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PROGRESS IN FIELD-FLOW FRACTIONATION: THEORY, METHODOLOGY AND APPLICATIONS

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INTRODUCTION

The history of Field-Flow Fractionation (FFF) began in 1966 when Giddings (1) described a new separation concept based on a coupling of solute concentration and fluid flow nonuniformities inside a narrow channel, which cause the differential migration of the solute species and thus the separation. Physical or chemical lateral field acting across the channel composed usually of two planparallel walls (e.g., temperature gradient, electrical, magnetic or gravitational forces, chemical potential gradient etc.), interacts with molecules or particles of the solute and compresses them to one of the channel walls in the direction of x-axis, perpendicular to this wall. This concentration gradient induces a diffusion flux in the reverse direction. After certain time a steady state is reached and the distribution of the solute across the channel can be characterized by a mean layer thickness l. The velocity of the flow of the fluid inside the

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channel also varies in the direction across the channel. This velocity profile is caused by viscosity effects, accompanying the flow processes. At a laminar isothermal flow of a Newtonian fluid along a narrow channel, usually a parabolic velocity profile is formed inside the channel. The molecules or the particles of the solute are transported in the direction of the longitudinal axis of the channel at varying velocities, depending on the distance from the channel walls, in which they occur.

FFF is similar to chromatography in many aspects. However, all of the processes associated with the separation take place in the fluid phase and there is no stationary phase which would play an active role. This simpleness is, however, characteristic of classical FFF only and not of its combination with the chromatographic technique, e.g., with the use of a channel packed with a chromatographic bed. This is why FFF is sometimes classified as a one-phase chromatography (2-6) or polarization chromatography (7). The absence of the stationary phase that has a large surface area can be of fundamental significance for fractionations of the materials of biological origin. These materials often are very sensitive to a type and intensity of interactions with active surfaces of the packings of chromatographic columns and on contact with large surface areas of these packings they can denaturate in an undesirable way. The total surface area of the FFF channel plays no active part in separation process and is lower by several orders of magnitude than active surface area of the chromatographic column with a comparable separation capacity. Moreover, it can be deactivated so that undesirable interactions with the substances under separation are suppressed to the minimum. The strength of the

physical field applied can be varied within a very wide range continuously, starting from very fine up to very strong fields that induce intensive transversal migration of substances under separation. Consequently, the range of retentions is fairly variable, theoretically from zero retention, determined by a mere passage of the solute through the channel, to a total retention when the solute is completely compressed to the channel wall. The retention in the range from zero to the retention determined by a ten up to approximately fifty-fold of the void volume of the channel comes into account in practice. A simple geometry of the fractionation channel, permitting a lucid mathematical description of the separation process, also belongs to the advantages of FFF.

Giddings (1) assumed the possibilities of programming the field strength, programming the flow velocity, forming the shape of the velocity profile by varying the geometric characteristics of the channel, using casually a channel packing and thus combining the separation based on the above concept with the chromatographic technique, and applying the gases and liquids as the fluids, etc. So far only some of these ideas have been verified experimentally in practice and associated problems have been solved to rather a limited extent even from the theoretical viewpoint.

FFF is important methodology for fractionation and separation of macromolecules (8) within a wide range of molecular weights, starting from several thousands of daltons up to 10^{12} and even perhaps to 10^{15} of daltons (9) even for particles in submicron and micron range and for organized structures, such as cells and microorganisms etc. (4,10-13).

None of the chromatographic techniques nowadays posesses such a flexibility.

Numerous reviews (13-19) appeared in the last years. Each latest one brings up, together with a historical overview, some reports concerning the newest findings and developments. This also demonstrates a revolutionary growth of the FFF methodology and technology.

THEORY

Retention

Nonequilibrium theory of FFF (20) was elaborated on the principles parallel to the nonequilibrium theory of chromatography (21). The solute is displaced in a moving fluid by a combined action of the flow and the field applied. Velocity vector ν can be decomposed into two perpendicular vectors: U, induced by the applied field and flow induced component v

$$\nu = U + v \tag{1}$$

The flux of the solute j can also be written as a sum of the axial component along the longitudinal axis of the channel z and the lateral component in the direction of the x-axis

$$j = j_x + j_z \tag{2}$$

$$j_x = -D_x \, (dc/dx) + Uc \tag{3}$$

$$j_z = -D_z \, (dc/dz) + vc \tag{4}$$

where $D_{x,z}$ are the diffusion coefficients in the

direction of the corresponding axes, c is the concentration. It follows that

$$dc/dt = -\nabla j = -(\nabla j_x + \nabla j_z) \quad (5)$$

where ∇ is a gradient operator. As long as no axial flow occurs in the system, the concentration gradient induced by the field will be balanced by the diffusion, which will lead to a steady state or to a quasiequilibrium concentration, c'. As soon as the flow starts its action, the quasiequilibrium will permanently be disturbed. The deviation from the equilibrium can be characterized by the coefficient ε

$$c = c' (1 + \varepsilon) \quad (6)$$

It holds for the lateral flux at equilibrium

$$D_x (dc/dx) - Uc' = 0 \quad (7)$$

By solving this differential equation we obtain the distribution of solute concentration across the channel

$$c'(x) = c'_o \exp(xU/D) \quad (8)$$

In the present coordinate system, x designates the distance from the wall at which the solute accumulates, U is then a negative magnitude. Equation (8) can thus be rewritten

$$c'(x) = c'_o \exp(-x|U|/D) \quad (9)$$

Constant c'_o is the solute concentration at the

coordinate $x = 0$. On defining $l = D/|U|$, Equation (9) can be written as

$$c'(x) = c'_o \exp(-x/l) \qquad (10)$$

where l is the mean layer thickness defined above. The average velocity of the zone in the axial direction z is given by

$$V = \langle c'(x) \cdot v(x) \rangle / \langle c'(x) \rangle \qquad (11)$$

where $v(x)$ is the actual velocity of the streamline at the coordinate x, brackets $\langle\ \rangle$ designate the average values. Retention ratio R is then defined by

$$R = V/\langle v(x) \rangle = \langle c'(x) \cdot v(x) \rangle / \langle c'(x) \rangle \langle v(x) \rangle \qquad (12)$$

For the isothermal, isoviscous flow of a Newtonian liquid between the two parallel infinite planes that is not affected by any outer field, it applies that

$$v(x) = \Delta P x(w-x)/2L\mu \qquad (13)$$

where ΔP is the pressure drop along the channel of the length L, the thickness w is the distance between the walls of the channel, and μ is the viscosity of the fluid. For the average velocity it holds

$$\langle v(x) \rangle = \Delta P w^2/12\,L\mu \qquad (14)$$

The solution of the Equation (12) gives (22)

$$R = 6\lambda\left[\coth(2\lambda)^{-1} - 2\lambda\right] \qquad (15)$$

where $\lambda = l/w$. This equation is a basic theoretical relationship that describes quantitatively the

retention in FFF. In the limit when λ approaches to zero it holds that

$$\lim_{\lambda \longrightarrow 0} R = 6\lambda \qquad (16)$$

In a number of practical applications of FFF the approximation given by Equation (16) is fully justified. Hence it can be seen that the relationship between R and λ is very simple. For even a better illustration of the physical meaning of λ , it can be written (4)

$$U = F/f \qquad \text{and} \qquad D = R^{o}\,T/f \qquad (17)$$

and hence

$$\lambda = R^{o}T/Fw \qquad (18)$$

where F is the effective field force acting on a mole of the solute, f is the friction coefficient, R^{o} is the gas constant, and T is the absolute temperature. Equation (18) demonstrates that λ is the ratio of the thermal energy and the energy related to the effect of the field.

Zone spreading

Zone spreading is characterized quantitatively by the height equivalent to a theoretical plate (22)

$$H = \zeta^{2}/L = 2D/R<v(x)> + \chi w^{2}<v(x)>/D + \Sigma H_{i} \qquad (19)$$

where ζ is the standard deviation of the concentration zone at the end of the channel and χ is a dimensionless parameter. The first term in Equation (19) describes the longitudinal diffusion, the second one the nonequilibrium effects and the third one the sum of different contributions following from

relaxation processes, finite width of the injection etc. Equation (19) is analogous to the description of chromatographic processes. However, with respect to the character of FFF a term corresponding to eddy diffusion in the classical packed chromatographic column is not included in the above equation. The term that describes nonequilibrium processes (23), derived theoretically (20) is the most significant contribution to the total value of H in Equation (19). As follows from the nonequilibrium theory (20,22,23), the dimensionless parameter χ is expressed

$$\chi = 2D \langle c'(x).\varepsilon.v(x)\rangle / [\langle c'(x).v(x)\rangle w^2.\langle v(x)\rangle.(d\ln c'/dz)] \quad (20)$$

The complex relationship for χ was discussed in detail (23). In the limit when λ tends to zero, it holds simply (22)

$$\lim_{\lambda \to 0} \chi = 24\,\lambda^3 \quad (21)$$

if λ tends to infinity, then it holds (23)

$$\lim_{\lambda \to \infty} \chi = 1/105 \quad (22)$$

Relaxation

Immediately after the injection of the sample into the channel, the solute is distributed across the channel homogeneously. Only due to the action of the field does the concentration gradient start its formation until the steady state is reached. The time period till the establishment of the quasi-equilibrium is called the relaxation time. Relaxation

processes contribute to zone spreading according to the relationship (12,22)

$$H_r = (17\ n/140\ L)\ (\lambda w^2 <v(x)>/\ D)^2 \qquad (23)$$

where n is an effective number of relaxation processes along the channel, usually equal to one. While increasing the total zone spreading undesirably, this contribution (often negligible) can be eliminated by stopping the flow through the channel after the injection for the time period that makes it possible to obtain the quasiequilibrium.

Relaxation processes occurring after the injection of the solute obviously affect also the retention. If the relaxation time of the solute, t_r, is defined as the time necessary for overcoming the distance between the centre of the channel and the centre of gravity of the quasiequilibrium zone, then the relationship between the apparent retention ratio, R′, and theoretical R, i.e., not including the relaxation processes, is given by (22)

$$1/R' = 1/R - n<v(x)>t_r(1-R)/RL \qquad (24)$$

and the relaxation time is given by (22)

$$t_r = \frac{w^2\lambda}{D} \left[\frac{1}{2} - \lambda + (\exp(1/\lambda) - 1)^{-1}\right] \qquad (25)$$

The choice of the above relaxation distance is rather arbitrary. In practice, it depends also on the arrangement of the given FFF experiment. Actually, in another paper by Giddings and co-workers (24), this distance is defined as the whole thickness of the channel w, and consequently t_r is expressed as

$$t_r = \frac{w^2\lambda}{D} \qquad (26)$$

by neglecting the second and the third terms in rectangular brackets of the right hand side of Equation (25). In fact, each of the above simple equations is only an approximation of the real situations after the injection of the sample into the channel. Kirkland and co-workers (25) investigated the time necessary to obtain the retentions not influenced by relaxation when using the stop-flow technique. Their experimental results indicated much longer times than expected on the basis of simple equations.

Quite obviously, an additional study, both theoretical and experimental, is needed to explain these differences.

Resolution and peak capacity

The resolution R_s of two solutes 1 and 2 has been defined by the known equation

$$R_s = \frac{|2(t_{R1} - t_{R2})|}{t_{W1} + t_{W2}} \qquad (27)$$

where t_{Ri} are the retention times of solutes 1 and 2 and t_{Wi} are the widths of the elution curves of solutes 1 and 2 expressed in time units. Alternatively, an expression in retention volume units instead of time t, can be used in Equation (27). An almost complete separation is obtained when $R_s = 1$ (ca. 95 % separation for Gaussian elution curves). It also holds for Gaussian elution curves that $W = 4\zeta$ (W being the width of the elution curve expressed consistently in the same units as ζ). By using the definition Equation (27) and the definition equations for retention and zone spreading (e.g. Equations (12) and (19)) Martin and Jaulmes (26) expressed the retention R_s as

$$R_s = \frac{\left| \frac{1}{R_1} - \frac{1}{R_2} \right|}{2 \left(\frac{1}{R_1\sqrt{N_1}} + \frac{1}{R_2\sqrt{N_2}} \right)} \tag{28}$$

where N_i is the number of theoretical plates for solutes 1 and 2 calculated simply from

$$N_i = L/H_i \tag{29}$$

The peak capacity is defined as the maximum number of components which can be resolved, usually at unit resolution. It depends on a large number of experimental variables and solute characteristics. In order to generalize the conclusions concerning the peak capacity of various FFF techniques, Martin and Jaulmes (26) used several dimensionless parameters such as the reduced plate height h, the reduced velocity ν, and the reduced channel length ξ

$$h = H/w, \qquad \nu = \langle v(x) \rangle w/D, \qquad \xi = L/w \tag{30}$$

The use of the reduced parameters leads to the following relationships

$$N = \xi/h \tag{31}$$

and

$$h = \frac{2}{R\,\nu} + \chi\nu \tag{32}$$

In Equation (32), which follows from Equation (19), the contribution due to relaxation and extra-channel zone spreading is neglected.

Martin and Jaulmes (26) used the above relationships and a set of typical values characterizing the parameters of actual FFF experiments and analyzed the

peak capacity of some of the FFF techniques with respect to the maximum accessible retention volume, the channel length, eluant velocity, detectability, and analysis time. The maximum accessible retention volume comes from the fact that at a certain point the solute cannot approach closer to the wall and the normal order of elution is disturbed by this steric effect. The details will be discussed below. The theoretical analysis (26) indicated that while the maximum peak capacity increases with increasing field strength, the size of solute which is retained before the occurence of the steric effect, decreases. Consequently, the maximum retention volume under typical conditions is about 24-100 times the channel void volume. The variation of the peak capacity with retention volume for a constant plate number is faster in FFF than in chromatography (26).

The peak capacity as well as resolution is proportional to the square root of the reduced channel length ξ, and decreases with increasing reduced eluant velocity in the practical retentions range.

Under the typical experimental conditions the peak capacity is not limited by detectability because the concentration of the solute at the maximum of the elution curve either increases or remains approximately constant with increasing retention volume (26). Providing the time allowed for the fractionation is limited, the highest peak capacity is obtained at the maximum retention volume.

All of the results of the theoretical analysis by Martin and Jaulmes (26) are, however, limited by the validity of Equations (15) and (19). It follows from the practical experience (26) as well as from the theoretical analysis (27) that some deviations have to be taken into consideration.

Optimization of FFF

By analyzing Equation (19) it was found (28) that the minimal obtainable value of H, i.e., the maximal efficiency with regard to the flow-rate, is given by an approximative relationship

$$H_{min} \doteq R\ w \tag{33}$$

and the optimal flow-rate corresponding to this efficiency is

$$\langle v(x) \rangle_{opt} = \sqrt{18}\ .\ D/R^2 w \tag{34}$$

The above relationships show that H_{min} decreases as the retention increases, i.e., with a decreasing retention ratio R, and hence the best resolution will be obtained for solutes that are retained most. For these solutes the optimal flow-rates will be the highest ones. It follows from the above that flow programming, i.e., a gradual increase of the flow rate, would make it possible on the one hand to work in the range of the optimal parameters permanently and to decrease the time of the analysis on the other. The speed of the FFF analysis can be expressed as the maximal number of theoretical plates generated per unit of time (28)

$$\dot{N}_{max} = D/4w^2\lambda^2 \tag{35}$$

It is obviously desirable to minimalize the values of w and λ, i.e., to increase the field strength and to bring up D to the maximum.

Deviations from idealized FFF model

All the theoretical relationships described above were derived considering some assumptions and

simplifications. Firstly, it was expected that the concentration profile is fully developed during elution. Secondly, the channel was considered to be formed between two infinite parallel planes (i.e., its width being infinite) but in reality it has finite all three dimensions.

Krishnamurthy and Subramanian published an exact theoretical analysis of FFF (29), based on their generalized dispersion theory. They formulated a model of FFF for a two-dimensional system with transverse flow of solute between parallel planes. It means that the effect of the side walls was neglected. Their results can be used to describe the zone spreading as well as the retention for all values of time since the injection of the solute into the channel. For the large values of time the results asymptotically approach those described by previous equations of the nonequilibrium theory by Giddings. The solution of Krishnamurthy and Subramanian (29) makes it possible to explain some experimental artefacts in detail. These artefacts could not be explained by means of the nonequilibrium theory. Perhaps the most important discrepancy between the nonequilibrium theory and the experimental data is that the zone spreading that is observed is considerably larger than the predicted one. Similar results were obtained by Doshi and Gill (30).

Jayaraj and Subramanian (31) further extended the original analysis (29) by a detailed theoretical study of relaxation phenomena in FFF. Using a numerical solution, they were able to model the processes occurring in the FFF channel in various phases of the development. According to this model the zones of the high concentration are situated in the vicinity of the channel centre, however, also in

the vicinity of its walls. In the vicinity of the channel centre the velocity gradient is low and thus axial dispersion is minimal. Although the velocity gradient is high in the vicinity of the wall, the actual velocity is very low and for this reason the axial dispersion is also relatively low. As a result, the concentration distributions in various cross-sections of the channel are rather complex functions. The results of this work (31) demonstrated a complex dependence of the concentration profiles across the channel on the axial coordinate during the relaxation.

Later on, Takahashi and Gill (32) analyzed quantitatively the problem of retention and dispersion in rectangular channel of finite dimensions. They found out that both the retention and dispersion are the functions of the aspect ratio a, that is, of the ratio of the width to the thickness of the channel. The higher is a, the closer is R to the asymptotic value obtained by neglecting side walls effect. On the other hand, the dispersion is allways higher in a real rectangular channel than in hypothetical one without wall effect. The comparison of the theoretical analysis with experiments confirmed the good agreement.

Janča (33) discussed the influence of various factors, like some experimental variables and the channel design, influencing the retention R, the dispersion characterized by χ, and the resolution R_s. The ratio R_{true}/R_{∞} (where R_{true} and R_{∞} are the retention ratios in real and hypothetical channel, respectively) approaches to unity with increasing a value. From the practical point of view when $a \geq 20$ there is no substantial difference between R_{true} and R_{∞}. The ratio $(\chi_{true}/\chi_{\infty})_{opt}$ derived for optimal flow velocity $\langle v(x) \rangle_{opt}$ varies within

1.7 to 71 for the practically exploited region of the λ values ($\lambda = 0.1$ to 0.01). For the velocities higher than $\langle v(x) \rangle_{opt}$, the ratio (X_{true}/X_{∞}) is higher. Also the influence of extra channel dispersion was discussed (33) with respect to the minimum attainable total zone width under the real conditions of a rectangular channel of finite dimensions. It is advantageous to operate in the region of higher retention values as in this case relatively larger volumes of solute solutions can be injected (33). The relative percentual distinguishable difference $(\Delta\lambda/\lambda)x100$ calculated for $R_s=1$ is lower in the case of a real rectangular channel than it is in the hypothetical case. However, relative resolution increases with the decreasing value of λ. Consequently, the required resolution can be achieved in higher retentions region.

