and XIIIα isomers of each compound, on the other hand, are very similar. Their separation is unimportant in faecal bile pigment determination. The heptane-methyl acetate- diethylamine system (6,7) is therefore recommended for the quantitative analysis of faecal bile pigments. A combination of the two systems, however, is necessary for the isolation of pure compounds for physico-chemical characterization.

COMPARISON OF HPLC SYSTEMS FOR BILE PIGMENT SEPARATION

It is well known that adsorption chromatography has the unique ability to obtain class separations and to resolve isomers. This is also true for bile pigment separation.

A complication in bile pigment chemistry is that naturally occurring IXα bile pigments such as bilirubin and urobilinogens are partially rearranged in acid solution to IIIα and XIIIα isomers (3,4). The ability to separate these isomers as dimethyl esters is invaluable in chemical synthesis and in the characterization of pigments isolated from natural sources. Silica is the only adsorbent used in the separation of bile pigment methyl esters. Chemically bonded normal phase packings like amino- and cyano-bonded silica have not been investigated. It is likely that they are equally good for the separation of bile pigment methyl esters and in addition may possess special selectivity for a particular group of pigments.

Reversed-phase chromatography is well suited for the separation of underivatised bile pigments but is generally unable to resolve the isomers except for bilirubin isomers. The best reversed-phase system for the resolution of bilirubin isomers is by use of a ternary solvent mixture (acetonitrile- dimethyl sulphoxide-water) on C1, C8 or C18 bonded silica (10). The separation of IIIα, IXα and XIIIα isomers is unimportant in clinical bile pigment analysis. Reversed-phase chromatography is therefore useful for the rapid group separation of bile pigments. The group separation of verdins, violins and urobilins, for example, is useful for the preparative isolation of bile pigments in haem turnover studies using labelled precursors.

Reversed-phase (19) and adsorption chromatography (18) have both been used for determination of conjugated and unconjugated bilirubin in serum. The reversed-phase isocratic system, which is simpler and faster, is preferred.

Reversed-phase ion-pair chromatography is the method of choice in the separation of bile pigments with conjugated groups including the azodipyrroles of conjugated bilirubin. It has been successfully applied to the analysis of conjugated bilirubin in bile (14-16) and to the estimation of the activity of UDP-glucuronyl transferase (16,17).

The normal phase separation of conjugated bilirubin on a μ-Bondapak carbohydrate column is possible (13) but efficiency and reproducibility are poor.

CONCLUSION

HPLC has become an important technique for bile pigment studies. In chemistry it is ideal for the separation and elucidation of structures, particularly for bile pigments with limited stability such as the photobilirubins. In biochemistry and physiology it provides a fast and sensitive method for the study of bile pigment function and metabolism. It has important applications in clinical chemistry and clinical medicine, allowing the identification and quantitation of conjugated and unconjugated bilirubin and differentiation of bilirubin from photobilirubin.

The use of HPLC for bile pigment analysis will no doubt be increased. One area not yet been fully exploited by HPLC is the study of plant bile pigments; another is the used of HPLC for optical resolution of the pigments. The study of conjugated bilirubin other than sugar conjugates will also be greatly facilitated by HPLC.

REFERENCES

1. Schoch,S.,Lempert,U.,Wieschhoff,H.,and Scheer,H., J.Chromatog.,,357,1978.

2. Rasmussen,R.D.,Yokoyama,W.H.,Blumenthal,S.G.,Bergstrom,D.E., and Ruebner,B.H.,Anal.Biochem.,101,66,1980.

3. McDonagh,A.F.,and Assisi,F., J.Chem.Soc.Chem.Commun.,3,117,1972.

4. Stoll,M.S., and Gray,C.H., Biochem.J.,163,59,1977.

5. Stoll,M.S., Lim,C.K. and Gray,C.H., in "High Pressure Liquid Chromatography in Clinical Chemistry", Dixon,P.F., Gray,C.H., Lim,C.K. and Stoll,M.S.,eds.,Academic Press, London, New York and San Francisco,97,1976.