Rigorous theory undoubtly brings a valuable contribution to the exact mathematical description of FFF. On the other hand, it is necessary to understand that in actual experimental conditions a number of nonidealities doubtlessly exist, such as imperfect smoothness of the surface of the FFF channel walls and some others, which can cause fundamental deviations of the experimental data from the theory. The above and a number of other possible nonidealities are treated by none of these theories. It is this simpleness and an easy telling physical conception of the derived relationships that are, in spite of some simplifying asymptotic assumptions, an advantage of the nonequilibrium theory.

FFF TECHNIQUES

While the principal arrangement of the contemporary experimental equipment for FFF is, except the

FFF channel proper, identical with the arrangement for liquid chromatography, various FFF techniques differ from each other by the character of the field applied. Their survey, including the main applications in the analysis of macromolecules and particles is given in the following paragraphs.

Thermal FFF

Thermal field-flow fractionation (TFFF) belongs to the historically oldest techniques of FFF. A channel for TFFF is relatively simple. It is usually composed of two metallic blocks with high-polished surfaces, which clamp a spacer. The upper of the two blocks is heated electrically, the lower one is cooled with water. The channel shape proper is cut into the spacer. A temperature gradient between the walls or rather the thermal energy flux causes the non—selective thermal diffusion of the solute species as a consequence of entropy production leading to the accumulation of solute usually near the cold wall.

In early works (34,35) a basic experimental arrangement was described and a successful fractionation of polystyrene (PS) standards with narrow distribution of molecular weights was demonstrated. Some fundamental theoretical and experimental aspects of TFFF were studied in papers by Giddings and co-workers (22,36). The value of λ is expressed by (22)

$$\lambda = \left[w(\alpha_T/T) \, . \, dT/dx\right]^{-1} \qquad (36)$$

where α_T is dimensionless thermal diffusion factor, associated with the thermal diffusion coefficient, D_T

$$\alpha_T = D_T \cdot T/D \qquad (37)$$

From the theoretical viewpoint TFFF is the most complex technique. Owing to the temperature gradient across the channel, the flow is not isoviscous and, consequently, the velocity profile is not parabolic (36). An exact analysis of the shape of the velocity profile in TFFF with regard to nonisothermal flow was presented by Westermann-Clark (37).

Several applications of TFFF were oriented toward the separation of synthetic polymers. TFFF measurement of thermal diffusion factors (38) was demonstrated. Theoretical comparison of TFFF and size exclusion chromatography (39) for the separation of polymers pointed out a number of advantages of TFFF. It was further used to study thermal diffusion of PS in various solvents differing in their thermodynamic quality (40). The use of the pressurized system operating at elevated temperatures provided an effective fractionation of low-molecular weight PS (41). Potentialities of this technique were shown even for the fractionation of polymers with extremely high molecular weights (9), up to 10^{12} daltons. Temperature gradient programming made it possible to fractionate PS standards in a wide range of molecular weights from 4000 up to 7 000 000 daltons in a single experiment (42).

Miniaturization of the channel for TFFF and some other design modifications made it possible to reduce the time of analysis to several tens of seconds up to several minutes (43), and to increase the resolution (44).

The subsequent studies were oriented at the explanation of the factors that cause and affect zone spreading in TFFF (45) and at the determination of the precise polydispersity of the polymer samples

(46) by measuring at various solvent velocities and by extrapolating to zero velocity. An improved separation in TFFF can be obtained by using thermogravitational effect, i.e., by using thermal convection in a nonhorizontal channel. The resulting velocity profile formed under such conditions has a more complicated nonparabolic shape (47). All of the experimental results mentioned above were obtained with the use of PS samples. Only recently TFFF was used for the fractionation of other polymers (48) including polyethylene and polypropylene (49) that represent an experimental problem due to the solubility of these polymers at high temperatures. Martin (50) demonstrated an advantage of a coupling of the TFFF channel with the photogoniodiffusometer for low-angle laser light scattering for the analysis of polymers. Janča and Klepárník (51) showed potentialities of TFFF for the determination of molecular weight distribution of polymers and a procedure for an exact interpretation of experimental results of TFFF by using a calibration method.

The conditions of the effective separation by TFFF were discussed in relation to the matter of Soret effect (thermal diffusion) (52) and to the possibility to fractionate polyelectrolytes (53). Theory of retention in TFFF of macromolecules was progressed (54). In a critical review on polymer analysis by TFFF, Martin and Reynaud (55) specified the requirements for successful separations and demonstrated that samples of polymethylmethacrylate accumulate at the cold wall in given conditions.

Sedimentation FFF

Sedimentation field-flow fractionation (SFFF) belongs, besides TFFF, to the oldest techniques of

FFF. It was predicted conceptually already in Giddings' work (1), Berg and co-workers (56-58) published their results independently. Either natural gravitational or centrifugal forces in the centrifuge serve here as an effective field.

For the value of λ of spherical particles it holds (59)

$$\lambda = 6kT / \pi d_p^3 gw\Delta\rho \quad (38)$$

g is centrifugal acceleration, k is Boltzmann constant, d_p is particle diameter, and $\Delta\rho$ is the difference in the densities of the particles and the solvent used. Berg and Purcell (56) presented the elementary theoretical analysis of fractionation of particles by using gravitational or centrifugal forces in the centrifuge. In their first experimental paper (57) they described the fractionation of PS latex with the particle size of 0.796 μm and 1.305 μm. Their experimental arrangement was quite simple, but the time of the analysis was very long, 76-125 hours. In their subsequent paper (58) they described the separation of R 17 E. Coli bacteriophage, having the molecular weight of 4×10^6 daltons, in the centrifuge. The time of the analysis was substantially shorter in this instance, approximately 4 - 12 hours.

Giddings and co-workers (59) described the device in which the channel was coiled along the internal wall of the centrifuge basket. The basic theoretical and experimental aspects of SFFF were discussed and the fractionation of a series of monodisperse spherical PS latex was demonstrated (59). In the following paper (60) a theory of programmed SFFF using the programming of the intensity of centrifugal force and the programing of the density of the solvent was elaborated. Utilization of

programming expands considerably the range of molecular weights which can be fractionated in a single run. The programming of the field strength in SFFF was effectuated by decreasing gradually the number of revolutions of the centrifuge in the course of the separation of PS latex samples and the programming of the solvent density by increasing gradually concentration of saccharose in water which was used as a solvent.

Another type of programming, the flow-rate, was elaborated theoretically and verified experimentally (61). Extension and narrowing of the channel, slowing down the flow, field programming and an increase in the field strength applied (62) provided a high resolution of particle separation by SFFF in a thousand-fold range of masses in a single measurement.

Yau and Kirkland (25,63-65) also dealt with the programming of SFFF in the analysis of the particle size distribution. They also used the time-delayed exponential-decay programming of the intensity of the centrifugal forces which allowed to linearize the dependence of retention time on a logarithm of dimensions of fractionated solutes. Moreover, the total analysis time was shortened without sacrificing the resolution.

A new design of the equipment for SFFF (25) allowed to work with very high intensities of the centrifugal field, up to 15 000 G at 12 000 r.p.m. Innovations in technology of a rotor for SFFF permit further increase in rotational speed up to 32 000 r.p.m. corresponding to 100 000 G (66). The absolute dimensions or molecular weights of the particles under analysis can be calculated from the retention data (67). In such a way molecular weight

of T 2 bacteriophage was determined. According to the Equation (38), the retention is a function of the product $d_p^3 \Delta\rho$, among other parameters. Thus it is possible to determine the size and the density of the solute particles independently, providing the fractionation is made in various solvents differing in densities, e.g. in aqueous sucrose solutions (68). When the dilute sample of solute is injected during rotation it concentrates at the begining of the channel due to the fact that the average volume flow-rate of the retained solute is lower than the average one of the injected solution. This effect was actively used to concentrate the dilute colloidal samples by SFFF (24). One can even operate in such a way that the injection is run at higher field forces and after the whole quantity of the solute solution is injected, the field force is decreased to the required value. Of course, this procedure could be used in principal not only for SFFF but also for another FFF techniques. Above a certain limit of retention, determined by the size of the solute macromolecules or particles, there is no effective separation because the steric effect comes to be operative, as mentioned in theoretical part. This limit was found theoretically for SFFF (69). Accordingly, chain molecules larger than about 10^{10} in mass would difficult to separate (69).

The character of SFFF makes this technique very attractive, particularly for biological applications, but several other uses were described (70). SFFF was used for the characterization of liposomes (71), BSA microspheres (72), various viruses (73,74), colloidal particles in river water (75) and nuclear energy related materials (76,77). The consistent methodology of colloid characterization by SFFF was

developed, concerning the analysis of monodisperse populations (78), particles having size distributions (79), and emulsions (80). An experimental verification of theory indicated good agreement (81). SFFF was compared with several competitive chromatographic methods and conventional non-chromatographic methods, like exclusion chromatography, hydrodynamic chromatography and disc centrifugation. The comparison was made on the basis of resolution, accuracy, resolving power, peak capacity and dynamic range. SFFF provides superior or at least the same above parameters over the compared methods (82).

Sedimentation-Flotation Focusing FFF

The principle of a newly proposed (83) sedimentation-flotation focusing field-flow fractionation (SFFFFF) is substantialy different. Solute particles or macromolecules sediment or float in the density gradient of a liquid phase according to the differences between the local density of the liquid and that of the particle or macromolecule. At quasiequilibrium, the solute species are focused in a thin layer at the position where the density of the environment is the same as the solute density. The distribution of the solute can thus be characterized by a Gaussian function (83)

$$c(x) = c_{max} \exp\left[-\vartheta \omega^2 x_{max} (d\rho/dx)(x_{max}-x)^2/2R^{o}T\right] \quad (39)$$

where ϑ is the molar volume of the solute, ω is the rotational velocity of the rotor, and x_{max} is the coordinate of the maximum solute concentration c_{max} where the densities of solute and environment are identical. Originaly it was proposed to use a

rectangular cross-section of the fractionation channel so that the formed fluid velocity profile is parabolic (83). In a recent paper, Janča and Jahnová (84) proposed the new-shaped cross-section of the channel which is either trapezoidal or parabolic. In such a case the channel has a modulated permeability resulting in the variation of the fluid flow velocity across the channel width. A theoretical analysis of such parameters like the efficiency, the resolution and the retention was given (83,84). SFFFFF is anticipated for the separation of particles or macromolecules differing in their densities. For example, polymers could be analyzed from the point of view of tacticity because of differences in densities for chains having the same molecular weight but different structures. In a similar way, biological macromolecules or particles could be effectively fractionated. These substances are very often monodisperse with respect to molecular weight, but exhibit differences in structure which decide on density. The principle of the channel with modulated cross-sectional permeability can also be applied to other techniques of FFF (84).

Electrical FFF

Electrical field-flow fractionation (EFFF) belongs to experimentally most sophisticated techniques of FFF. This may also be the reason why a relatively less number of papers has been published, in spite of the first work (85) having been published as early as in 1972. The altogether homogeneous field is induced by electrical current across the channel. The walls of the channel for EFFF are composed of two semipermeable membranes permitting the passage

of small ions and separating the channel space from the electrode compartment. Dimensionless quantity λ is determined by the electrophoretic mobility, μ_e, electrical field strength, E, diffusion coefficient and channel thickness according to (86)

$$\lambda = D/\mu_e E\,w \qquad (40)$$

In the first paper (85), the principle of EFFF was described qualitatively and the method was applied to the analysis of some proteins - albumin, lysozym, haemoglobin and γ-globulin. In a subsequent paper (86) the theory of EFFF was elaborated in more detail, the experimental arrangement using regenerated cellulose as semipermeable flexible membrane that composed the channel walls was described and, again, some proteins were fractionated.

An explanation of the deviations, observed during the fractionation of the above proteins was presented by Subramanian and co-workers (87) as a result of the electrical field gradient in the vicinity of the membrane interface.

In order to eliminate the disadvantages following from the use of flexible membranes, Giddings et al. (88) designed a new channel in which both of the semipermeable membranes were carried by polyethylene frits. This gave rise to a better reproducibility and a better agreement between the theory and experiments for the separation of native proteins. The separations of denaturated proteins and PS latex, however, were not successful.

Reis and Lightfoot (7) also treated the separation of proteins by using a method from the EFFF class. The channel was composed of a circular

semipermeable tube and the electrical field was applied perpendicularly to the central axis of the channel. Recent studies of electroretention of proteins (89,90) have resulted in more detailed understanding of previously observed retention anomalies in EFFF. Homogeneous proteins were used to characterize the improvements of the separation system and a detailed critical review of both theoretical and experimental aspects of EFFF was presented (91). The solutes that show only minor differences in the mobilities, but differ substantially in D, can be separated by EFFF in spite that their electrophoretic resolution is poor. Hence EFFF and direct electrophoretic methods complement each other. The high voltage gradients per unit of length are obtained at low absolute values of the voltage across the EFFF channel. The heat, generated due to high voltage values impairs separation characteristics of direct electrophoretic methods.

Flow FFF

Flow field-flow fractionation (FFFF) is the most universal technique of FFF. The flow of the solvent, perpendicular to the flow of the basic medium in the channel, creates an external field. The earliest experimental works belonging to this class of FFF were published by Lee and co-workers (92,93), but were called one-phase chromatography (92) or ultrafiltration-induced polarization chromatography (93). They elaborated a basic theoretical model of the separation in circular semipermeable tubes and fractionated blue-dextran and human plasma (92), bovine serum albumin and some polydextrans (93).

Giddings and co-workers (94,95) designed the FFFF channel in a classical manner, i.e., of two

planparallel semipermeable membranes. They developed theoretical bases of FFFF and fractionated successfully a series of monodisperse spherical PS latex and a number of proteins. The separation in FFFF is determined only by the differences in the values of the diffusion coefficient, D, or the friction coefficient, f, because the perpendicular flow having the velocity U acts on all of the solutes uniformly. The retention parameter, λ, is then determined by (10)

$$\lambda = R^{o}TV^{o} \ / \ 3\pi N_{A}\mu V_{c}w^{2}d^{o} \tag{41}$$

where V_c is the volumetric perpendicular flow, μ is the viscosity of the medium, V^o is the dead volume of the channel, d^o is the effective Stokes′ diameter, N_A is Avogadro′s number.

The effect of relaxation on the retention and resolution in FFFF was studied (96). A substantial improvement of the fractionation of f2 virus was achieved by using the stop-flow technique (96). FFFF can be applied as a dialysation or ultrafiltration cell (97) to a continuous separation. The operation of one such unit was demonstrated for the isolation of low-molecular weight ethylene blue from bovine serum albumin (97). Various viruses (98) and a number of proteins (99) were separated, purified and characterized as well as colloid silica gel samples (100). FFFF of water-soluble polyelectrolytes, sulphonated polystyrene and sodium salt of polyacrylic acid proved its applicability to the separation of macromolecules (101). FFFF complements to advantage SFFF as far as size distribution analysis is concerned (11).

Magnetic FFF

Magnetic field-flow fractionation (MFFF) has been studied in the only work (102), dealing with theoretical principles of the separation and demonstrating retentions of bovine serum albumin in the presence of nickel(II) ions in a magnetic field of 400 G. A coiled Teflon capillary was used as a channel. In the absence of nickel(II) ions no retention was observed.

For the value of λ of spherical particles the relationship was derived

$$\lambda = (8r/w)(R^{o}T/N_{A}\,\mu_{p}\,\Delta H)^{2} \qquad (42)$$

where r is the particle radius, ΔH is the gradient of the magnetic field strength and μ_p is the magnetic moment of the particle. Equation (42) obviously represents an approximation valid for λ approaching to zero. Moreover, the comparison of experimental retentions with the theoretical model indicates that besides the described effect of magnetic field on solute molecules other phenomena also play an important role. This conclusion results from the fact that the observed retention at 400 G is higher than the calculated one by using the simplified model (102).

Concentration FFF

Concentration field-flow fractionation (CFFF) is the only technique of FFF that makes use of a concentration gradient of a mixed solvent across the channel in order to induce effective chemical forces or chemical field (103). When chemical

potential gradient is $d\mu^{o}/dx$, it follows from the theory (103) that the value of λ is

$$\lambda = R^{o}T/\Delta\mu_{c}^{o} \qquad (43)$$

where $\Delta\mu_{c}^{o} = (d\mu^{o}/dx).w$ is the total increment of the chemical potential across the channel. If the ratio of the concentrations near the both walls is $\alpha_c = c_o/c_w$, then it holds for the retention ratio, R,

$$R = (6/\ln\alpha_c)\left(\frac{\alpha_c + 1}{\alpha_c - 1} - \frac{2}{\ln\alpha_c}\right) \qquad (44)$$

It was found by analyzing Equation (44) that for an effective fractionation α_c must be at least 10 to 100.

CFFF represents the most difficultly realizable technique in classical arrangement, but there exists a prospect that, owing to its unique retention mechanism, the effort required for its practical realization and application will be made.

Steric FFF

Steric field-flow fractionation (steric FFF) occupies among other techniques of FFF an exceptional position. It represents the upper limit of the field strength applied. The particles are compressed closer to the wall as the field strength increases. When the mean distance of Brownian motion is less than the particle radius, **r**, steric FFF takes place. The mean layer thickness is thus controlled by steric exclusion. Hence larger particles migrate into the streamlines of higher velocities than smaller particles do and are eluated more rapidly.

Giddings (104) treated theoretical aspects of steric FFF and its comparison with the mechanism of hydrodynamic chromatography. He derived limit relationship for the value of R, and for $\alpha \longrightarrow 0$

$$R = 6\alpha(1 - \alpha) \tag{45}$$

where $\alpha = r/w$. Of course, α in brackets can be neglected for $\alpha \longrightarrow 0$ and consequently

$$R = 6\alpha \tag{46}$$

When taking into consideration both normal and steric FFF, R was defined (105) as

$$R = 6\gamma\alpha + 6\lambda \tag{47}$$

where $\gamma \doteq 1$ is a dimensionless factor allowing for some nonidealities. λ is related to the solute diameter, d, for TFFF, SFFF, and FFFF by

$$\lambda = \lambda_o/d^n \tag{48}$$

where the exponent n has different typical values for various FFF techniques. It is obvious from Equation (47) that R will increase with increasing d in the region of normal TFFF, SFFF, and FFFF, and will decrease with increasing d in steric FFF. Hence it exists the inversion value R_{inv} for some of the FFF techniques for which $dR/dd = 0$. This was derived (105) as

$$R_{inv} = 3(n+1)(\gamma/nw)^{n/(n+1)}(2\lambda_o)^{1/(n+1)} \tag{49}$$

Theoretically, any effective field may be applied to the steric FFF mode. However, gravitational field represents the most practical means of the

utilization of the principle of steric FFF for fractionations of 1 - 100 μm particles. Experimental evidence for the applicability of steric FFF was presented by Giddings and Myers (106), by an example of the fractionation of glass beads having 10 - 32 μm in diameter. The column was composed of a spacer clamped between two glass plates. The channel proper was cut into this spacer. Various types of the chromatographic spherical packings were fractionated and characterized from the viewpoint of dimensions in the subsequent work (107). In this case, a dependence of the retention ratio, R, on the flow-rate, which was not predicted by the theory, was observed. Caldwell and co-workers (108) explained the dependence of R on the flow-rate observed previously (107) by the existence of lift forces.

By inclining the transversal axis of the channel and by injecting the sample into the upper part of the channel, it was provided that particles under separation were carried and slid towards the lower part of the channel where collection "pockets" were placed along the channel (109). The particles that were carried along the channel slid at the same time to lower parts of the channel and were trapped in the "pockets". Larger particles were trapped in a shorter distance from the injection port, smaller particles were trapped further from the injection port. Continuous fractionation of particles could be obtained in this manner by selecting the channel design properly. The distance from the injection point Z at which the particles are trapped into the "pocket" can be calculated from

$$Z = \frac{27\, Q \mu}{w^2\, r\, \Delta\rho G \cos\varphi} \qquad (50)$$

where Q is the fluid flow-rate, G is the gravity force, and φ is the inclination angle. Hence, both steric and sedimentation effect decide on separation.

Steric FFF represents a further principal progress in the methodology of FFF, and permits a simultaneous extension of applications into the range of the large-diameter particles.

PROSPECTS OF FFF

Several recent papers have treated both theoretically and practically further possibilities than can be provided by FFF.

An increase in the retention and capacity of the FFF channel, and an increase in the selectivity can be obtained by modifying surface of the channel accumulation wall with the aid of transversal barriers (110). These barriers form the grooves in which the solvent does not move and where the solute can penetrate both in and out by diffusion only. The grooves could be used to trap the second phase and to combine the action of the field and the partition between the phases. Preliminary results were obtained in experiments with the fractionation of PS standards by TFFF method using the channel with transversal grooves (110).

Subramanian (111) proposed that a perpendicular field across a short part of the channel establishes the concentration distribution without the flow and, later on, separation proceeds with the aid of the flow without the field action. By selecting properly the experimental conditions, i.e., the intensity and the time of the field action, the channel length and the solvent flow-rate, a high efficiency of fractionations can be reached within a relatively short time (111).

Except TFFF and SFFFFF all of the other techniques of FFF involved until now the establishment of a parabolic velocity profile. Theoretical analysis of the retention and the zone spreading when the velocity profile is asymmetric with regard to the longitudinal central axis of the channel and can be described by a general function of a polynomial type, was performed by Martin and Giddings (112). Janča and Giddings (113) showed a prospective possibility of utilizing non-Newtonian behavior of some liquids. They used flexible Ellis′ three-parameter equation, describing non-Newtonian phenomena, to derive the dependence of R on λ for different conditions of the non-Newtonian flow. This phenomenon could be utilized to increase the selectivity of strongly retained solutes. The variation in the shape of the velocity profile in a single separation run was also suggested (113), i.e., programming of the properties that are decisive for non-Newtonian behavior of the liquid applied.

An extension of FFF to the separation of nonspherical particles and the influence of the wall effect were studied both theoretically and experimentally by Gajdos and Brenner (114).

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CALIBRATION OF SIZE-EXCLUSION CHROMATOGRAPHY SYSTEMS WITH POLYDISPERSE STANDARDS

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Dedicated to Prof.O.Wichterle on the occasion of his 70th birthday

1 INTRODUCTION

Soon after its introduction to polymer science (1), size-exclusion chromatography (SEC) matured into an important tool for the determination of molecular weights and, in particular, molecular weight distributions (MWD) of diverse synthetic macromolecules, and superseded almost completely the older, tedious and less reliable fractionation methods. Different aspects of SEC have been covered in many monographs - the more recent are, e.g., (2 - 4) - and reviews including articles that deal specifically with calibration and data evaluation procedures (5 - 8).

Data evaluation via the calibration dependence molecular weight - retention volume remains an important aspect of SEC (3, 8, 9) in spite of a growing interest in dual detectors concentration/molecular weight. If the simplest method of calibration by means of narrow-distribution polymer standards is not applicable, procedures that rely on characterized polydisperse standards represent a useful alternative, as evidenced by the fact that new methods are constantly being proposed and the older recommendations are modified, improved (and sometimes reinvented).

It is the aim of the present article to summarize and critically evaluate the available information on calibration

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of SEC columns by means of characterized polymer standards having a broad molecular weight distribution.

2 BASIC CONCEPTS AND RELATIONS

It is known that under specific conditions (temperature, type of mobile phase, flowrate etc.) and on a given set of SEC columns there exists for each polymer of a given chemical and physical structure a unique relation between the molecular weight, M, and the retention volume, v, which we shall write as

$$\ln M = f(v) \tag{1}$$

and call the molecular weight calibration dependence. The retention volume is usually defined as the elution volume at the peak maximum, but it is more correct (9 - 14) to define v as the coordinate of the centroid of the peak, i.e., its first statistical moment.

The early hopes (15) that SEC with packings having known pore structure could be developed into an absolute method for measuring polymer molecular weight and MWD have not been substantiated; it is necessary to determine the shape of f(v) in Eq. (1) experimentally by means of a suitable calibration method.

As SEC separates solutes according to their size, and the size of polymer coils in solution depends on many factors which include molecular weight, chain stiffness, segment-solvent interaction, excluded-volume effects etc., the calibration dependence must be found for each polymer separately. Several attempts have been made (16 - 20) to construct a universal calibration, valid for all polymers, by plotting a suitable measure of polymer size against the retention volume. The proposal (21, 22) to use the product $[\eta] . M$ as a universal calibration parameter has gained the most general acceptance; it has a sound physical basis, being proportional to the hydrodynamic volume of the solute, and is easy to apply as the intrinsic viscosity $[\eta]$ can be readily determined.

The molecular weight calibration could be found in a straightforward manner if truly monodisperse polymer

standards were available. As this is not the case, however, one of the following procedures is usually used for establishing the molecular weight calibration f(v):

(i) A number of very narrow fractions ($M_w/M_n < 1.1$, where M_n and M_w are the number- and weight-average molecular weight, respectively) of the polymer in question are available, which span a sufficiently broad interval in M. These fractions are successively chromatographed and, as they are narrow, it is assumed that for the i-th standard the respective retention volume v_i corresponds to the molecular weight M_i which must be known from independent measurements by an absolute method. So far only the commercially available anionically polymerized polystyrenes fulfil the above requirements on primary calibration standards for SEC.

(ii) The system is calibrated according to the method (i) by primary standards of an auxiliary polymer (usually polystyrene) and a universal calibration is constructed as

$$\ln([\eta].M) = f_u(v) \qquad (2)$$

where the intrinsic viscosity $[\eta]$ of each standard is either directly measured (in the same solvent and at the same temperature as in the actual SEC experiment) or calculated from its molecular weight using published values of parameters K_s and α_s in the Mark-Houwink equation

$$[\eta]_s = K_s M_s^{\alpha_s} \qquad (3)$$

where the subscript s refers to the auxiliary standard. According to the principle of universal calibration (21,22) it holds that

$$[\eta]_p.M_p = [\eta]_s . M_s \qquad (4)$$

at the same retention volume (the index p refers to the polymer to be analysed). It is then easy to show by combining equations (3) and (4) that the sought calibration dependence f(v) can be determined from the universal function $f_u(v)$ as

$$\ln M_p = f(v) = f_u(v)/(\alpha_p+1) - (\ln K_p)/(\alpha_p+1) \qquad (5)$$

provided the Mark-Houwink parameters K_p and α_p for the polymer in question are known.

Very often one encounters a situation where the above methods of calibration are not applicable: narrow-distribution fractions of the polymer to be analysed are not at one's disposal and the universal calibration based on polystyrene primary standards cannot be used either because information on the appropriate Mark-Houwink constants K_p and α_p is lacking or the mobile phase used is a non-solvent for polystyrene. Methods that rely on secondary calibration standards (i.e. broad-distribution samples of the polymer to be analysed with one or more molecular weight characteristics known, such as M_n, M_w, $[\eta]$) must be then employed for establishing the calibration dependence; these methods will be discussed in detail below.

Separation of solutes is in real SEC systems always accompanied by processes that lead to axial dispersion (spreading) of the initially narrow bands, so that even a strictly monodisperse sample appears at the column outlet as a peak of final width. Prior to evaluating the data in terms of correct MWD and/or molecular weight averages, it is desirable to correct the chromatogram for axial spreading by solving numerically the integral equation derived by Tung (23)

$$g(V) = \int_{-\infty}^{\infty} w(v)\ G(V,v)\ dv \qquad (6)$$

where g(V) is the normalized, experimental (uncorrected) chromatogram, w(v) is the chromatogram corrected for imperfect resolution (chromatogram which would have been observed in the absence of spreading), and G(V,v) is the so-called spreading function which must be determined experimentally by calibration; G(V,v) is defined as the response of the chromatograph (as a function of elution volume V) to the injection of unit amount of a strictly monodisperse polymer having retention volume v. Calibration for spreading is then equivalent to the determination of the shape of a function of two variables G(V,v). In most instances the spreading function is assumed to be Gaussian,

$$G(V,v) = (h/\pi)^{1/2} \exp\ [-h(v - V)^2] \qquad (7)$$

and the spreading calibration then consists in determining the spreading factor h which is in general a function of retention volume v,

$$h = h(v) \tag{8}$$

(More general forms of the spreading function have been proposed (24 - 27) in order to account for skew etc.)

The most general and exact method of calibration for spreading is the reverse-flow procedure proposed by Tung, Moore, and Knight (28), but this has been shown to be inapplicable (29) with the modern high-performance, high-speed SEC systems that utilize microparticulate packings in columns of relatively small volume, because of extracolumn spreading in the additional tubing and valve required for the flow reversal. Other methods that rely on manufacturer's data on M_n and M_w of primary polystyrene standards have been proposed (29 - 32); in this review some published methods of spreading calibration which employ characterized polydisperse standards will be discussed in Sections 4.1 and 4.2.

It follows from the definition of the spreading-corrected chromatogram that the product w(v) dv represents the weight fraction of the polymer eluting between volumes v and v+dv from an ideal column; accordingly, w is simply related to the molecular weight distribution F(M),

$$F(M)\ dM = -w(v)\ dv \tag{9}$$

(The sign in Eq. (9) reflects the fact that in SEC the molecular weight decreases with increasing retention volume.) Combining Eqns (1) and (9) one arrives at

$$F(M) = -w(v)/(dM/dv) = -w(v)\exp[-f(v)]/(df/dv) \tag{10}$$

which shows that MWD can be calculated from the corrected chromatogram, the molecular weight calibration dependence f(v) and its derivative. Once F(M) is known, the various molecular weight averages (M_n, M_w, the viscosity-average M_η etc.) can be calculated from their definitions; alternatively, they can be found directly from the corrected function w using the relationships

$$M_n = \left[\int_0^{\infty} \frac{F(M)\ dM}{M}\right]^{-1} = \left[\int \frac{w(v)\ dv}{\exp\ [f(v)]}\right]^{-1} \qquad (11)$$

$$M_w = \int_0^{\infty} M\ F(M)\ dM \quad = \int \exp\ [f(v)]w(v)\ dv \qquad (12)$$

$$M_\eta = \left[\int_0^{\infty} M^{\alpha}\ F(M)\ dM\right]^{1/\alpha} = \left[\int \exp[\alpha f(v)]\ w(v)\ dv\right]^{1/\alpha} \qquad (13)$$

(where α is the exponent in the Mark-Houwink equation (3) for the polymer in question). The quantity M_{GPC} calculated from the calibration dependence - Eq. (1) - for the elution volume corresponding to the peak maximum is an average of unknown type and its use for polydisperse samples is strongly discouraged.

3 MOLECULAR WEIGHT CALIBRATION

3.1 Secondary standards with known MWD

As shown in Section 2, the distribution of a sample is unequivocally connected with the corrected chromatogram and the molecular weight calibration function. In principle, it is therefore possible to determine f(v) from the chromatogram of a polydisperse polymer with known MWD, assuming that the axial spreading can be neglected. (This assumption seems to be reasonable in view of the necessity to use samples of very broad distribution).

The procedure is illustrated in Fig.1. The total area under the normalized, uncorrected chromatogram plotted in Fig.1a represents all the polymer; the fractional area A from the final elution volume V_2 at the end of chromatogram to a selected volume V_i corresponds to a weight fraction of the polymer for which $V > V_i$ (axial spreading is neglected) or, considering the integrated form of Eq. (9) with w replaced by g,

$$\int_{V_2}^{V_i} g(v)\ dv = \int_0^{M_i} F(M)\ dM \qquad (14)$$

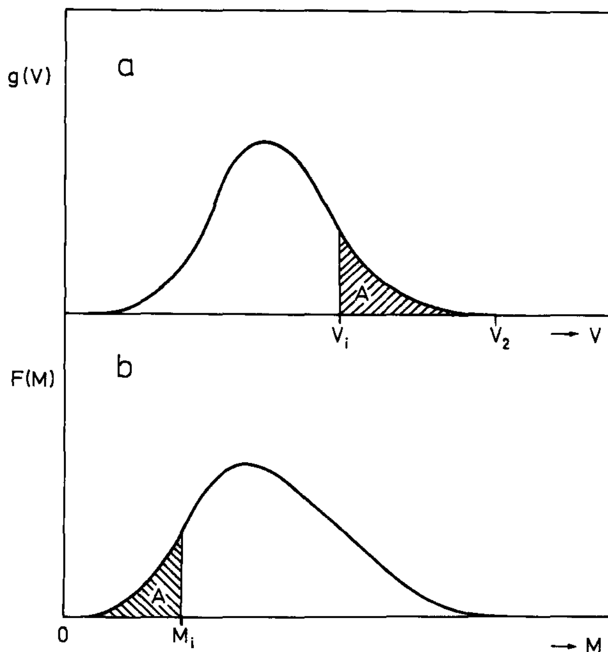

FIGURE 1

Estimation of molecular weight calibration dependence from normalized chromatogram g(V) of a polydisperse sample with known molecular weight distribution F(M). The shaded areas are equal.

for which the molecular weight is lower than M_i. One simply assigns to each V_i the corresponding abscissa M_i in Fig.1b such that the shaded areas A in both Figures are equal. In this way a series of corresponding pairs (v_i, M_i) is generated which define the calibration curve.

The method has been successfully used (33) in 1967 with broad-distribution polyisobutylenes of known MWD. The molecular weight distribution is either determined experimentally (33) (e.g., by a careful fractionation) or a commercially available sample with a known MWD is used (34 - 36). (A polyethylene sample with known MWD, SRM 1475, is available from the National Bureau of Standards, Washington; a polydisperse polystyrene standard with known MWD can be

purchased from the National Physical Laboratory, Teddington, Great Britain. Dextran samples similarly characterized are available from Pharmacia Fine Chemicals, Uppsala, Sweden).

Other authors (37 - 39) assumed the distribution to be known on the basis of thoroughly studied kinetics of polymerization. It is also possible to assume the form of the distribution function in advance (e.g., the Schulz-Zimm function has been made use of by Weiss and Cohn-Ginsberg (40)) and to calculate its parameters from measured molecular weight averages.

If a suitable broad-distribution sample with known MWD is available, the procedure is very simple, but one must keep in mind the following limitations:

(i) The reliability of the resulting calibration dependence is directly given by the accuracy with which the molecular weight distribution of the standard has been determined.

(ii) The calibration curve is determined only in the interval of M spanned by the distribution (34); it is known from experience that any extrapolation of the calibration dependence is a very dubious procedure. This limitation has been overcome is some papers (35, 40) by applying the method to several samples with partially overlapping MWD's and combining the results; the coincidence of superimposed curves was surprisingly good (35, 40).

(iii) At both extreme ends of the chromatogram the error in the area A can be very large (34, 38); in addition, the difference between the corrected and uncorrected chromatogram is most pronounced in these two regions.

Some authors (37, 38) claim that because uncorrected chromatogram is used in Eq. (14), the resulting calibration dependence has the property that the calculated molecular weight averages are automatically and effectively corrected for zone broadening. This will be discussed (and mostly disproved) in the next Section in connection with the effective linear calibration.

3.2 Secondary standards with known molecular size characteristics

3.2.1 Calibration when information is scarce

If only a small number of broad-distribution secondary standards are available, one has to resort to the assumption

that the sought calibration dependence can be adequately described by a linear equation of the form

$$\ln M = A - B\,v \tag{15}$$

In principle, two characteristics are then sufficient for the determination of the constants A and B, such as the number- and weight-average molecular weight of a single standard. It follows from Eqns (11), (12), and (15) that in this case

$$M_n = e^{A} / \int e^{Bv}\, w(v)\, dv\ ,\quad M_w = e^{A} \cdot \int e^{-Bv}\, w(v)\, dv \tag{16}$$

Balke et al. (41) replaced in these equations the unknown corrected chromatogram w by the experimentally available chromatogram g of the secondary calibration standard in question; the equations then define not the true constants A and B, but some effective values A^* and B^*:

$$M_n = e^{A^*} / \int e^{B^* v} g(v)\, dv\ ,\quad M_w = e^{A^*} \cdot \int e^{-B^* v}\, g(v)\, dv \tag{17}$$

Balke et al. solved equations (17) by means of a two-dimensional search algorithm for the constants A^* and B^* of the effective linear calibration

$$\ln M = A^* - B^*\, v \tag{18}$$

More efficient and rapid methods have been later proposed (42 - 47) for solving numerically equations (17).

It has been claimed (38, 41, 44, 48, 49) that the effective linear calibration automatically corrects the molecular weight averages calculated from the experimental chromatogram g(v) for zone broadening. This is certainly and obviously true for the standard used in establishing the effective linear calibration (see equations (17)); it is also true that for any sample the ratio M_w/M_n calculated from the uncorrected chromatogram with Eq. (18) is always lower than if the actual calibration line - Eq. (15) - established, e.g., by means of primary standards were used, because the slope B^* is always smaller (47, 49, 50) in absolute value than B. An analysis proves (51) that the ex-

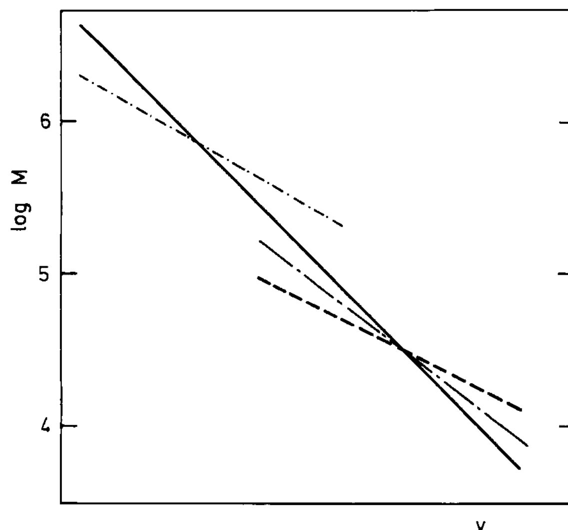

FIGURE 2

Effective linear calibrations
Full line is the true calibration. Effective linear calibration dependences from samples with
---- $M_n=5.10^4$, $M_w=6.10^4$;
— - —$M_n=4.10^4$, $M_w=7.10^4$;
-.-.-$M_n=6.10^5$, $M_w=1.10^6$.

tent of this "correction" depends both on the separation efficiency of the SEC system and on the width of the distribution of the calibration standard. The situation is illustrated in Fig.2 which shows that the difference between the effective and true calibration lines increases with decreasing width of the standard MWD and also that the effective linear calibration can lead to biassed molecular weights calculated for an unknown polymer if its mean molecular weight is sufficiently removed from that of the original calibration standard - see also (52).