6. Stoll,M.S.,Lim,C.K., and Gray,C.H., in "Chemistry and Physiology of Bile Pigments",Berk,P.D.,and Berlin,N.I.,eds.,U.S.Dept. of health, education and welfare, 483,1977.

7. Gray,C.H.,Lim,C.K.,Rideout,J.M.,Skacel,P.O., and Stoll,M.S., in "Current Development in the Clinical Applications of HPLC, GC and MS", Lawson,A.M.,Lim,C.K.,and Richmond,W.,eds.,Academic Press,London, New York,Toronto,Sydney, and San Francisco,75,1980.

8. Wooldridge,T.A.,and Lightner,D.A., J.Liq.Chromatog.,1,653,1978.

9. Onishi,S.,Itoh,S.,Kawade,N.,Isobe,K.,and Sugiyama,S., Biochem.Biophy.Res.Commun.,90,890,1979.

10. Lim,C.K.,Bull,R.V.A.,and Rideout,J.M., J.Chromatog.,204,219,1981.

11. Little,G.H., J.Chromatogr.,163,81,1979.

12. Onishi,S.,Itoh,S.,Kawade,N.,Isobe,K.,and Sugiyama,S., J.Chromatogr.,182,105,1980.

13. Lim,C.K., J.Liq.Chromatogr.,2,37,1979.

14. Jansen,P.L.M.,and Tangerman,A., J.Chromatogr.,182,100,1980.

15. Jansen,P.L.M., Clin.Chim.Acta,110,309,1981.

16. Onishi,S.,Itoh,S.,Kawade,N.,Isobe,K.,and Sugiyama,S., Biochem.J.,185,281,1980.

17. Chowdhury,J.R.,Chowhury,N.R.,Wu,G.,Shouval,R.,and Arias,I.M., Hepatology,1,1981.

18. Blanckaert,N.,Kabra,P.M.,Farina,F.A.,Stafford,B.E.,Morton,L.J., and Schmid,G.J.Lab.Clin.Med.,96,198,1980.

19. Bull,R.V.A.,and Lim,C.K., Anal.Proceedings, in press.

20. Lauff,J.J.,KasM.E.,and Ambrose,R.T., J.Cromatogr.,226,391,1981.

21. Lightner,D.A.,Wooldridge,T.A.,and McDonagh,A.F., Proc.Natl.Acad.Sci.,U.S.A.,76,29,1979.

22. McDonagh,A.F.,Lightner,D.A.,and Wooldridge,T.A., J.Chem.Soc.Chem.Commun.,110,1979.

23. Stoll,M.S.,Vicker,N.,Gray,C.H.,and Bonnett,R., Biochem.J.,201,179,1982.

24. Onishi,S.,Kawade,N.,Itoh,S.,Isobe,K.,and Sugiyama,S., Biochem.J.,190,527,1980.

25. Onishi,S.,Isobe,K.,Itoh,S.,Kawade,N.,and Sugiyama,S., Biochem.J.,190,533,1980.

26. Rosenthal,P.,Blanckaert,N.,Kabra,P.M.,and Thaler,M.M., Clin.Chem.,27,1704,1981.

27. Bull,R.V.A.,Lim,C.K.,and Gray,C.H., J.Chromatogr.,218,647,1981.

28. Brockmann Jr.,H.,Knoblock,G.,Plieninger,K.Ehl.,Ruppert,J., Moscowitz,A.,andWatson,C.J., Proc.Natl.Acad.Sci.,U.S.A.,68,2141,1971.

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OLIGOMER ANALYSIS BY HPLC AND GPC

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INTRODUCTION

When a substance, such as styrene, is polymerized, a mixture of oligomers is formed. Even though one can calculate an average molecular weight on the order of 2000, oligomers of much smaller molecular weight, some of them fairly small, are always found in the product. Because many physical properties change with the molecular weight, the properties of the polymer preparation may depend to an important extent on the percentages of the small oligomers that are present.