Yau et al. (49) suggested an improved method which takes into account the effect of axial spreading and reduces the difference between the effective and true calibration lines.

Their procedure requires the spreading factor to be estimated beforehand; however, the method the authors suggested (49) for calculating the spreading factor from a chromatogram of a narrow-distribution standard is liable to overestimate the spreading unless the standard used is very narrow indeed (M_w/M_n < 1.005, see Knox and McLennan (53)). It was later shown by Pollock et al. (44) that from the point of view of error propagation the original method (41) and the improved version (49) are roughly equivalent.

Tsvetkovskii et al. (54) used another approach to the linear calibration which also respects peak spreading; it requires at least two secondary standards (each characterized by any combination of two values from the set M_n, M_w, $[\eta]$), but its basic simplicity is offset by the inherent assumption that the molecular weight distribution of the standards must be logarithmic-normal.

In assessing merit of the above methods it must be borne in mind that although the restriction of linearity seems to detract much from their general applicability - see, e.g., (51, 52) - with the limited amount of information one can hardly expect to do better. Moreover, with the so-called "linear" SEC columns marketed by DuPont and by Toyo Soda, one can be confident that the accuracy of a straight-line calibration dependence will be sufficient for most practical purposes. Finally, the effective linear calibration forms the starting point of some more sophisticated calibration procedures to be described below.

3.2.2 Universal calibration in combination with secondary standards

Very often the column set can be calibrated by means of polystyrene primary standards (the universal calibration dependence $f_u(v)$ is known), but this information is useless for the problem at hand because the Mark-Houwink constants for the polymer to be analysed in the given mobile phase are not known. If one or several broad-distribution samples of the polymer with at least two molecular size characteristics (such as M_n, M_w, or $[\eta]$) are at disposal, the situation can be rectified by procedures that are based on a derivation first published by Weiss and Cohn-Ginsberg (55).

Recalling equations (2), (4), and (5), we can express the molecular weight M_p by means of the known function $f_u(v)$ and the as yet unknown Mark-Houwink constants of the polymer in question:

$$M_p(v) = \exp[f_u(v)/(\alpha_p + 1)]/K_p \tag{19}$$

(As in the following derivation only the constants K_p and α_p will be required, we shall drop the subscript for simplicity). Assuming again that for the polydisperse standard(s) it is legitimate to replace the spreading-corrected chromatogram w by the experimentally available function g, we have from Eqns (11) and (12)

$$M_n = K^{-\frac{1}{\alpha+1}} / \int \exp\left[-\frac{f_u(v)}{\alpha+1}\right] g(v)\ dv \tag{20}$$

$$M_w = K^{-\frac{1}{\alpha+1}} \cdot \int \exp\left[\frac{f_u(v)}{\alpha+1}\right] g(v)\ dv \tag{21}$$

and for the intrinsic viscosity $[\eta]$ of the standard we obtain similarly

$$[\eta] = K^{\frac{1}{\alpha+1}} \cdot \int \exp\left[\alpha \cdot f_u(v)/(\alpha+1)\right] g(v)\ dv \tag{22}$$

The required Mark-Houwink constants can now be found by solving numerically two of the above equations using two experimentally determined characteristics. The solution is considerably simplified by forming groups of the molecular size characteristics such that the parameter K in the resulting equation cancels; the most useful of these are

$$[\eta] \cdot M_n = \frac{\int e^{\alpha f_u(v)/(\alpha+1)}\ g(v)\ dv}{\int e^{-f_u(v)/(\alpha+1)}\ g(v)\ dv} \tag{23}$$

$$M_w/M_n = \int e^{f_u(v)/(\alpha+1)}\ g(v)\ dv \cdot \int e^{-f_u(v)/(\alpha+1)}\ g(v)\ dv \tag{24}$$

and, if intrinsic viscosities of two polydisperse samples are known, the ratio

$$\frac{[\eta]_1}{[\eta]_2} = \frac{\int e^{\alpha f_u(v)/(\alpha+1)} g_1(v)\, dv}{\int e^{\alpha f_u(v)/(\alpha+1)} g_2(v)\, dv} \tag{25}$$

where g_i is the uncorrected chromatogram of the i-th sample. Depending on the available characteristics, one of the last three equations is solved for α by a one-dimensional search and K is then calculated from (20), (21), or (22), as the case may be. The calibration dependence for the unknown polymer is then determined according to Eq. (5).

The method and its modifications have been extensively tested with many polymers (52, 56 - 61). The investigators agree that although the Mark-Houwink parameters obtained in this manner vary from sample to sample, a high value of K is always compensated by a low value of α so that the calculated molecular weights are in reasonable agreement with values measured by absolute methods. The possible source of error due to the uncertainty in Mark-Houwink constants determined in this manner can be diminished by combining chromatograms of several characterized samples and determining K and α by some least-squares optimization (60). Kato et al. (62) recommend to use a Θ-solvent as the mobile phase where the exponent α is known ($\alpha = 0.5$). Dobbin et al. (63) compared several of the possible combinations of molecular size characteristics and concluded that the best values of K and α are obtained when the ratio of two intrinsic viscosities - Eqn. (25) - is used.

Some proposed modifications of the method introduce additional assumptions (linearity of calibration (64, 65), Gaussian shape of chromatograms (65)), which are unnecessary and limit the scope of the original method, but facilitate the evaluation of data.

Hamielec (66) and Taganov et al. (67) proposed generalizations of the above procedure which take into account spreading and skewing.

3.2.3 Calibration with a series of secondary standards

If a sufficient number of characterized, polydisperse standards are available, the SEC system can be calibrated without recourse to the universal calibration and also without the awkward restriction of linearity. As early as in 1968 Frank et al. (68), faced with the task of calibrating SEC columns for polyethylene, developed a somewhat cumbersome graphical-numerical procedure, where the calibration dependence was defined by a number of its enveloping curves and in the final stage had to be drawn by hand (see also Dawkins (69)).

Barlow et al. (36) extended the calibration dependence for polyethylene obtained by the method of Section 3.1 into the high-molecular-weight region (not covered by the polyethylene standard NBS 1475) by an iterative procedure that seeked to obtain coincidence between the measured intrinsic viscosities of polyethylene samples and values calculated from their chromatograms.

A number of investigators proposed calibration procedures that can be described in general terms as follows. Calibration standards with arbitrary MWD are available, characterized by N molecular weight averages $\bar{M}_{\beta}^{(i)}$ ($i = 1,2,..N$, β = n or w). The molecular weight calibration dependence is described by a mathematical function $f(v;c_1,c_2,..c_n)$ with n adjustable parameters, where $n \leq N$. The values of the parameters in the model function are then determined from the requirement that the observed (i.e. calculated from SEC data) averages $M_{\beta}^{(i)}$ agree with the corresponding "true" values $\bar{M}_{\beta}^{(i)}$.

Thus, Szewczyk (70), McCrackin (71), Vrijbergen et al. (43), Chaplin and Ching (61) all used polynomials in v,

$$\ln M = \sum_{j=1}^{n} c_j v^{j-1} \qquad (26)$$

for modelling the molecular weight calibration dependence. Nilsson and Nilsson (72) employed a modified polynomial function

$$\ln (M - c_5) = c_4 + c_1 v + c_2 v^2 + c_3 v^3 \qquad (27)$$

with five parameters. Cardenas and O'Driscoll (48) proposed to describe the calibration dependence by a four-parameter function

$$v = c_1 + c_2 \{\pi^{-1/2} \psi^{-1} [1 - \exp(-\psi^2)] + \operatorname{erfc}(\psi)\} \qquad (28a)$$

where

$$\psi = M^{c_4} / c_3 \qquad (28b)$$

originally derived by Yau and Malone (73) in their diffusion theory of separation in SEC (see also (12)).

All these methods, however, suffer from one deficiency, viz., the number of adjustable parameters n is set in advance. (This is obviously less important if n is sufficiently large, as in Eqns (28) or (27)). It has been repeatedly stressed (31, 48, 74, 75) that considerable errors can arise if a distinctly curvilinear calibration is forcibly fitted to a linear equation of the type (15); by the same token the number of unknowns (e.g., the degree of approximating polynomial) should be also optimized by a suitable statistical procedure. This has been recognized by Andersson (76) who tested several types of model functions and optimized also the number of parameters by minimizing the modified weighted sum of squared deviations

$$X^2 = (N - n)^{-1} \sum_{i=1}^{N} [M_\beta^{(i)} - \bar{M}_\beta^{(i)}]^2 \cdot W_i \qquad (29)$$

where the weights W_i are in the simplest case given by $W_i = [\bar{M}_\beta^{(i)}]^{-2}$ – see (18, 76) for a detailed discussion. Andersson recommends to use cubic splines for modelling the calibration dependence.

All the above authors substituted the experimental, uncorrected chromatogram into formulae that are strictly speaking valid only for the corrected function w. As a consequence, the results bear the character of effective

and not true calibration dependences in the sense discussed in Section 3.2.1.

McCrackin (71) in his Method II respected the effect of axial dispersion in an elementary manner: although uncorrected chromatograms of the calibration standards were still used for calculating the average molecular weights, the known characteristics $\bar{M}_n^{(i)}$ and $\bar{M}_w^{(i)}$ were first multiplied by the appropriate correction factors derived (32) for the case of constant spreading and linear calibration dependence. In view of the latter two conditions the method is restricted to standards of moderately broad MWD, for which both $\bar{M}_n$ and $\bar{M}_w$ are known, but along with the true calibration dependence (modelled by a polynomial of a second degree) the spreading factor (assumed constant) is also determined.

The most recent approaches that respect the imperfect resolution of real SEC columns and yield the true (in general non-linear) molecular weight calibration together with the spreading calibration h(v) will be discussed separately in Section 5.

3.3 Calibration in SEC with multiple detectors

In recent years the dual detectors concentration/molecular weight find many applications in SEC. So far, viscometers (either discontinuous (77 - 85), measuring the viscosity of separate portions of the eluate, or continuous (86 - 88)) and the low-angle laser light scattering photometer - LALLSP (89 - 92), now marketed as KMX-6 by Chromatix, U.S.A, have been used in combination with a concentration detector (mostly differential refractometer). The molecular weight of the fraction leaving the column can be determined directly (LALLSP) or indirectly (automatic viscometer) by combining the data of the two detectors.

It would seem that in this case the relationship molecular weight - retention volume looses much of its importance, and that at any rate it can be readily established for any polymer simply by plotting the data obtained by chromatographing a broad-distribution sample. This is, however, not completely true. First, the calibration dependence and in particular the universal function $\ln([\eta]\cdot M) = f_u(v)$ is

necessary for data evaluation when complex macromolecular systems (branched structures, chemically heterogeneous copolymers) are investigated - and here lies the true realm of SEC with dual detection. Secondly, it is now definitely established that the correction for axial spreading is necessary if meaningful estimates of the calibration curve are to be obtained (93) from the data of the LALLSP system, and this is even more important with the automatic viscometer if one considers the problem of backmixing in the tubing and in the siphon.

Zone broadening has been recognized (94) as the source of observed deviations (81, 84) between the molecular weight calibration obtained by classical methods using narrow fractions and that resulting from the uncorrected data of an automatic viscometer coupled with SEC. Park and Graessley (85) obtained correct molecular weight calibration dependence from SEC/viscometry applied to broad-distribution samples by taking into account the transport lag between the detectors, the zone spreading in the column, and post-refractometer mixing in the tubing and in siphon. The problem of finding the molecular weight calibration for the given polymer from the universal function $f_u(v)$ using the data of the SEC/viscometer system with correction for zone broadening has been investigated by Taganov (93).

Recently, Kim et al. (96) utilized the new, generalized analytical correction for imperfect resolution (93) and developed a valid method for the determination of the molecular weight calibration function (and, incidentally, the spreading factor - see Section 4.2) from a single chromatogram of a polydisperse polymer using the SEC/LALLSP detection system. In another article Hamielec (97) outlined the methodology for the determination of the molecular weight calibration, together with the Mark-Houwink constants and the elution volume dependence of spreading, from the universal curve $f_u(v)$ using the data of the dual detector (see also (98)).

4 SPREADING CALIBRATION

Calibration for spreading in SEC is seriously hindered by the fact that (with the exception of some naturally occurring macromolecules) strictly monodisperse polymers simply do not

exist. As shown by Knox and McLennan (53) the contribution of polydispersity to the total peak width is considerable even for very narrow fractions. Accordingly, it is not justified to estimate the spreading directly from the variance of normal-flow (as opposed to reverse-flow) uncorrected chromatograms of primary calibration standards (25, 27, 49, 99), unless special measures (100) are adopted to ensure that the quotient M_w/M_n is lower than about 1.005.

Standards characterized as to their polydispersity are required for the spreading calibration, and it can be expected that for accurate results samples having narrow MWD will be preferable; even here, however, the scatter in the experimentally determined spreading factors is often quite high (29). Fortunately, the correction for imperfect resolution is not very sensitive to the accuracy of h, as demonstrated by the following argument. Consider the spreading correction according to Balke and Hamielec (101) to be $\exp(-B^2/4h) = 0.9$ (the corrected M_w will be then lower than the uncorrected average by some 10 %). If the value of h is now varied by ± 20 %, the correction factor changes from 0.92 to 0.88, well within the limits of accuracy of SEC results. It is therefore not surprising that the calibration for spreading with polydisperse secondary standards often yields reasonable results.

4.1 Spreading calibration with secondary standards

Taganov et al. (102) and later Berger (103) suggested "refractionation" procedures for the estimation of the shape of G(V,v) that require the chromatogram of a standard and also chromatograms of several of its fractions isolated with (and recorded on) the same set of SEC columns. The methods are tedious but require no additional information on the standard. A method of spreading calibration by means of a polymer sample with accurately determined MWD has been outlined by Berger (100) - see also (104).

Several groups (105 - 107) used recycle SEC to determine the spreading; the methods have been mostly tested with narrow-distribution samples, but there is no reason why they could not work with moderately broad polymers as well.

The method of moments (29) developed for spreading calibration with a Gaussian $G(V,v)$ can utilize both narrow- and broad-distribution standards.

Additional methods developed recently and capable of determining the functions $f(v)$ and $h(v)$ simultaneously in a once-through process from the chromatograms of characterized standards with an arbitrary shape of MWD will be discussed separately in Section 5.

4.2 Spreading calibration using dual detectors

Park and Graessley (85) showed how to estimate the extent of spreading from the data of an automatic viscometer coupled with SEC: assuming that the true molecular weight calibration dependence is known, $h(v)$ can be determined by trial and error from the chromatogram and from measured intrinsic viscosities of fractions using the Tung equation (6) for calculating the distribution of various molecular weight species among the fractions.

Berger (108) developed a systematic method for correcting the data of the dual detector for imperfect resolution by solving simultaneously the Tung equation (6) and the integral equation

$$[M^*(V)]^\alpha = \frac{\int [M(v)]^\alpha w(v)\, G(V,v)\, dv}{\int G(V,v)\, w(v)\, dv} \qquad (30)$$

where α is the Mark-Houwink exponent for the automatic viscometer and $\alpha = 1$ for the LALLSP. $M^*(V)$ is the uncorrected molecular weight at the elution volume V as determined from the combined signals of the detectors, $M(v)$ is the true (spreading-corrected) molecular weight. A fairly complicated matrix algebra is involved but, provided again that the true molecular weight calibration dependence is known, the treatment yields both the corrected molecular weight $M(v)$ of the species leaving the column at v and the spreading function (assumed to be uniform, i.e. independent of M in Berger's treatment).

This procedure has been simplified by Netopilík (109) who recognized that the two integral equations (6) and (30)

can be rewritten in a form which makes them basically equivalent and amenable to be solved successively by any of the numerous methods originally developed for handling the Tung equation (6). The correct spreading factor is then found as that value of h which makes the corrected function M(v) to coincide with the molecular weight calibration established independently. Hamielec (97) outlined a procedure wherein the new analytical solution (93) of the Tung spreading equation (6) is employed and the spreading factor h(v) is determined from the data of SEC with an on-line molecular weight detector, assuming that either the molecular weight calibration f(v) or the universal function $f_u(v)$ is known.

5 SIMULTANEOUS MOLECULAR WEIGHT AND SPREADING CALIBRATION

Hamielec (97) maintains that in order to obtain accurate molecular weights, the data of SEC must be corrected for axial spreading even for broad-distribution samples chromatographed with a high-performance equipment. Several methods for evaluating normal-flow chromatograms of primary, narrow-distribution standards, as obtained in the process of molecular weight calibration, also in terms of the spreading factor and its dependence on elution volume have been proposed and tested (29, 30, 31, 101, 110). The problem of a comprehensive, once-through calibration in SEC using secondary standards having MWD of an arbitrary shape has been seriously attacked only recently. (Already in 1968 Almin (111) outlined a method for the determination of both the true molecular weight calibration (assumed to be linear) and the spreading factor as a function of retention volume, h(v), from chromatograms of polydisperse standards, but his procedure was based on rather artificial assumptions concerning the shape of experimental chromatograms.)

Andreetta and Figini (112) proposed a method for a simultaneous determination of the functions ln M = f(v) and h(v), based on the equation for the general β-average molecular weight derived by Figini (113):

$$M_\beta = \{\int M^\beta \cdot F(M)\, dM\}^{1/\beta} = \{\int g(v) \cdot [M(v)]^k\, dv\}^{1/k} \cdot L^{-1} \quad (31)$$

where k is a function of β and of the parameters (say c_i) that define the molecular weight calibration dependence - $f(v,c_1,c_2,..)$ - as well as of the coefficients (say b_i) which enter into the elution volume dependence of spreading - $h = h(v, b_1, b_2,..)$; thus

$$k = k(\beta, c_1,c_2,...;b_1,b_2,...) \tag{32}$$

Similarly, it holds for L that

$$L = L(c_1,c_2,...;b_1,b_2,...) \tag{33}$$

Andreetta and Figini recognized that in many instances of practical interest the functional dependences in Eqns (32) and (33) can be explicitly derived by direct integration of the Tung's spreading equation (6). In these cases the sum of squared deviations

$$X^2 = \sum_{i=1}^{N} [M_\beta^{(i)} - \bar{M}_\beta^{(i)}]^2 \tag{34}$$

(where $\bar{M}_\beta^{(i)}$ are again the known molecular weights of N secondary standards employed) can be minimized by Gauss iteration as the required partial derivatives can be evaluated analytically. So far only results with a constant spreading factor have been published, but the method is capable of further generalization.

Kubín (47) analysed theoretically the relationship between the true - Eqn (15) - and effective - Eqn (18) - linear calibrations for the case of Gaussian spreading and ascertained that the two straight lines intersect at the properly defined retention volume (centroid of chromatogram); as the constants A^* and B^* can be readily found by solving the equations (17) (assuming that M_n and M_w are known for each calibration standard), and the centroid of the chromatogram, μ^*_1, is easily accessible, one obtains for each standard the coordinates (μ^*_1, $A^* - B^* \mu^*_1$) of the intersect, i.e., of one point on the true calibration dependence. With a number of calibration standards the

resulting points can be processed by some standard correlation procedure to yield the best-fit functional dependence for the true calibration f(v), e.g., in the form of a polynomial of statistically correct degree.

The slopes of Eqns (15) and (18) are related (47) through the equation

$$1 + (B^*)^2\mu_2 = \exp(-B^2/2h)\ (1 + B^2\ \mu_2) \qquad (35)$$

which also contains the spreading factor h and the variance (second statistical moment) of the respective peak,

$$\mu_2 = \int (v - \mu_1^*)^2\ g(v)\ dv \qquad (36)$$

As B^* is known and B can be taken as the slope of the true calibration dependence determined e.g. by the above method of intersects (if this is curvilinear the local slope at $v = \mu_1^*$ is assigned to B), Eqn (35) can be directly solved for the spreading factor h.

This method yields reliable molecular weight and spreading calibrations for moderately broad standards (75). For very broad polymers where the accuracy of corrected molecular weight averages calculated from the above calibration dependences is less satisfactory, an iteration loop can be initialized which improves the initial estimates of f(v) and h(v); for details see (47, 75).

6 CONCLUSION

Reliable calibration is an absolute necessity in contemporary SEC and apparently will remain so in the foreseeable future. The studies reported in this review should convince the reader that it is feasible to establish reliable molecular weight and spreading calibration dependences using normal-flow chromatograms of polydisperse, characterized polymer standards. The techniques developed differ greatly in scope and in their requirements on the extent of necessary input information; the most powerful methods involve considerable computation and require the

use of a computer. It is expected that new procedures will emerge in the future in spite of the growing interest in the so-called absolute detectors in SEC.

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ANALYSIS OF ANTITUMOR ANTIBIOTICS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

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Cancer chemotherapy is a concern of many people today: laboratory scientists, clinicians, cancer victims and news reporters. Their hopes for a cancer cure are always raised when a new chemotherapeutic agent is discovered in the laboratory and shows some promising characteristics towards clinical application. Some of the agents that have been discovered are of natural origin[1], and are complex structures of unknown composition; others are enzymes or antibiotics. The antibiotics are obtained mostly from fermentations, but are also derived from plants or marine animals. Considerable efforts are being made in laboratories to purify these antibiotics to homogeneity and to determine their structure, mode of action, toxicity and applicability to clinical cancer chemotherapy. All these complex functions require analytical support. One of the most modern analytical tools, high pressure liquid chromatography

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(HPLC), is used with increasing frequency to analyze these antitumor antibiotics in different media and for different reasons.

Before the advent of HPLC, analytical methods used in connection with different antibiotic assays relied on liquid chromatography, countercurrent distribution[2], thin layer chromatography[3] and gas chromatography[4]. Liquid chromatography, in general, is a time-consuming, slow technique. Thin layer chromatography is much faster and uses inexpensive instrumentation but lacks resolution and reproducibility for complicated, large compounds and especially for mixtures. Gas chromatography is a fast chromatographic system, with good reproducibility, precision and specificity at relatively modest instrumentation costs. However, many antibiotics cannot be analyzed by gas chromatographic techniques because they cannot be volatilized.

In the last decade or so, HPLC has been developed into an analytical tool that combines the advantages of all previous chromatographic techniques. Most of the details of the techniques of HPLC are well described in recent literature[5][6], and there is no need to deal with them here. Briefly, HPLC uses a narrow column with small diameter column packing particles, high pressure to obtain the flow rate necessary for short analytical time and highly sensitive detectors. The short analytical time is an important factor in

analyzing antibiotics, because some antibiotics decompose during lengthy analytical manipulations. Also, analytical results may be needed urgently, e.g., for assessment of antibiotic composition in fermentation broth or in human or animal tissues. This short analytical time is achieved by high volume of solvent flow, which in turn is achieved by high column-inlet pressure. This analytical arrangement is very seldom a problem in connection with antibiotic analysis.

The separation of antibiotics is monitored with ultraviolet absorption detectors in most cases, since many of the antibiotics absorb in the ultraviolet range. However, some antibiotics, e.g., the aminoglycosides, have no characteristic ultraviolet absorption above 210 nm. In such cases pre- or postcolumn derivatization is performed to provide antibiotic derivatives which can be monitored by fluorescence or ultraviolet detectors.

HPLC has been used increasingly in the antibiotic field in research, quality control and manufacturing environments. For many antibiotics the official or most accepted assay is now performed by HPLC. However, some of the HPLC assays lack precise characterization of the chromatographic system, mostly because the system was applied to non-routine testing. For example, no precise control of the temperature is mentioned in most literature although work done with the vinca alkaloids demonstrated the importance of this parameter[7]. Precise

characterization of the columns used in connection with antibiotic work is almost entirely lacking. Today HPLC is used in the isolation studies of new antibiotics, in preparation of large quantities of antitumor antibiotics for biological studies and, if the assay proves to be sensitive enough, for quantitative estimation of antibiotics in biological fluids and in various drug preparations.

Because some of the work was done with equipment not used any more, these systems need to be translated to more current conditions. Routine assays should undergo collaborative studies before wide-scale introduction, and such studies were done for several antibiotic assays as discussed below.

This review summarizes and comments on the HPLC systems used in connection with antitumor antibiotic analysis. It is intended to alert the reader about possible use of HPLC in connection with studying antibiotics rather than as a critical evaluation of work done in other laboratories. Discussions are grouped according to the most important or most frequently analyzed antibiotics and the use of HPLC in isolation studies of new antitumor antibiotics. Moreover, the literature on determination of these compounds in biological fluids has been selected so that similar analytical details are not repeated too frequently. Also, methods older than 8 to 10 years are not reported in this discussion. It will be apparent to the reader that most of the HPLC processes discussed below use reversed

phase systems. The most frequently used column is μBondapak C_{18}. Some other columns used are Lichrosorb, Porasil, Ultrasphere ODS, Nucleosil C_{-18} and Durapak. Occasionally the application of a guard column like CO:Pell ODC or RSiL had to be employed. For eluting solvents combinations of methanol and acetonitrile with phosphate buffers are used most frequently.

It should be mentioned that in this review the HPLC nomenclature used is that of the original authors. It was felt that an attempt to modify their descriptions for the purpose of uniformity would create a problem for those who want to refer to the original paper. Also, no attempt was made to describe the origin and quality of reagents used in the experiments. It was assumed that all solvents were filtered and degassed before use. Structures of the discussed antibiotics can be found in the Merck Index, Handbook of Antibiotic Compounds (J. Berdy, A. Aszalos, M. Bostian and K. L. McNitt eds., CRC Press) and in the original articles cited, and have not been duplicated in this paper. Many papers describe the use of HPLC in conjunction with the objective of that paper, e.g., isolation of an antibiotic, metabolic studies, etc. In each case only the HPLC portion of the paper and the objective of the study have been described; details and results of the study were not reviewed here.

ACTINOMYCINS

One of the earliest antibiotic groups discovered was the actinomycins. This family of antibiotics comprises closely

related chromopeptides which differ only in their amino acid composition[8]. Different chromatographic systems applied to separate these closely related antineoplastic antibiotics helped in the elucidation of their structure[9] and in their biological evaluation[10]. It was realized in the early separation attempts that distribution chromatography is an excellent method for separation of these partially lipophilic antibiotics[11] [12]. As a natural consequence, separation of the actinomycin mixtures was attempted by reversed phase HPLC.

In a successful HPLC separation of the actinomycins, Rzeszotarski and Mauger[13] used the following conditions: chromatograph, Waters Associates AL C202/6000 psi; UV detector, operated at 254 nm; column, 6 ft. x 1/8 in., filled with μBondapak C_{18}/Corasil or Bondapak phenyl/Corasil, stationary phases covalently bound to Corasil, columns drypacked by vibration and topping; mobile phase, water-acetonitrile (1:1); flow rate, 1 ml/min (1000 psi).

The above experimental conditions provided almost complete baseline separation for the actinomycin mixture C_1, C_2 and C_3 with symmetrical peaks and for the actinomycin mixture whose members contained cis-4-chloro-L-proline. This latter complex was obtained by using cis-4-chloro-L-proline as precursor in the fermentation medium of the microorganism of Streptomyces parvullus. This HPLC method provided evidence for the formation of the two new types of actinomycins, CP_3 and

CP_2. By using chromatographic recycling techniques, the major components of the latter complex could be prepared in sufficient quantities for chemical characterization.

A recent method used in several laboratories to determine quantitatively the actinomycin D content of bulk preparations (unpublished) utilizes the following conditions: chromatograph, Waters Associates Model 244; UV detector, operated at 254 nm; column, 30 cm x 4 mm, packed with μBondapak C_{18}; mobile phase, acetonitrile-water (6:4); flow rate, 2.5 ml/min; samples, about 0.25 mg/ml standard or bulk preparation of actinomycin D; calculations, actinomycin D content of bulk preparations calculated on the basis of comparison of the area under the peak of standard and samples. The above procedure was the subject of collaborative studies (unpublished). Statistical evaluation of these studies indicated the acceptance of this HPLC system for official analytical purposes.

The two HPLC systems described above make it possible to detect and separate common actinomycins and to quantitate actinomycin D in bulk preparations. For other determinations, such as quantitations of actinomycin in different tissues, no accepted method has yet been reported in the literature. Such assays are currently done by different techniques, like fluorescence spectroscopy. However, with the present sensitive detectors an HPLC assay may be developed for the estimation of such low level actinomycin concentration.

VINCA ALKALOIDS

Important antineoplastic antibiotics are obtained from the plant Catharanthus roseus (Vinca rosea). Two members of this family of alkaloid-type antibiotics, vinblastine and rincristine, are used clinically[14]. Both the estimation of the useful vinca compounds in the different plant extracts and the separation and identification of metabolites require reliable quantitative analytical methods. Such methods are required to estimate the individual alkaloids in the presence of structurally related compounds and in the presence of other unrelated materials. Methods which rely on thin layer chromatography, spectrophotometric or colorimetric measurements do not fulfill all these analytical requirements. With this background in mind Gorog et al.[15] developed a method for these alkaloids that includes an HPLC system which can separate 26 related vinca alkaloids. The structures of all 26 compounds used in this study in relation to the basic structures are shown in Figure 1. The retention times obtained in the HPLC system are described below are also shown in Table 1. The chromatographic conditions were as follows: Instrument, Hewlett-Packard 1010 B; UV detector, operated at 298 nm; column, 250 cm x 4 mm, packed with Lichrosorb RP-8 (Merck, Darmstadt); solvent, acetonitrile-0.01 M ammonium carbonate (47:53); flow rate, 1.5 ml/min; injector, Valco loop, 25 μl.

In the process of assigning retention times to individual compounds of the alkaloid mixture, scanning was also done at

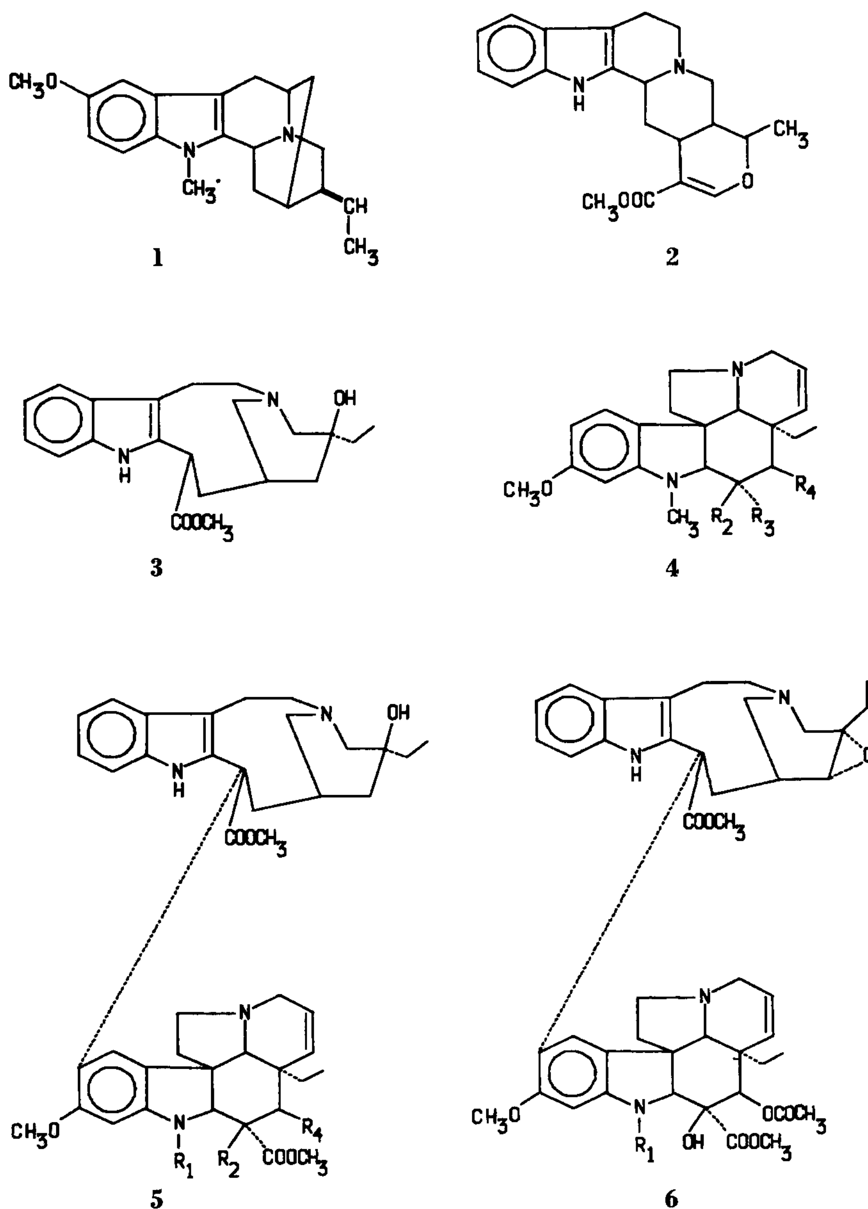

Figure 1. Structures of the vinca alkaloids studied by Gorog et al.(15) by HPLC.

Table 1. Retention data for vinca alkaloids [15]

Compound	Retention time (min)	Structure[a]	Functional group[b]			
			R_1	R_2	R_3	R_4
Ajmalicine	7.90	2				
Catharanthine	9.75	3				
Desacetoxyvinblastine	17.15	5	G	J		H
Formylleurosine	9.75	6	F			
Leurosine	15.65	6	G			
	11.89	6	H			
Lochnerine	3.29	1				
Tetrahydroalstonine	14.65	2				
Vinblastine	12.37	5	G	J		B
	7.04	5	G	J		J
	12.52	5	G	J		C
	10.25	5	G	J		D
	10.09	5	G	J		E
	48.10	5	G	B		B
	6.18	5	H	J		J
	10.04	5	H	J		B
Vincristine	7.22	5	F	J		B
	4.87	5	F	J		J
Vindoline	6.16	4		J	A	B
	4.71	4		J	A	J
	9.39	4		J	A	C
	5.84	4		J	A	D
	4.30	4		J	A	E
	23.22	4		B	A	B
Vindolinol	4.44	4		J	K	J
Vindorosine[c]	7.90					

[a] From Fig. 1.

[b] A=$COOCH_3$; B=$OCOCH_3$; C=$OCOCH_2Cl$; D=$OCOCH_2N(CH_3)_2$; E=$OCOCH_2NH(CH_3)$; F=CHO; G=CH_3; H=H; J=OH; K=CH_2OH.

[c] Vindoline without the aromatic methoxy group.

lower wavelengths so that structural assignments could be made from UV data obtained at two wavelengths. Although some of today's equipment (e.g., Waters Associates Model 440 UV detector) can be operated at two wavelengths simultaneously, Gorog et al. had to perform these operations in succession.

Much later, in 1981, Verzele et al.[16] published an HPLC system with gradient elution for the separation of vinca alkaloids. The specific objective of these authors was to assess the composition of crude *Vinca rosea* plant extracts. The crude solvent extract of a plant was extracted with an acid-water mixture and then subjected to chromatography. The following conditions were used: Instrument, Varian LC-5020; UV detector, operated at 280 nm, Varichrom; column, LiChroma filled with 10 µm RSiL-C_{18}-HL-D octadecyl silica gel; precolumn, 10 cm, filled with 20 µm RSiL or 20 µm RSiL-C_{18}-HL-D or with a mixture of the two to prevent dissolution of column material by the solvent; solvent, water-methanol with a gradient in methanol 50 to 85% (both solvents contained 0.1% ethanolamine; no gradient time was specified); flow rate, 2 ml/min; injector, Valco 7000 psi loop injector.

The resolution of this gradient technique is not much superior to that obtained by Gorog et al. by their isocratic system described above. However, by interruption of this gradient system the dimeric vinca alkaloids could be separated into two groups and the separation of some individual components

could be achieved better this way. This interrupted gradient technique seems to be a good method to fractionate complicated mixtures even at large scale.

Verzele et al. used the above HPLC technique to distinguish between monomeric and dimeric alcohols based on plate number (N) calculations. The authors observed that the required number of plates for separation of the dimeric alkaloids is smaller than that for separation of the monomeric alkaloids. To ascertain that this observation is correct for all temperatures the required N was determined between 5 and 50°C for each compound. The authors observed that changing the temperature not only changes N by a factor of 2 for monomerics and by a factor of 2.5 for dimerics, but simultaneously changes the capacity factor k' also. However, the average ratio of N between monomeric and dimeric alkaloids did not change greatly with changing temperature. To be certain that N values were not influenced by temperature changes, which would result in k' value changes, N value measurements were made at different temperatures at constant k' values by using different composition of eluants.

Measurements made with similar k' values indicated that the average N value ratios of monomeric and dimeric alkaloids do not change greatly with the temperature. From these studies the authors concluded that N values are indicative for structures of vinca alkaloids independently of the temperature of the

chromatography, and that the diffusion rates of these different alkaloids change parallel with temperature. They have also concluded that to obtain consistent k' values for identification purposes the temperature of the chromatography has to be closely controlled.