In some cases, low molecular weight oligomers are deliberately used in some of the newer coating systems that are required by government regulations (1,2). For example, in powder coatings, a low molecular weight polymer is reacted with an oligomeric cross-linking agent. Similarly, in UV-curable coatings, low molecular

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Wilmington, DE 19897

weight multi-functional oligomers are mixed with reactive monomers and a photosensitizer. In both cases, the molecular weight distribution of the mixture of oligomers used in the synthesis must be controlled carefully so as to obtain the desired mechanical properties in the finished coating. Because a small difference in the distribution of a low molecular weight oligomer represents greater relative changes in the molecular weight than in a high molecular weight polymer, the physical properties are much more sensitive to changes in distribution. In addition to properties such as mechanical strength and softening point, solubility as reflected by the leaching of smaller polymers by a liquid in contact with the plastic, can be a serious problem (3).

Before discussing the separations carried out by High Performance Liquid Chromatography (HPLC) and Gel Permeation Chromatography (GPC), it is important to note that other chromatographic systems have been applied to fractionations of oligomers. In an early example of oligomer fractionation using capillary columns and gas chromatography, silicone oligomers were shown to be separable by Averill (4). Although that separation was spectacularly good, gas chromatography is generally of limited applicability compared to HPLC due to restrictions related to volatility.

Supercritical Fluid Chromatography (SFC) is a technique that has been shown to overcome the problem of volatility. Ten years ago, Jentoft and Gauw (5) in a classic study, demonstrated that a 2100 molecular weight polystyrene sample could be fractionated into more that 25 oligomers in a relatively short period of time by programming the pressure to change the density of the mobile phase and, hence, the "solubility" of the partitioning species. More recently, Hartman, et al, (6) have shown that 45 species could be isolated by having a further refinement that the flow was main-

tained constant as the pressure increased, thereby gaining efficiency. The power of the technique was also demonstrated by Nieman and Rogers (7) who were able to fractionate a methylphenyl silicone into two series of oligomers, cyclic and linear. It was later shown (8), through the isolation of pure cyclic tetramer, pure silic pentamer, pure linear tetramer and pure linear pentamer that the partitioning behavior of volatile species into those phases was quite similar for three but markedly different for the cyclic pentamer. This served to emphasize that structure as well as molecular weight of the oligomer affects certain physical properties signficantly.

Finally, thin layer chromatography (TLC) is obviously applicable to oligomers having low volatility (9,10). In general, the application of TLC to mixtures of oligomers has been less widely applied than HPLC though high performance TLC may increase its use in the future.

GEL PERMEATION CHROMATOGRAPHY

GPC has an important advantage in that it can be performed relatively quickly. All compounds should, in theory, elute at or before the total permeation (internal) volume. If a column has been calibrated with standards of the same material or the hydrodynamic radius can be estimated, characterization of polymer preparations as a whole can be carried out easily. However, when fractionating a mixture of oligomers, one encounters the very limited peak capacity of GPC because of the limited elution range of the retained species. Although recycling (11) can be used to overcome this drawback to some extent, the improved separation is obtained at a cost of increased time for the analysis. Another way to extend the apparent peak capacity is to use larger columns,

thereby otaining larger permeation volumes and providing the opportunity for better resolution. The larger column volumes also tend to minimize the effects of dead volume, which further improves the resolution. However, the use of longer columns entails considerable increases in the amount of solvent and stationary phase used as well as increased analysis time. Another problem that may be encountered in GPC is the absorption of components by the stationary phase. This can be particularly important when using silica packings though it can be minimized by modifying the surface chemically.

High Performance GPC (HPGPC) (12,13) involves the use of rigid microparticles having narrow pore size distributions that are also well defined. The rigidity of the particles permits higher pressures to be used thereby insuring faster flow rates and rapid analysis times while still obtaining high resolution due to the small particle size. Cross linked organic polymers as well as porous silicas have been used as packings. Cross linked polystyrenes are used with organic solvents while crosslinked polyethers containing hydroxyl groups (TSK-type PW) (14) or porous silicas derivatized with hydrophilic functional groups (TSK-type SW) (15) are used with aqueous mobile phases Kato, et al, (16) have shown that type PW gel provides superior resolution for lower oligomers (MW 500) due to its smaller pore size relative to type SW. The increased column efficiency obtained with these newer packings has made HPGPC a practical method for the analysis of oligomer mixtures.

The fact that smaller (3-10 micron), rigid particles, are now available makes possible the use of micro-packed columns for GPC. Ishii, et al, (17) have shown that one can use injection volumes of 0.02 ul or less with a detector cell having a volume of 0.1 uL or

less. The small extra column volumes are necessary because of the relatively low retention of GPC as compared to HPLC. The technique was applied to polystyrene and polyepoxide oligomers. Promising results were obtained in that initial report.

Among the applications of High Performance GPC are those to fractionations of polyethylene glycols (18) on TSK type PW gel with water as the eluent as well as polystyrene, polyethylene glycols, PCR novolaks (phenol-formaldehyde polymers), and epoxy resins (19), all on cross linked polystryene gels. In the latter work, elution volumes and the gel capacity (pore volume divided by exclusion limit) for all samples except the epoxy resins were increased upon changing from THF to chloroform as the mobile phase. For the epoxy resins, the reverse was observed. These phenomena have been attributed to solute-solvent interactions increasing the apparent molecular size. In other HPGPC work, polyethylene terpthalate cyclic oligomers up to the pentamer (20) and polysulfones (21), specifically the cyclic dimer residue, have been separated on cross linked polystyrene gels using THF and chloroform, respectively, as eluents. Applications of HPGPC in the coatings industry have been reviewed by Kuo and Prouder (2).

The analysis of oligosaccharides is of considerable importance to the food and beverage industry. Size exclusion chromatography has long been used to fractionate such mixtures. One approach has been to use ion exchange resins. Kesler (22) and Lee (23) used anion exchange resins with a borate buffer. Fitt, et al, (24) used a cation exchange resin in the calcium form with water as the mobile phase. Separations of oligosaccarides from corn syrup up to degree of polymerigation (DP) 8 were achieved. Scobel and Brobst (25) later showed that cation exchange resins in the silver form gave much better results, resolving corn syrup oligosaccharides up

to DP 15. In all of these separations, size exclusion is the dominant mechanism as shown by the elution order which follows decreasing molecular size.

Gel filtration packings have also been used for oligosaccharide fractionations. John, et al, (26) separated glucose oligomers up to DP 13 on polyacrylamide gel. Brown (27) separated cellodextrins on both dextran and polyacrylamide gels. It was shown that both steric exclusion and solute gel interactions play a role in these separations.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC, like High Performance GPC, has benefited greatly by the use of small-diameter rigid particles which lead to highly efficient, rapid separations. The fact that different types of derivitized silica as well as conventional silica, alumina and other packings can be used contributes to the already broad selectivity that is available through the use of different solvents including mixed solvents. In the case of oligomers there is also a general elution problem which results from the broad range of solubilities within a given series of oligomers. Although limited success can be obtained using isocratic conditions, as Kirkland showed by separating 12 polystyrene oligomers (28), gradient elution, in which a changing composition forms the eluent, provides a convenient means for solving that problem. Snyder and Kirkland (29) have shown that convex gradients are preferred for optimum resolution of homologous series such as oligomers. Although flow programming has been used to speed up the fractionation of oligosaccharides (30), it appears to be of more limited applicability.

As indicated earlier, a series of oligomers can represent a very broad range of solubilities. If too "strong" a solvent is used for the sample, it can noticeably degrade the resolution (31,32). Clearly, the larger the volume of sample solution injected, the greater the effect.

However, it should be noted that the important feature is the difference in strength between the eluent and the sample solvent. Gradient elution reverse phase separations of polystyrene and epoxy oligomers by Curtis (32) and Shiono (33), respectively have shown no sample solvent effect. In both cases, the initial eluent had a rather high solvent strength. The closer the strength of the eluent to that of the sample solvent, the less the effect of using the strong solvent. Often a compromise must be reached between sample solubility and compatibility of the sample solvent with the initial mobile phase.