HPLC was also used to assess the composition of different plant extracts of vinca alkaloids in conjunction with radioimmunoassay[17]. HPLC served as the fractionation method and the fractions thus obtained were subjected to radioimmunoassay for identification of the individual vinca alkaloids. Conditions of the HPLC system were as follows: Instrument, Waters Associates, Model AC202; UV detector, operated at 254 μm; column, two reversed phase μBondapak C_{18} (3.9 mm x 30 cm), connected in series; solvent, acetonitrile-0.01 $\underline{M}$ Na_2HPO_4 pH 7.4 buffer (1:1); flow rate, 10 ml/min; injector, Model U6K. The extract injected into the above system was prepared by a process that resulted in an acetonitrile solution of a material to be separated further by HPLC. The extraction process assured that all bioactive alkaloids were retained and that most of the materials harmful for the HPLC column were eliminated.

Besides estimation of composition of extracts or synthetic products of vinca alkaloids HPLC was also shown to be useful in the separation and identification of metabolites of these vinca-type antitumor antibiotics[18]. In this study

radiolabeled vinca alkaloids were injected into rats, and tissue samples were taken after certain time periods. These samples were prepared for HPLC injection by extraction-purification procedures. The HPLC system applied here was very similar to that discussed above[17] except that a gradient technique was used. Instrument, Waters Associates, Model 202; UV detector, operated at 254 µm; column, µBondapak C_{18}; solvent, acetonitrile-0.001 M K_2HPO_4, pH 7.5 buffer, linear gradient 20 to 80% acetonitrile, program time 40 min; flow rate, 2.5 ml/min. With this system vincristine has a retention time of 25 min. Fractions were collected for identification by taking eluates corresponding to UV-absorbing materials. Since the injected vinca alkaloids were radiolabeled the collected HPLC fractions could be checked for metabolites by scintillation counting technique.

NEOCARCINOSTATIN

Neocarcinostatin is a chromo-protein [19] with considerable antitumor activity[20]. Its mode of action is connected to DNA strand scission[21]. Recently the chromophor portion of this antibiotic was separated from the apo-protein portion and was shown to possess all DNA-related biological activity previously attributed to the original antibiotic[22]. Separation of the chromophor and its different forms was achieved by HPLC. The intact antibiotic was extracted with 0.1 M acetic acid in methanol or 0.1 M HCl in

methanol or glacial acetic acid, in each case with an antibiotic concentration of 1.4 mg/ml. This extract was injected directly (50 µl) into the following HPLC system: Instrument, Waters Associates Model A2 C/GPC-204; detectors, UV, operated at 254 nm and a Schoeffel Model SF 970 fluorescence detector with 340 nm excitation and 418 µm emission cutoff filter; column, µBondapak C_{18}; solvent, 56 to 84% methanol in 0.01 M ammonium acetate, pH 4, concave gradient run for 50 min period; flow rate, 2 ml/min.

Fractions were collected by the above method and were analyzed for their relative DNA scission activity. Three UV active peaks, associated with biological activity were identified as the major chromophor and its chemical derivatives which formed during the hydrolysis of the antibiotic.

The above HPLC process could be extended for the collection of larger quantities of the chromophor[23]. For this purpose 500 ml methanolic solution, equivalent of 6.5 mg neocarcinostatin chromophor, was injected. The mobile phase was modified and consisted of 35 to 100% methanol containing 0.1% triethanolamine and 0.1% acetic acid in the solvent of 20% aqueous methanol containing 0.1% triethanolamine and 0.1% acetic acid delivered with a concave gradient program.

BLEOMYCINS

Bleomycins are a family of glycopeptide antibiotics[24] and are used effectively against various human neoplasms,

especially against squamous cell carcinoma and sarcoma[25]. Pharmaceutical preparations used in treatment of these neoplasms contain several members of the bleomycin family of antibiotics. Because of the different toxicity of these different bleomycins the composition of the clinically used preparations must be controlled. To assess the exact composition of the preparations, various analytical methods were developed. Until recently the accepted method was based on a lengthy CM-Sephadex column chromatographic procedure[26]. This method has been replaced by a much faster and more precise HPLC method[27] which is able to separate 10 components of the clinically used bleomycin preparation. The method proved to be suitable for quantitation of most of these components. Some analytical values obtained with this HPLC method are shown in Table 2, in comparison with the CM-Sephadex column chromatographic method. A typical separation profile of the bleomycins is shown in Figure 2. The chromatographic conditions were as follow: Instrument, Waters Associates Model 6000, equipped with a valve type injector and constant flow pump; UV detector, Model 440, operated at 254 μm; column, μBondapak C_{18} or Chromegabond MC-18; solvent, methanol-0.005 M 1-pentanesulfonic acid in 0.5% acetic acid, pH 4.3, linear gradient in methanol 10 to 40%, gradient mixing time 60 min; chromatographic time, 75 min; flow rate, 1.2-1.8 ml/min.

A previous attempt to analyze pharmaceutical preparations of bleomycin was described by T. T. Sakai[28]. This HPLC system

Table 2. Comparison of analyses of bleomycin samples by HPLC method[27] and by Sephadex column chromatography (CFR method)[26]

	Bleomycin A, % in sample			Bleomycin B, % in sample		
Sample	CFR Method	HPLC Method	SD	CFR Method	HPLC Method	SD
1	68.43 (64.15)[a]	62.30	0.46	28.33	28.10	0.80
2	67.60 (63.82)[a]	63.16	1.12	28.54	29.49	1.25
3	66.52	59.50	1.26	30.85	28.48	0.77
4	65.78	60.33	0.85	31.39	29.89	0.66
5	69.27	65.12	--	26.63	29.30	--
Av. SD			0.92			0.87

[a]Calculation after HPLC separation.

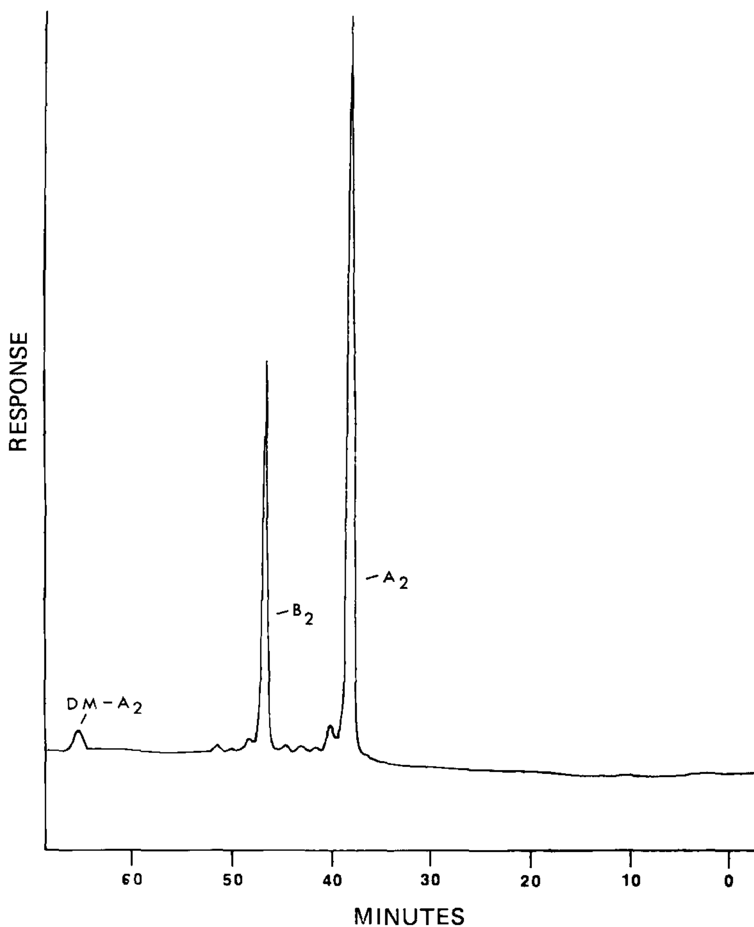

Figure 2. Separation profile of a commercial bleomycin preparation as performed by the method of Aszalos et al.[27].

could not resolve the minor components of such preparations. The described HPLC system was as follows: Instrument, Waters Associates Liquid Chromatograph Model ALC-202/6000; UV detector, Model 440, operated at 254 or 280 nm and a variable wavelength absorbance detector; column, μBondapak C_{18}; solvent, 5 m*M*

1-heptane sulfonic acid in 50% aqueous methanol, pH 8.3; flow rate, 0.7 ml/min (1800 psi). This system provided an inferior chromatographic profile to that shown at Figure 1.

Vos et al.[29] used HPLC for the analysis of synthetic products of bleomycins. These authors prepared different demethyl bleomycin A_2 cobalt complexes. Products of individual reactions were analyzed for comparative yields by HPLC, and were also separated for chemical analyses by this technique. Experimental conditions given in the literature were as follows: Column, Nucleosil C_{18} (Chrompack); solvent, 1% ammonium acetate-methanol (6:4); flow rate, 2 ml/min; injected amount, 1 mg.

Another previous HPLC method designed to isolate individual components of the bleomycin family of antibiotics was described by Rzeszotarski et al.[30]. This described system was used to separate bleomycin A_1, B_2 and A_2 components. The separation time was about 4.5 hours and a flow rate gradient was employed to accelerate the elution of the last component, bleomycin A_2, from the column. The bleomycins could also be analyzed in biological fluids, e.g. in urine[31]. To separate interfering materials present in urine, a 1.0 ml urine sample containing bleomycin was passed through a Sep-Pak C_{18} cartridge and the cartridge was washed successively with water, acetone, water and methanol. The bleomycins were then eluted with 2 ml of 0.02 M sodium heptane sulfonate in methanol.

Aliquots of this eluate were injected into the analytical HPLC column. Chromatographic conditions were as follows: Instrument, Waters Associates Model M-6000A, with a loop type injector (Model U6K; UV detector (Model 440) operated at 254 μm; column, μBondapak C_{18}; guard column (3.9 x 60 mm), filled with pellicular reversed-phase packing of CO:Pell ODS; solvent, methanol-acetonitrile-0.0085 M sodium heptane sulfonate-acetic acid (30:10:59:1); flow rate, 2.0 ml/min (2500 psi). The recovery of bleomycin A_2 and B_2 from spiked urine samples was very good, with an average coefficient of variation of 7.4% and a relative error of 5.6%. The recovery from the cartridge was, at maximum, 85% if the cartridge was reconditioned with 0.02 M sodium heptane sulfonate in methanol.

MITOMYCIN

Mitomycins are produced by a number of strains of streptomycin[32] and one member of this antibiotic family, mitomycin C, is of clinical interest as an antineoplastic agent[33]. Different chromatographic systems were developed for separation of the members of this antibiotic family, for quantitation of mitomycin C in biological fluids, for studying its mode of action, and for its quantitative determination in different pharmaceutical preparations.

Mitomycins and their chemical conversion products were successfully separated by HPLC technique in the course of

studying their interaction with nucleophiles in aqueous medium[34]. The following chromatographic conditions were used: Instrument, Waters Associates Model ALC/GPC 242, equipped with a Model 660 solvent flow programmer; UV detector, Model 202, operated at 245 μm; columns, Corasil II (61 cm x 2 mm) and μPorasil (30 cu x 4 mm); solvent, chloroform-methanol (9:1); flow rate, 1 ml/min (100 psi). For preparative work the μPorasil column was used with the solvent chloroform-methanol (92:8).

With the above analytical system, mitomycin A eluted at 2 min, mitomycin B at 10.5 min, mitomycin C at 18.3 min and sodium-7-amino-mitosane-9a-sodium sulfonate at 40 min. Several reduced mitomycin derivatives could be collected for analysis from the preparative HPLC system. The retention time of these compounds correlated well with their polarity, indicating the presence of an ideal distribution system.

A quantitative HPLC assay was worked out for the determination of mitomycin C in serum by Kono et al.[35]. Samples were prepared by ethyl acetate extraction of the serum, urine or ascites, followed by evaporation to dryness and injection into the chromatograph in methanol solution. Standard curves were prepared by using spiked body fluid samples. A linear relation was obtained between 1 and 25 μg mitomycin injected and the detector response. Chromatographic conditions were as follows: Instrument, Waters Associates Model 204; UV

detector, Model 440, operated at 365 nm; column, μBondapak C_{18} (8-10 μm particle size), 300 x 3.8 mm; solvent, methanol-water (35-65); flow rate, 1 ml/min (1800 psi). The retention time for mitomycin C was 7 min. Using this sytem the authors could determine mitomycin C, at concentrations as low as 40 μg/ml concentration in biological fluids and could follow the concentration of this compound in serum of treated patients.

An excellent biochemical work establishing the mode of action of mitomycin C was published by Tomasz and Lipman[36], who used an HPLC method to separate nanomole quantities of the different metabolites and derivatives of mitomycin C[37]. Conditions were as follows: Instrument, Waters Associates Model 204; UV detector, operated at 254 μm; column, Ultrasphere-ODX (Beckman) 10 x 250 mm (semi-preparative); solvent, acetonitrile-0.03 M potassium phosphate (12.5:87.5), pH 6.0; flow rate, 2 ml/min (1100 psi). The separation profile of these compounds is shown in Figure 3.

Two metabolism studies of mitomycin C also used HPLC separation methods[38, 39]. These studies employed the following HPLC conditions: UV detector, operated at 365 and 313 nm; column, 100 x 3 mm C_{18} (10 μm) radial compression cartridge filled with a 70 x 21 mm guard column packed with CO:Pell ODS; solvent, linear gradient of 0 to 50% methanol in 0.01 M phosphate, pH 7.0, gradient time 13 min; flow rate, 3

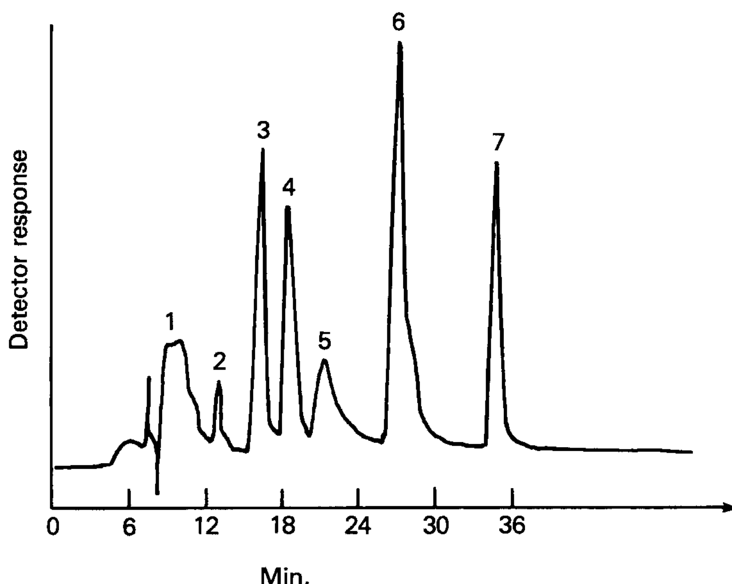

Figure 3. Separation profile of mitomycin and its metabolite by the method of Tomasz and Lipman(37). 1: Mixture of 1,2-cis- and 1,2-trans-2,7-diaminomitosene 1-phosphate; 2: 1,2-trans-1-hydroxy-2,7-diamino-10-decarbamoyl mitosene; 3: 1,2-cis-1-hydroxy-2,7-diamino-10-decarbamoyl mitosene and 1,2-trans-1-hydroxy-2,7-diaminomitosene; 4: 2,7-diamino-10-decarbamoyl mitosene; 5: 1,2-cis-1-hydroxy-2,7-diaminomitosene; 6: 2,7-diaminomitosene and 10-decarbamoyl mitomycin C; 7, mitomycin C. Quantities are in the 1-10 nanomole range.

ml/min. With this technique 9 mitomycin derivatives and mitomycin C could be resolved.

For the purpose of quantitatively assaying the mitomycin C content of pharmaceutical preparations an HPLC assay was developed by Aszalos et al.[40]. Bulk mitomycin C preparations were dissolved to a concentration of 0.4 mg/ml. Injectable samples were dissolved in water and extracted with

ethyl acetate, the ethyl acetate solution was taken to dryness and the residue was dissolved in methanol. From these methanol solutions of mitomycin C, 1 to 8 ng quantities were injected into the chromatograph and the areas under the peak, obtained by a microprocessor, were compared to those obtained with standard mitomycin C solutions. The assay has a standard deviation of less than 1%. Chromatographic conditions were as follows: Instrument, Waters Associates Model 6000 equipped with valve type injector; UV detector, Model 440, operated at 254 nm; column, µBondapak C_{18}; solvent, methanol-water (35-65); flow rate, 1 ml/min (1200 psi). This HPLC system is designed to replace the present microbiological method which was adopted for official use earlier.

A detailed paper by Tjaden et al.[41] deals with the comparison of several normal phase and reversed phase HPLC systems for the use of pharmacokinetic studies of mitomycin C. The compound was detected by ultraviolet and polarographic detectors and a simple procedure for its isolation from plasma, serum and urine was described. This isolation process was based on adsorption of mitomycin C on Amberlite XAD-2 resin and elution by methanol after a water wash of the resin. The dried sample was dissolved in mobile phase; porfiromycin (1 µg/ml) was added as internal standard, and the solution was injected into the chromatograph. Chromatographic conditions were as follows: Instruments, Waters Associates 6000A and Spectra Physics Model

740B; injector Model U6K and Model 7125 (Rheodyne); detectors, variable wavelength detector, LC-UV3, Pye-Unicam operated at 360 nm and polarographic detection system, PAR-310 (EG and G Instrument) cell, polarograph Model E100 (Bruker) hanging mercury drop electrode, 600 mV versus the silver/silver chloride reference electrode; columns, stainless steel, 3 x 100 mm, filled with Hypersil MOS as silica SI-60 (63-200 μm); solvents, ethyl acetate-methanol-water-dichloromethane (97:2:1:1) for normal phase and 10-12% acetonitrile in 0.05 M phospate buffer, pH 7.0, or 10% acetonitrile in water for the reversed phase column; flow rate, 0.5 to 2.0 ml/min.

The sensitivity of the above system was 5 mg/ml. The normal phase column was found more stable, less expensive and more suitable for electrochemical detection, and was used for detecting mitomycin C metabolites. The reversed phase system was used for rutin analysis.