Many years ago, Billiet (34) showed when using conventional ion exchange for the fractionation of polyglycine oligomers, that the elution times increased with oligomer size only up to a certain point before size exclusion produced a reversal. The counterpart of this effect in HPLC has been reported by Van Der Maeden, et al, (30) who observed steric exclusion for higher oligomers when eluting polystyrene standards with THF on a C-18 column. The size exclusion effect is particularly noticeable on long chain reverse phase packings since derivatization with alkyl chains reduces the average pore size, the effect being greater with longer alkyl chains (35). While the use of packings having a larger average pore sizes will tend to overcome the size-exclusion effect, it should reach the point of diminishing returns due to the reduced surface area of packings with larger pore sizes.

A number of typical applications can be cited for the use of HPLC to fractionate oligomers. In one example, linear and cyclic PET (Polyethylene Terephthalate) oligomers were separated on silica using a hexane/chloroform gradient (36). Zaborsky (37) separated polyester prepolymer oligomers on silica using ethanol modified chloroform. Polycaprolactam oligomers have been fractionated using silica in combination with butanol, acetic acid, and water (38). Shiono, et al, (33) have separated oligomers of epoxy by-products on an octadecyl silica packing, with an acetonitrile/ water gradient using acetonitrile/tetrahydroforan as the sample solvent. Van Der Maeden, et al, (31) have separated novolaks using tetrahydrofuran/water, and poly (2,6-diphenyl-p-phenylene oxide) oligomers using dioxane/water, both on an octadecyl bonded phase as well as ethoxylated octylphenols using a hexane/THF gradient on an amino bonded phase. Molnar and Horvath (39) have separated alanine oligomers using an aqueous phosphate buffer and phenylalanine oligomers using a water/acetonitrile gradient containing perchloric acid, both on octadecylsilica.

HPLC has also been applied to the analysis of oligosaccharide mixtures. Amino bonded phase columns using acetonitrile/water eluents have been used by Linden and Lawhead (40) to fractionate maltose oligomers of up to DP 8 and by Schwarzenbach (41) to resolve dextrose oligomers of up to DP 7. Rabel and coworkers (42) obtained excellent separations of oligosaccharide of up to DP 10 using an amino/cyano mixed bonded phase column with an acetonitrile/ acetate buffer eluent. Aitzetmuller (43,44) has shown that ordinary silica columns can be used for oligosaccharide separations by adding a polyfunctional amine modifier to the mobile phase.

Chetham, et al, (45) have recently shown the utility of a new reverse phase packing for oligosaccharide analysis using a commer-

cially made octadecylsilica designed specifically for carbohydrate separations. This material uses water as the mobile phase and thus avoids the solubility problems of higher oligosaccharides in acetonitrile/water mixtures. It is also free from the column degradation associated with amino bonded phase columns (43). This reverse phase technique also resolves anomers having of the same degree of polymerization.

Finally, it is important to note that isomers of synthetic polymeric species having the same degree of oligomerization have been resolved by two different groups. Scheuing (45) separated isomers of polyester oligomers using a C-8 derivitized silica packing in conjunction with acetonitrile/water gradient at 70^{o}C. Van Der Maeden, et al, (30) showed an even more spectacular fractionation involving the isomers produced by O-cresol and formaldehyde using an octodecyl derivatized silica and a tetrahydrofuran/water gradient. In these cases, there is no question that the larger peak capacity and greater selectivity of HPLC compared to high performance GPC is advantageous.

CONCLUSION

The studies reported in this review were selected as representative ones that should serve to convince the reader that High Performance Liquid Chromatography and, to a lesser extent High Performance Gel Permeation Chromatography, are capable of performing fractionations of oligomers for a variety of species. This review was not intended to be exhaustive.

REFERENCES

1. Chemical Week, 1981, 129, 64-67.
2. Kuo, C. Y.; Prouder, T. "High Performance Gel Permeation Chromatography Characterization of Oligomers Used in Coatings Systems." ACS Symposium series no. 138:Size Exclusion Chromatography (GPC), 1980, 207-204.
3. Shiono, S., Anal. Chem., 1979, 51, 2380-23.
4. Averill, W., Analabs Research Notes, 1972, 12, 7.
5. Jentoft, R. E.,; Gauw, T. H., J. Polym. Sci. B., 1969, 7, 811-813.
6. Klesper, E.; Hartmann, W., Eur. Polym. J., 1978, 14, 77-88.
7. Nieman, J. A.; Rogers, L. B., Sep. Sci., 1975, 10, 517-545.
8. Van Lenten, F. J.; Conaway, J. E.; Rogers, L. B., Spe. Sci., 1977, 12, 1-28.
9. Belenky, B. G.; Valchekhena, M.D.; Vakhtina, I. A.; Gankina, E. S., J. Chromatogr., 1976, 129, 115-124.
10. Belenky, G. B.; Gankina, E. S., J. Chromatogr., 1977, 141, 13-90.
11. Karger, B. L.; Snyder, L. R.; Horvath, C. "An Introduction to Separation Science," Wiley Interscience, New York, NY, 1973, Chapter 15.
12. Kirkland, J. J.; Antle, P.E., J. Chrom. Sci., 1977, 15, 137-146.
13. Mori, S. J. Appl. Poly. Sci., 1977, 21, 1921-1932.
14. Hashemoto, T.; Sasaki, H.; Aiura, M.; Kato, Y., J. Polym. Sci. Polym. Phys. Ed., 1978, 16, 1789-1800.
15. Kato, Y.; Kido, S.; Manabu, Y.; Hashimoto, T., J. Polym. Sci. Part C, 1974, 12, 1339-1345.
16. Kato, Y.; Komiya, L.; Hiroo, S.; Hashimoto, T., J. Chromatogr. 1980, 193, 311-315.
17. Ishii, D.; Hibi, K.; Asai, K.; Jonokuchi, T., J. Chromatogr. 1978, 151, 147-154.
18. Kato, Y.; Sasaki, H.; Aivra, M.; Hashimoto, T., J. Chromatogr. 1978, 153, 546-549.
19. Mori, S.; Yamakawa, A., J. Liquid Chrom., 1980, 3, 329-342.
20. Shiono, S., J. Polym. Sci. Polym. Chem. Ed., 1979, 17, 4123-4127.
21. Brydia, L. E.; Garty, O. M., J. Polym. Sci. Polym. Chem. Ed., 1980, 18, 1577-1586.

22. Kesler, R. B., Anal. Chem., 1967, 39, 1416-1422.

23. Lee, F. C., Anal. Brochem., 1971, 43, 640-643.

24. Fitt, L. E.; Hassler, W.; Just, D. E., J. Chromatogr., 1980, 187, 381-389.

25. Scobell, H. D.; Brobst, K. M., J. Chromatogr., 1981, 212, 51-64.

26. John, M.; Trenel, G.; Dellweg, H., j. Chromatogr., 1969, 476-484.

27. Brown, W., J. Chromatogr., 1970, 52, 273-284.

28. Kirkland, J. J., Chromatographia, 1975, 8, 661-668.

29. Snyder, L. R.; Kirkland, J. J., "Introduction to Modern Liquid Chromatography," 2nd ed. Wiley Interscience: New York, NY, 1979, Chapter 16.

30. "Rapid and Reliable Analysis of Beverages," Brochure N67, Waters Associates, Milford, MA, 1976.

31. Van Der Meaden, F.P.B.; Biemond, M. E. F.; Janssen, P. C. M., J. Chromatogr., 1978, 149, 539-552.

32. Curtis, M. A.; Webb, J. W.; Warren, D. C.; Brandt, V. O.; Gerberich, F. G.; Raut, K. B.; Rogers, L. B., Separ. Sci. Technol., 1980, 15, 1413-1428.