STREPTOZOTOCIN

Streptozotocin, 1-methyl-1-nitrosourea-2-deoxyglucose, shows promise in the treatment of pancreatic islet cell carcinoma[42]. During the clinical trials of this drug, the amount of unchanged streptozotocin in serum had to be assessed[43]. One of the analytical methods used for this purpose was HPLC. In this method identification of the streptozotocin peak was based on specific colorimetric analysis

of the eluted materials, ^3H and ^{14}C-labeled streptozotocin, and UV analysis. Performance of the HPLC system was checked each time by measuring retention time of a drug standard (t_R = 17 min). Samples were prepared for analysis as follows: plasma proteins were removed by ethanol precipitation, the supernatant was adjusted to pH 4, and urine was lyophilized, taken up in methanol-acetone (3:1) and centrifuged. From the plasma or urine samples prepared in this manner, 10 μl was injected into the high pressure liquid chromatograph. Conditions were as follows: Instrument, not described; UV detector operated at 254 nm; column, 3,3'-oxypropionylnitrile (Durapak); solvent, hexane-isopropanol (3:1). The above system gave a linear relation from injected amount of streptozotocin and detector response from 3 to 90 μg/ml.

RIFAMYCINS

Some rifamycin-type antibiotics are reverse transcriptase inhibitors and were implicated in cancer chemotherapy. Below are given some examples of the use of HPLC in the determination of rifamycin and its metabolites in human plasma and in separation of chemical conversion products of rifampicin.

One method described quantitation of rifamycin and its main metabolite, 25-desacetylrifampicin, in plasma by an HPLC system with a silica gel column[44]. An extension of this method described by Lecaillon et al.[45], who were using the same

technique, achieved the same level of detection (0.1 μg/ml), but could also simultaneously assay another metabolite, 3-formyl rifamycin SV. Conditions of this HPLC method were as follows: Instrument, Hewlett-Packard, Model 1011, equipped with Waters Associates U6K valve injector; UV detector, Hewlett-Packard Model 1036, operated at 254 nm; column, 100 x 7.5 mm stainless steel column filled with LiChrosorb Si 60 (5 μm), by a special filling technique. The column efficiency was estimated to be 2000-3000 theoretical plates for the third peak appearing around 8 min elution time; solvent, dichloromethane-isooctane-ethanol-water-acetic acid (36.6:45:16.8:1.65:0.002); flow rate, 3 ml/min. For HPLC analysis, rifamycin and its metabolites were extracted from plasma, urine or saliva and the quantities injected (5-200 μl) were selected according to the approximate concentration of the drug in these body fluids. Precision of the measurements was ± 5%. Calibrations with all four compounds were done every day.

It was believed that this technique would be improved by introducing a reversed phase column for the analysis of the above compounds and an additional metabolite, N-desmethyl rifampicin[46]. Good baseline separation could be obtained for most of the compounds except for rifamycin and N-desmethyl rifamycin. Therefore the system was used to quantitate only rifamycin and 25-desacetylrifamycin. Two chromatographic conditions were used. Conditions of the first system:

Instrument, Hewlett-Packard Model 1084B; UV detector, Model 79870A, operated at 254 nm; column, PP-8, 10 μm, 250 x 4.6 mm, Brownlee Labs; solvent, 0.1 M KH_2PO_4, pH 3.5 with 0.2 M H_3PO_4-acetonitrile (62:38); flow rate, 2 ml/min; temperature, 30°C. Conditions of the second system: Instrument, Waters Associates 6000A; UV detector Model 440, operated at 254 nm; column and flow rate as in the first system; solvent, 0.1 M KH_2PO_4, pH 3.5, 0.2 M H_3PO_4-acetonitrile-water (60:36:4); temperature, 20°C. With the second instrument a Waters Associates W.I.S.P. Model 710A sample programmer was used for rutin analysis. The standard deviations of these determinations were between 3 and 5.8% depending on the compound and its concentration. Limit of detection was 0.2 mg/ml.

An interesting HPLC system was developed for the separation of rifamycin and its metabolites from other drugs, using a single column and sequential elution with different solvents[47]. Depending on which of the solvent was used first, the different drugs present on the column could be eluted and quantitated. With one of the solvent systems, (A), 4,4'-diaminodiphenyl sulfone and its metabolites could be analyzed; with the second solvent, (B), rifamycin and clofazimine could be analyzed simultaneously; with a third solvent, (C), clofazimine could be quantitated alone. Chromatographic conditions were as follows: Instrument, Waters

Associates Model 6000A, with injector Model U6K; UV detection, Jesco Uridec 100 II spectrophotometer; column, μBondapak C_{18}; solvent A, acetonitrile-water (20:8); solvent B, tetrahydrofuran-0.5% acetic acid (40:60); solvent C, tetrahydrofuran-water (50:50, containing 0.0025 M 1-pentanesulfonic acid); flow rate, 2.0 ml/min with solvent system A, 1.5 ml/min with solvent systems B and C. The temperature was controlled in each case at 20 ± 2°C. After solvent A was used for the analysis of the different sulfones, solvent B could be introduced into the system directly. However, solvent B could be followed by solvent A only if methanol water (1:1) was pumped through the system after solvent B. All chromatographic profiles shown in this detailed paper indicate good baseline separation.

With the aim of controlling the hydrogenation reaction of rifamycin an HPLC system was developed by Vlasakova et al.[48]. The system proved to be useful to quantitatively assess rifamycin, the quinone form of rifamycin and their dihydro and tetrahydro derivations. Chromatographic conditions were as follows: Instrument, Varian Model 4100; UV detector, Variscan UV spectrophotometer (Varian), 8 μl cell, operated at 334 nm; column, 250 x 2 mm, filled with Micro Pak NH_2 (10 μm); solvent, chloroform-methanol (97:3); flow rate, 0.2-0.7 ml/min. Two other columns tried for the above purpose were found not to be suitable: Micro Pak Si-10 and Pmicro Pak CN

with chemically bonded alkyl nitrile groups. During this study capacity factor and theoretical plate height (H) calculations were made. The formula for the calculation of H was $H=(L/16)(W_t/t_R)^2$ where L is the column length and W_t the peak width at the baseline. Optimal plate heights were 0.5-1.9 mm.

ANTHRACYCLINE ANTIBIOTICS

The most important members of this antibiotic family are the clinically useful adriamycin (doxorubicin)(49) and daunorubicin (daunomycin)(50). Most HPLC studies were therefore done with these two antibiotics, their degradation products and metabolites. A large number of papers have been published on HPLC analysis of these drugs. They can be divided into four groups: quantitation in pharmaceutical preparations, quantitation in body fluids, metabolite studies and study of chromatographic conditions of these drugs.

The official method accepted by the Food and Drug Administration for determination of adriamycin in bulk pharmaceutical preparations is described in the Code of Federal Regulations(51). The method is as follows: Instrument, Waters Associates Model 244 (or equivalent); UV detector, operated at 254 nm; column, μBondapak C_{18}; solvent, water-acetonitrile (69:31), adjusted to pH 2.0 with phosphoric acid; flow rate, 1.5 ml/min; internal standard, 2 mg/ml solution of 2-naphthalene sulfuric acid in the solvent mixture.

This HPLC system was not designed to determine adriamycin and daunorubicin simultaneously. Also the possible impurities, the aglycones adriamycinone and daunorubicinone, could not be assayed. For these reasons Haneke et al.[52] designed an HPLC system in which all four of these compounds could be quantitated simultanenously. The system is not designed to use an internal standard but relies on standard curves obtained with each new chromatographic setup. Conditions were as follows: Instrument, Waters Associates 6001A; UV detector, Model 240, operated at 254 nm; injector, Model U6K, valve type; column, μBondapak C_{18}; solvents, best solvent methanol-0.1 M $NH_4H_2PO_4$ (65:35), pH 4.0, other useful solvent methanol-0.005 M 1-heptanesulfuric acid (62.5-37.5), pH 3.5; flow rate, 1 or 2 ml/min (1000 or 1600 psi). The aglycones could be estimated as low as 0.5 relative % in preparations by this method. Area under the peak was calculated by a microprocessor (integrator 3380A, Hewlett-Packard).

Good separation could be obtained for adriamycin, daunorubicin and a third anthracycline antibiotic, carminomycin, when acetonitrile-0.025 M camphorsulfuric acid, pH 3.8, was used as solvent with a column of 5 μm C_8 Lichrosorb[53].

An assay was worked out for anthracycline concentration in formulated drug products by Averbuch et al.[54], using an aminocyanosilica column. The sensitivity of the assay was about 5 ng/ml. Six anthracycline antitumor agents, all from the

bohemic acid complex, were isolated from fermentation broth by preparative HPLC using a normal phase column[55]. HPLC was shown to be useful in determining stability of anthracyclines in infusion fluids[56]. One other study aimed to determine adriamycin in pharmaceutical preparations was presented by Barth and Conner[57]. A method based on electrochemical detection, applying 0.65 V oxidative potential, to monitor daunorubicin and its metabolite in different body fluids was designed by Akpofure et al.[58], and compared with fluorescence detection. It was found that the electrochemical detection was more sensitive than fluorescence detection and that a sensitivity of 10 ng/ml of compound could be achieved.

Adriamycin and daunorubicin are determined in fermentation broth at different fermentation times in order to determine maximum production yield. For this purpose Alemanni et al.[59] extracted the broth at pH 1.5, and analyzed the extract on a μBondapak C_{18} column using acetonitrile-KH_2PO_4 buffer, pH 3 (citric acid), (7:18) and UV detection at 254 nm. Similar systems were developed by Stroshane et al.[60] using direct injection of the acidified, heated and filtered fermentation broth.

For assaying daunorubicin hydrochloride content in different forms of pharmaceutical preparations the U. S. Pharmacopeia adopted the following HPLC method.[61] Samples are dissolved in a 2 mg/ml solution of 2-naphthalene sulfuric acid containing

a mixture of water-acetonitrile (62:38), adjusted to pH 2.2 with phosphoric acid. Other conditions are as follows: Column, μBondapak C_{18} or equivalent column; detector, UV, operated at 254 nm; solvent, water-acetonitrile (62:38), pH 2.2 (phosphoric acid); flow rate, 1.5 ml/min; resolution factor between daunorubicin and 2 naphthalene sulfuric acid, 2.0; calculation of potency as μg daunorubicin per mg: (25 C/W) (Ru/Rs) where C is the concentration in μg in the standard preparation, W is the weight in mg of daunorubicin hydrochloride and Ru and Rs are the ratios of peak responses of daunorubicin peak to 2-naphthalene sulfuric acid peak obtained with the unknown preparation and the standard preparation, respectively.

An efficient extraction and separation method was worked out by Pandey and Toussaint[62] for the detection of different anthracyclines in fermentation broth. The new extraction method relied on extraction of the mycelium at pH 1.5 into aqueous solution. After this aqueous layer is filtered, it is applied directly to the high pressure chromatographic system. The HPLC system was optimized to separate and quantitate several components of this type of fermentation: daunorubicin, baumycin A_2, daunorubicinone, 7-deoxydihydrodaunorubicinone, E-rhodomycinone and an unknown structure 30-8-1M. The optimized HPLC system was as follows: Instrument, Waters Associates 6000A, with Model 660 solvent programmer and U6K universal injector; detector, Schoeffel SF 770 Spectroflow, operated at

254 nm; column, μBondapak C_{18}; solvent, water (pH 2.0, H_3PO_4)-methanol (35-65 or 40-60), solvents filtered separately and mixed afterwards; flow rate, 2 ml/min. A typical separation profile is shown in Figure 4.

The determination of anthracycline antibiotics in body fluids was described in several papers. One of the earliest papers was that by Hulhoven and Desager[63] who used a quaternary solvent system and adriamycin as internal standard to estimate daunorubicin in plasma. Later Pierce and Jatlow[64] provided an assay for adriamycin and adriamycinol and the two aglycones in human plasma. For internal standard these authors used daunorubicin and daunorubicinone (2 μg/ml each, in methanol). Samples were mixed with internal standards, made alkaline and extracted with five volumes of chloroform-isopropanol (2:1). After separation of the phases the organic phase was taken to dryness and the residue was dissolved in methanol. From this solution an aliquot was injected into the chromatograph. Chromatographic conditions were as follows: Instrument, Perkin-Elmer dual pump Model 601; fluorescence detector, Perkin-Elmer Model 204-S, or Model 650-10LC, operated at 465 nm excitation and 580 nm emission wavelength; column, ODS "Hi-Eff" C_{18}, 5 μm, reversed phase; solvent, acetonitrile-0.01 M phosphoric acid, pH 2.3 (40:60) (for measurements of the aglycones the solvent was acetonitrile-0.01 M phosphoric acid (36-40:64-60)); flow rate, 1 ml/min; temperature, 25°C.

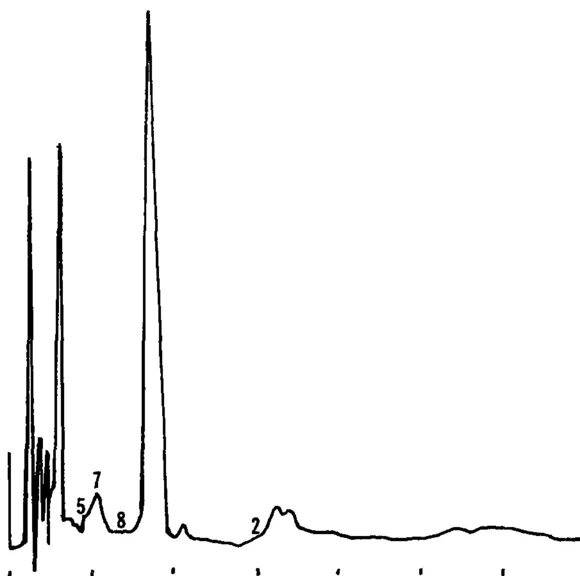

Figure 4. Separation profile of extracted anthracyclines from fermentation broth as described by Pandey and Toussaint(62). 2: daunorubicin; 5: daunorubicinone; 7: 7-deoxydihydrodaunorubicinone; 8: E-rhodomycinone.

Quantitation was based on peak height ratios. In this system t_R for daunorubicin was 18 min and adriamycin, its metabolite and two aglycones eluted before 18 min. It was shown that many other drugs, if also present in the plasma, would not interfere with this assay. Because of the use of the fluorescence detector the sensitivity of this determination could be increased to 2 ng/ml.

Eksborg et al. provided a similar determination for adriamycin and adriamycinol simultaneously[65]. A LiChrosorb RP-2, 5 µm column and the same solvent system as in reference 54

was used. The sensitivity of their measurement was somewhat less than described in reference 54, perhaps because a slightly modified extraction process and different setting of the fluorescence detector (Schoeffel Instrument FS-970) were used.

Another HPLC assay system was aimed to determine adriamycin, adriamycinol, aclacinomycin A and two 4''' isomers of aclacinomycin A, MA-144-171 and MA-144-N1, in rabbit plasma[66]. The extraction of the plasma was very similar to those described in the above HPLC systems used in connection with assays of adriamycin. HPLC conditions were as follows: Instrument, not described; detector, spectrophotofluorometer, operated at 475 nm excitation and 580 nm emission wavelength; column, 5 µm LiChrosorb SI 60; solvent, chloroform-methanol-ammonium hydroxide-water (855:130:10:5) for adriamycin and adriamycinol and chloroform-dimethylsulfoxide-ethylene glycol (975:20:5) for the carminomycin-type compounds; flow rate, 1.1 ml/min; lower limits of sensitivity of the assay, 2-4 ng adriamycin/ml and 10-20 ng/ml aclacinomycin. It is interesting to note that the silica gel adsorption system gives almost equivalent sensitivity to the reversed phase as shown in the above two examples.

Tissue distribution of liposome-entrapped adriamycin was followed by HPLC[67]. A simple extraction followed by HPLC provides quantitation for daunorubicin and adriamycin in tissues[68]. Similarly HPLC assays were developed for

estimation of adriamycin and daunorubicin in urine[69] in lymph and gall[70], carminomycin in human plasma[71] and adriamycin in biological fluids[72].

Two other studies described the determination of daunorubicin and daunorubinicol in plasma of humans administered the parent compound[73] or its DNA complex[74].

Some other very good work should be mentioned here briefly. Brown et al.[75] determined daunorubicin and 7-con-o-methyl nagarol in plasma, using a fluoresence detector, a Pye LC-XPD pump and a LiChrosorb RP-2 (5 μm) column, capable of estimating daunorubicin at 5 μg/ml. Moro[76] et al. quantitated 4'-epiadriamycin and its 13-dihydro derivative in human plasma, also using a fluorescence detector, a Spectra-Physics Model SP 3500 B chromatograph, a Partisil ODS column and a CO:Pell ODS Whatman precolumn, 7 cm x 2.1 mm. S. E. Fandrich[77] developed a system to determine carminomycin in serum. In connection with this assay a very good extraction process developed, starting from human serum and allowing carminomycin and carminomycinol to be measured at 2 μg/ml.

An analytical HPLC system was devised to follow the fate of adriamycin in vivo[78]. This system involved two HPLC columns, one reversed phase and one normal phase. Drug and metabolites were determined in plasma, bile and urine. Sample preparation for HPLC was about the same as in the studies described above, except that a protein precipitation step

(methanol) was introduced before chloroform extraction of the anthracycline compounds at pH 8.5. The chromatographic system was as follows: Instrument, Waters Associates Model ALC/202 and Model ALC/244 for the reversed phase studies; fluorescence detector, Schoeffel Instrument Model SF-970, operated at wavelengths of 482 nm for excitation and 550 nm for emission; column, Partisil-10 PAC for normal phase and Bondapak phenyl for reversed phase; solvent, chloroform-methanol-acetic acid-water (850:150:50:15); initial convex gradient (profile α4) set for 2 min starting with 10% of the above solvent in chloroform and finishing with 100% of the above solvent system, the initial 2 min followed in 6 min with the final solvent to elute all compounds (normal phase system); linear gradient starting at 30% acetonitrile (containing 2% buffer) and 70% pH 4.0 ammonium formate buffer, finishing at 35% acetonitrile and 65% pH 4.0 ammonium formate buffer, gradient time 5 min (reversed phase); flow rate, normal phase 2 ml/min, reversed phase 3.5 ml/min. In each case the column was re-equilibrated with the starting solvent system. The peaks could be quantitated by peak height measurements with the reversed phase system but the "cut and weigh" method had to be employed with the tailing peaks of the normal phase system. Sensitivity of the reversed phase system was 3.0-0.5 μg/ml depending on the compound assayed. The use of the two chromatographic systems was thought to provide an unequivocal identification basis for adriamycin and its

metabolites. It should be mentioned here that compared to the other studies discussed above the design of this system is somewhat complicated, and equally good results were obtained by a single column isocratic solvent system described in the other studies.

Baurain et al.[79] studied the cellular uptake and metabolism of daunorubicin in L1210 leukemic cells and followed the events by HPLC. They were successful in determining concentrations at the level of 1.5 ng/ml. For internal standard adriamycin was used (10 μg/ml), from which 0.1 ml was added to a 2 ml cell suspension. Daunorubicin was extracted by chloroform-methanol (4:1) at pH 7.2 and the organic phase was injected into the chromatograph. The HPLC system was as follows: Instrument, Hewlett-Packard, Model 1084 with a 6-part injection valve, Rheodyne Model 7120, 20 μl loop; detector, Gilson Fl-1A/B fluorimeter, operated at 480 nm excitation and 560 nm emission wavelength; column, LiChrosorb Si 60; solvent, chloroform-methanol-acetic acid-water (720:210:35:30); flow rate, 1 ml/min. The system provided very good calibration curves for daunorubicin, daunorubicinol and the aglycone, with good reproducibility and very well-resolved peaks.