33. Shiono, S.; Karino, I; Ishimura, A.; Enomoto, J., J. Chromatogr., 1980, 193, 243-253.

34. Billiet, H. A., Chromatographia, 1972, 5, 275-277.

35. Halasz, I., Anal. Chem., 1980, 52, 1393A-1403A.

36. Hudgins, W. R.; Theurer, K.; Mariani, T., J. Appl. Poly. Sci. 1978, 34, 144-155.

37. Zaborsky, L. M., Anal. Chem., 1977, 49, 1166-1168.

38. Brodilova, J.; Rotschova, J.; Pospisil, J., J. Chromatogr., 1979, 168, 530-532.

39. Molnar, I.; Horvath, C., J. Chromatogr., 1977, 142, 623-640.

40. Linden, J. C.; Lawhead, C. L., J. Chromatogr., 1975, 105, 125-133.

41. Schwarzenbach, R., J. Chromatogr., 1976, 117, 206-210.

42. Rabel, F. M.; Caputo, A. G.; Butts, E. T., J. Chromatogr., 1976, 126, 731-740.

43. Aitzetmuller, K., J. Chromatogr., 1978, 156, 354-358.

44. Aitzetmuller, K., Jour. High. Res. Chrom. and Chrom. Comm., 1979, 9, 589-590.

45. Cheetham, N. W.; Sirimanne, P.; Day, W. R., J. Chromatogr., 1981, 207, 439-444.

46. Scheuing, D. R., Spectra Physics Chromatography Review, 1980, 6, 4-6.

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LC NEWS

LIQUID CHROMATOGRAPH/MASS SPECTROMETER INTERFACE continuously concentrates the effluent from a conventional liquid chromatograph and delivers the concentrated solution into the mass spectrometer. Combination with a mass spectrometer/data system provides capability for analysis of complex and intractable biological, environmental, and petrochemical samples. Extranuclear Laboratories, Inc., JLC/82/II, P. O. Box 11512, Pittsburgh, PA, 15238, USA.

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HIGH PRESSURE FLUID CELLS for UV monitors are rated at 1000 psi back pressure and incorporate enhanced chromatographic flow characteristics to optimize the plate count in microparticulate columns without sacrificing detector sensitivity. The cells also improve bubble clearing and allow additional detectors to be added downstream without excessive band spreading. LDC/Milton Roy Co., JLC/82/II, P. O. Box 10235, Riviera Beach, CA, 33404, USA.

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LC CALENDAR

1982

OCTOBER 4-6: Capillary Chromatography '82: International Symposium, Tarrytown, NY. Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX, 77004, USA.

OCTOBER 10 - 13: 21st Annual Mtg. of ASTM Committee E-19 on the Practice of Chromatography, Marriott Hotel, New Orleans. Contact Mr K. Riley, ASTM Headquarters, 1916 Race Street, Philadelphia, PA, 19103, USA.

OCTOBER 12 - 14: 3rd International Symposium On Chlorinated Dioxins and Related Compounds, International Congress Center, Salzburg, Austria. Contact: Prof. O. Hutzinger, University of Amsterdam, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands.

OCTOBER 14: Ninth ANACHEM Symposium, sponsored by The Association of Analytical Chemists, Dearborn Inn, Dearborn, MI, USA. Contact: J. W. Auld, Detroit Edison Co., Detroit, MI, 48226, USA.

OCTOBER 14 - 15: "New Perspectives in Racemic Coumpound Separation" sponsored by CNR-PF Chimica Fine e Secondaria, Societa Chimica Italiana, and Universita degli Studi di Roma, in Rome, Italy. Contact: Prof. Domenico Misiti, Inst. di Chimica Organica, Via del Castro Laurenziano 9, 00161 Roma, Italy.

OCTOBER 19 - 20: short Course on LC/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postal 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

OCTOBER 21 - 22: 2nd Workshop On LC/MS and MS/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l. Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postale 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

NOVEMBER 2 - 5: 1st Inter-American Congress in Forensic Medicine and Sciences, Pan-American Assoc. of Forensic Sci., Sacramento, CA. Contact: John D. DeHaan, Calif. Department of Justice Lab. Box 13337, Sacramento, CA 95813,USA.

NOVEMBER 11 - 14: Applied Seminar for the Association of Clincial Scientists, Chicago, IL. Contact: Dr. F. M. Sunderman, Jr., Dept. of Lab. Medicine, Univ. of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06032.

NOVEMBER 16 - 18: Medical and Laboratory Instrumentation Soc. Annual Int'l. Congress and Exhibition, Sheraton-Washington Hotel, Washington, DC. Contact: John Wolf, MLIS, 11310 Palisades Court, Kensington, MD, 20895, USA.

NOVEMBER 17 - 19: Eastern Analytical Symposium, Statler-Hilton Hotel, New York. Contact: Dr. H. Issag, Frederick Cancer Res. Facility, P.O. Box B, Frederick, MD, 21701, USA, or Dr. D. Strumeyer, Rutgers University, Chem. Dept, New Brunswick, NJ 08903.

DECEMBER 6 - 8: 3rd Biennial TLC Symposium-Advances in TLC, Hilton Hotel, Parsippany, NJ. Contact: J.C. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, 19104, USA.

1983

MARCH 20 - 25: National Amer. Chem. Soc. Meeting, Seattle, WA, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

MAY 2 - 6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17 - 23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BV, United Kingdon.

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OCTOBER 1 - 5: 15th International Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of

The Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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PRESS RELEASE FOR DR. MAURITS DEKKER

FOR IMMEDIATE RELEASE

Maurits Dekker, Ph.D., chairman of the board of Marcel Dekker, Inc., received the honorary degree of Doctor of Science from Polytechnic Institute on June 3, 1982 at ceremonies held at Carnegie Hall.

Dr. Dekker has spent all of his professional life - 59 years - in scientific and technical publishing. After his graduation in 1923 from the University of Amsterdam with a doctoral degree in chemistry, physics and microbiology, he joined a small scientific publishing firm. Five years later he founded Dekker and Nordemann in Amsterdam, specializing in translating and publishing works of the European "giants of science" for the international market.

In 1939 the Dekker family sailed for the United States. Dr. Dekker has gained unprecedented achievements in the field of technical and scholarly publishing, including the presidency of Interscience Publishers, Inc. Yet, his work at Marcel Dekker, Inc., as chairman of the board since 1966, has clearly been his highest professional distinction.

While at Interscience, Dr. Dekker collaborated with Dr. Ray Kirk, dean of the Graduate School at Brooklyn Polytechnic, for seven years, to bring forth "The Encyclopedia of Chemical Technology," edited by Kirk-Othmer. This monumental reference work, regarded as a classic in its field, is now in its third edition.

Dr. Guy Donaruma, provost of Polytechnic Institute, cites Dr. Dekker's lifetime contribution to scientific publishing, and particularly the renown which the Encyclopedia has earned, as the criteria for bestowing the honorary degree. He said, "Dr. Dekker's reputation in the field of scientific publishing and his long and close association with Polytechnic will be acknowledged by this scholarly award."

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(Chromatographic Science Series, Volume 16)

edited by
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Steroid Analysis by HPLC: Recent Applications is a laboratory guide for clinical researchers, pharmaceutical manufacturers, food and drug analysts, and steroid chemists who need to be able to perform successful separations and analyses of steroids and related compounds. This manual is written by leading chromatographers in the field who describe in precise detail the methodologies used in their laboratories. Scientists involved in the separation, isolation, or quantitation of steroids should take immediate advantage of the instructive material contained in this authoritative workbook.

Steroids are difficult compounds to isolate and analyze. The widespread use of HPLC for purifying and analyzing steroids is a recent development. *Steroid Analysis by HPLC: Recent Applications* covers the two major uses of HPLC in steroid separation and analysis: the analysis of specific steroid groups; and the utilization of HPLC in solving analytical problems common to all steroid-like compounds. Concise methods for the separation and analysis of bile acids, cardiac glycosides, progestins, adrenal and testicular steroids estrogens, D vitamins, steroid epimers, and hormones found in feed and food are presented. Complete procedures for the HPLC of sterol intermediates in cholesterol biosynthesis and the study of enzymatic steroid reactions are also included. Flow sheets and typical chromatogram scans accompany the articles. An appendix identifying various types of column packing will be especially helpful to scientists unfamiliar with the new column nomenclature.

The use of HPLC has developed rapidly over the past few years. This collection of papers by practicing chromatographers presents tested approaches to problems in steroid separation and quantitation. With *Steroid Analysis by HPLC: Recent Applications*, analytical chemists, clinical analysts, endocrinologists, organic chemists, and biochemists have at their disposal a practical, concise guide for successful isolation and analysis of steroids.

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