Many other pharmacological studies were conducted with HPLC, in addition to those described above. The HPLC methodologies of these studies are in large part similar to the ones detailed above and will not be quoted here. However, most of them are

listed below with the objective of the study. Metabolism of daunorubicin was studied in sensitive and resistant Ehrlich ascites tumor cells[80] and in rat liver microsomal preparation[81]. The pharmacokinetics of adriamycin were studied in Ehrlich tumor-bearing mice[82] and in gynecologic carcinoma patients[83]. The same type of study was made of 4'-epi-adriamycin in patients with impaired renal function[84] and for aclacinomycin A in cultured L1210 cells[85].

In an excellent paper Eksborg described optimization of the chromatographic conditions for adriamycin, daunorubicin and their 13-hydroxylated metabolites[86]. First the retention value of the compounds (expressed as log k') versus mobile phase composition (log (R), molar concentration of organic modifier) was studied. Using acetone, acetonitrile and ethanol as organic modifiers maxima were found for log k' between 0.8 log (R) and 1.1 log (R) for each modifier and for all four compounds studied. Next the selectivity was studied. For this purpose the log(R) was plotted against separation of the different compounds expressed as the log α (α is the separation factor relative to adriamycinol). Again for each solvent modifier very good separation could be obtained at relatively low concentrations of the studied compounds. Least separation was obtained with ethanol; other alcohols like methanol or propanol did not give any better results. A third factor, the length of the alkyl chain bound on the silica gel support, was studied in

relation to retention time and selectivity when acetonitrile was used as organic modifier. It was found that with increase of the length of the alkyl chain (RP-2, RP-8 and RP-18) the retention time of each component increases but no change in selectivity occurs.

It was deduced from the results of those experiments that the shortest separation time can be achieved according to the function

$$f(\alpha, k'_2) = (4R_s\ \alpha)^2(\alpha - 1)^{-2}(1 - k'_2)^3\ k'^{-2}_2$$

where R_s is chromatographic resolution and α and k' are as defined above. Further considerations yielded the necessary number of theoretical plates for the separation of each antibiotic from its 13-hydroxy derivative.

The chromatographic conditions of these experiments were as follows: Instrument, LDC-711 solvent delivery system pump; Rheodyne Model 70-10 injection valve with a 100 μl sample loop; detector, LDC Spectromonitor (8 μl) operated at 500 nm; column, one 150 and one 50 mm x 4 mm OD, filled with LiChrosorb RP-2, RP-8 or RP-18 (each 5 μm); solvents, 20 to 90% acetonitrile, acetone or ethanol in water; flow rate, 1.7 mm/sec.

USE OF HPLC IN THE ISOLATION STUDIES OF NEWLY DISCOVERED ANTITUMOR ANTIBIOTICS

There are many examples in the literature of the use of HPLC for new antitumor antibiotics, and not all of them can be cited

here. HPLC apparently has proved to be an important tool in the isolation of newly discovered antibiotics. A few typical examples are given.

One example is the work dealing with the detection of the gilvocarcin antitumor antibiotic complex[87]. The original problem concerned a fermentation broth containing an unknown antibiotic complex. To assess the quantity in the broth of the two major components of this complex, 2064A and B, an HPLC system was developed. Conditions were as follows: Instrument, Waters Associates 6000A; UV detector, operated at 254 nm; column, μBondapak C_{18}; solvent, methanol-water (70:30); flow rate, 1.5 ml/min. The retention times obtained for the two major components suggested the presence of gilvocarcin type antibiotics in the unknown fermented complex. Other identification methods were then used which required more material, and a preparative HPLC method was developed for this purpose. The following conditions were used: Instrument, Waters Associates 6000A; UV detector, operated at 254 nm; column, C_{18} Magnum semipreparative, 50 x 9.4 mm column (Whatman); solvent, methanol-water-tetrahydrofuran (40:45:15); flow rate, 5.0 ml/min.

Another example of the use of HPLC in isolation studies of antitumor antibiotics was described by Pandey et al.[88], in connection with the antibiotic fredericamycin. Very small quantities of this antibiotic complex were isolated by two

different methods from the fermentation broth. The most biologically active component in each complex was identified by HPLC studies. The t_R values of the most active component, fredericamycin A, with μBondapak C_{18} or μPoracil columns were 6.5 and 6.0, respectively. Conditions of the HPLC studies were as follows: Instrument, Waters Associates Model 6000A; UV detector, Schoeffel Model SF 770 variable wavelenth, operated at 254 nm; columns, μBondapak C_{18} and μPorasil; solvent, methanol-water-acetic acid (70:30:1) or chloroform-methanol-acetic acid (87:3:3); flow rate, 2 ml/min or 1 ml/min. These HPLC systems also could be used to isolate small quantities of the antibiotic for biological assays.

When concentrations of individual components in an isolated antibiotic complex have to be assessed, HPLC can be a very useful technique, as shown by Argoudelis et al.[89]. This research team had to evaluate a number of fermentation broths for yield and composition of the paulomycin antibiotic complex. In this investigation, a Hewlett-Packard instrument was used to determine ultraviolet spectra of components producing individual peaks in the HPLC chromatogram. These spectra indicated that four components of the isolated complex belong to the same antibiotic type, the paulomycins. Operational conditions were as follows: Instrument, Hewlett-Packard Model 1084B, operated with the dual pump mode; UV detector, HP Model 79875A variable wavelength detector, operated at 320 nm; column, Brownlee 100 x

4.6 mm packed with 10 μm reversed phase filling; solvent, acetonitrile-0.5 M potassium phosphate buffer, pH 7.0 (38:62); flow rate, 2 ml/min; temperature 30°C.

Another example of the use of HPLC in the isolation studies of antitumor antibiotics can be cited in connection with the first isolation of gilvocarcins[90]. These antibiotics were difficult to separate because of solubility problems. The preparative HPLC method designed to solve this problem had the following details: Instrument, Waters Associates Prep LC/system 500; detector, variable wavelength Schoeffel Spectroflow SF 770, operated at 400 nm; column, Prep Pak (two) 500 silica gel column with radial pressure of 40 atm, partially deactivated by passing through 2.5 l of solvent (ethyl acetate-isopropanol-water (87:13:0.5)); flow rate, 100 ml/min; sample 100 to 300 mg crude antibiotic in 500 ml eluting solvent. The antibiotic complex was recirculated 8-10 times until individual members of the complex separated. The fractions finally obtained were assayed for homogeneity by an analytical HPLC system.

A family of new antibiotics active against Ehrlich carcinoma of mice was isolated by Anke et al.[91] and named the deflectins. The initial chromatographic methods used to isolate a crystalline product led the investigators to believe that they obtained a homogeneous antibiotic. However, high pressure chromatography indicated the presence of five major and several minor components in the original crystalline isolate. Since

different fermentations produced deflectin complexes of different compositions, the following HPLC system was developed to analyze the crude products of these fermentations: Instrument, Waters Associates 6000A; detector, Waters 450 and R401, operated at 339 nm; column, reversed phase silica (RP-18); solvent, methanol-triethylammonium formate buffer, pH 6.0 (8:2). No other details were given for this system. Also, the preparative HPLC method used to isolate the individual members of the deflectin antibiotic family was omitted.

Many antitumor antibiotics are isolated from plants. A variety of separation and purification methods, including HPLC, are used in these isolation studies. A good summary of the use of HPLC in these isolation studies was given by Wall et al.[92].

HPLC proved to be an excellent tool in assessing the concentration of the antibiotic produced in a fermentation broth at different times, which is important for the selection of the harvest time. Examples of this approach were described by Tsuji and Goetz[93] who used HPLC to monitor erythromycin and tetracycline fermentations. In another study Ogasawa et al.[94] followed the fermentation of aclacinomycin and related compounds by the organism Streptomycos galilaens. The key factor in this HPLC monitoring study was that the selection of the detector wavelength, 436 nm, put all the cofermented related compounds, aclacinomycin B, MA144-L1, -MI, -NI, -S1, -T1 and

-Dl, on an equal molecular absorbance basis, and therefore on an equal quantitation basis by simple peak area determination. An internal standard was added to an aliquot of the fermentation broth to facilitate calculation of the recovery yield. The samples were prepared for HPLC by extraction of the broth with toluene at pH 7.5, centrifugation and filtration of the toluene layer. The chromatographic conditions were as follows: UV detection: 436 nm; column, μPorasil; solvent, chloroform-methanol-acetic acid-water-triethylamine (68:20:10:2:0.01, v/v); flow rate, 1.0 ml/min (1000 psi). The system provided baseline separation for six fermentation products with symmetrical peaks. Two new anthracycline antibiotics, auramycin and sulfurmycin, were isolated recently(95). To show the novelty of these antibiotics, their sugar moiety was hydrolyzed and the isolated aglycones were compared to those of known anthracyclines by HPLC. The chromatographic conditions of this study were: UV detection, 430 nm; column, μBondapak C_{18}; solvent, methanol-water (60:40), with heptanesulfonic acid; flow rate, 1 ml/min. After showing differences in migration rates of these two new aglycones and those of known ones, the additional physico-chemical studies revealed that these new anthracyclines differ from known anthracyclines by a new combination of side chains on the aglycone moiety.

Five endogenous growth inhibitors isolated from JB-1 ascites tumor were purified by a combination of several methods,

including HPLC[96]. These growth inhibitors were small glycopeptides and were extracted from the tumor by methanol. The methanol-extracted material could be separated into five components by LH-20 column chromatography. The HPLC conditions for the final purification were as follows: Instrument, Hewlett-Packard 1084B and Waters Associates 6000A; detector, Cecil CE 588 UV Scanning Spectrophotometer operated at 280 nm and Model 440; column, Nucleosil 5 μm C_{18} and Nucleosil 5 μm CN; solvents, water-acetic acid (96:4) or 0.1% trifluoroacetic acid in water for 2 min followed by a gradient of 2% acetonitrile per min up to 20% acetonitrile; flow rate, 0.5 ml/min or 1.0 ml/min; temperature, ambient or 40°C. These two HPLC conditions refer to the purification conditions of two growth inhibitors separated by the LH-20 column.

Cadequomycin, a novel nucleoside analog antibiotic, was isolated from fermentation broth by recycling preparative HPLC[97]. In another study[98] the chromophores could be isolated from two peptide-type antibiotics, macromomycin and auromomycin. Among other techniques, like UV spectroscopy and antibacterial spectra, HPLC was used to show that the two isolated chromophores are identical.

USE OF HPLC IN METABOLIC STUDIES OF VARIOUS ANTITUMOR ANTIBIOTICS

The use of HPLC in metabolic studies was already detailed in the discussion of the above antibiotics. However, some studies

along these lines with other antibiotics are discussed as a special group for reasons outlined below.

One assay concerns the analysis of 1 hexylcarbamoyl-5-fluorouracyl (HCFU) and its metabolites, 5-fluorouracyl (FU), 1-w-carboxypentylcarbamoyl-3-fluorouracyl (CPEFU) and 1-w-carboxypropylcarbamoyl-5-fluorouracyl (CPRFU). While FU is therapeutically advantageous and important, CPRFU and CPEFU are connected with side effects. To provide an optimal drug administration schedule, the pharmacokinetic studies required a fast and sensitive monitoring assay. Such an assay was worked out by Kono et al.[99], utilizing HPLC. Specimen samples were prepared by homogenizing the tissue, centrifuging at 7000 g, acidifying with 1 N HCl, extracting with ethyl acetate, taking to dryness and dissolving the obtained residue in methanol. Chromatographic conditions were as follows: Instrument, Waters Associates liquid chromatograph equipped with Model 6000 solvent delivery system and Model U6K injector; UV detector, Model 440, operated at 254 nm; column, µBondapak C_{18}/Porasil (10 µm particle size); solvent, water-tetrahydrofuran-acetonitrile (50:35:15) for the quantitation of HCFU, water-tetrahydrofuran (65:35) for separation and quantitation of CPEFU and CPRFU; flow rate, 1 ml/min (1500 psi). The two-solvent system was necessary because in the first system the retention times of CPEFU and CPRFU were too close for quantitation.

While aclacinomycin belongs to the anthracycline antibiotic family, one assay method connected with it will be described

here to emphasize the importance of HPLC in these studies. It was of importance to develop a sensitive method to assess the concentration of this drug and its metabolite in plasma and serum. A published method[100] for the determination of aclacinomycin in fermentation broth and drug preparations based on normal phase HPLC was found not to be sensitive enough for this purpose. A new method, based on reversed phase HPLC and fluorescence detection, was developed by Ogasawara et al.[101]. In these experiments standard solutions of aclacinomycin and its metabolites MA 144 S1, MA 144 T1 and MA 144 M1 were prepared in the range of 200 to 1000 ng/ml. Then 100 µl of each of the standards was added to 1 ml portions of plasma and these solutions were diluted with water to different concentrations. Efficiency and reproducibility of the extraction process were based on these samples. Aclacinomycin B, a compound not found among the metabolites, was used for the internal standard and was added to the plasma extracts before the HPLC assay. The extraction process consisted of two successive ethyl acetate extractions at pH 7.0, centrifugation, evaporation of the organic layer and addition of the internal standard. The following chromatographic conditions were used: Instrument, Shimadzu, Model LC-3A; detection, fluorescence detector Model RF-500LC, excitation wavelength 435 nm and emission wavelength 505 nm; column, Bondapak alkyl phenyl; solvent, acetonitrile-0.03 _M_ ammonium formate, pH 5.0, (1:1);

flow rate, 1 ml/min (1000 psi); temperature, 21 ± 1°C; recorder-integrator, Shimadzu Model C-RIA Chromatopak. The system provided baseline separation for all metabolites, straight line calibration curves and a sensitivity limit of 20 ng/ml aclacinomycin in plasma.

A highly sensitive assay was required to estimate the pharmacological behavior of the antitumor antibiotics etoposide and teniposide in humans. Such an assay was developed by Strife et al.[102] under conditions which allowed quantitation of these drugs up to 50 ng/ml serum. Fluorescence detection was used with 215 nm excitation wavelength and 328 nm emission wavelength. The detector was a Schoeffel SF-770 fluorescence detector used with cut-off filters at 300 and 320 nm, 7-54 broad band filter and a narrow band pass interference filter, 8.2 nm wide at half height and centered around 328 nm (Spectrofilm). With the solvent, methanol-water (60:40), good quantitation curves could be obtained despite the failure to achieve baseline separation. The two drugs were used as internal standards for each other.

In an elegant metabolic study, Dye and Rossomando[103] have shown that a salvage mechanism exists for the nucleoside antibiotic formycin A. This antibiotic was phosphorylated to the corresponding mono-, di- and triphosphates with the enzyme adenosine kinase. In this metabolic study HPLC played an important role by providing the possibility of assessing the

quantities of the different phosphorylated products of formycin A. Furthermore it was shown[104] that formycin A triphosphate is a substrate of adenylate cyclase enzyme of rat osteosarcoma. The substrate of this enzymatic reaction was separated from the product, 3',5'-cyclic formycin monophosphate and was quantitated by an HPLC procedure.

Biotransformation by microbiological means is a way to obtain novel derivatives of antibiotics. In such a study[105] the antitumor agent 9-methoxyellipticine was converted to the o-demethylated product and to other metabolites. Also, in another microbial biotransformation study[106] hydroxylation occurred and 8- and 9-hydroxy ellipticines were obtained. The yield of this biological reaction was monitored by an HPLC assay. The assay utilized a Bondapak phenyl column and acetonitrile-0.1% $(NH_4)_2 CO_3$ solvent.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-1), 127-204 (1984)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF METAL CHELATES

by

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1. INTRODUCTION

Liquid chromatography, especially ion-exchange chromatography, has been used very successfully for the separation of ionic metal chelates for many years. More recently, ion chromatography, an ion exchange method proposed by Small et al. (1) in 1975 for the determination of ionic species at low concentrations has undergone a period of rapid growth. Fritz (2) has recently published a book on ion chromatography which reviews work done in this field. Classical chromatographic methods for the separation of inorganic species and metal chelates has been reviewed by Michal (3) and, the somewhat more recently developed, reversed-phase extraction chromatographic methods by Cerrai and Ghersini (4) and Braun and Ghersini (5).

The first reported use of modern liquid chromatographic methods or high performance liquid chromatography, HPLC, of metal chelates is generally credited to Huber et al. (6). Rapid improvements in HPLC methodology over the past decade have significantly enhanced the potential usefulness of HPLC for the separation and determination of

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metal chelates and organometallic species. This is being recognized by a rapidly increasing number of scientists in many research areas including; analytical chemists interested in trace metal analysis; inorganic and coordination chemists interested in the separation of metal chelates and organometallic species; environmental chemists interested in speciation studies; biochemists investigating biologically active metal-containing species; and scientists in nuclear medicine trying to prepare organ selective imaging reagents, to name only a few.

Applications of HPLC to organometallic and metal coordination compounds have been reviewed by Willeford and Veening (7,8) and Schwedt (9). A book on liquid chromatography applied to environmental analaysis edited by Lawrence (10) is in press. Wong (11) has reviewed applications of HPLC to radiopharmaceuticals.

The present review stresses applications of HPLC to the separation and determination of metal chelates. Organometallic compounds are not included except where the work pertains to the separation or detection of metal chelates. An example might be the study of a metal specific detector which could be used for both types of compounds. Only selected papers on ion-exchange methods are included which, for example, might discuss a metal selective or specific detection system or involve chelating reagents in the separation process. No attempt was made to cover the field of ion chromatography.

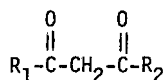
The author has attempted to include all the work reported on applications of HPLC to the separation of metal chelates through 1982 and more recent papers which came to his attention. Because work in this area is published in so many diverse journals, and the emphasis in many papers is not on HPLC, some very relevant work may have been overlooked. To partially compensate for these possible omissions, a

number of papers are cited which bear only indirectly on the HPLC of metal chelates. These include, for example, selected papers where metal chelates are used as modifiers or ion-pairing reagents for the separation of organic solutes. This particular area is growing rapidly and full coverage is outside the scope of the review. It is hoped only that the papers cited will introduce readers not familiar with this area to its potential usefulness.

2. METAL CHELATE SYSTEMS

2.1 β-Diketones

General Structure



Name	Abbreviation	Structure R_1	Structure R_2
1. Acetylacetone	H(acac)	methyl	methyl
2. Trifluoroacetylacetone	H(tfa)	trifluoromethyl	methyl
3. Dipivaloylmethane	H(dpm)	tert-butyl	tert-butyl
4. 1,1,1,2,2,3,3-Hepta-fluoro-4,6-octanedione	H(fod)	n-heptafluoropropyl	ethyl
5. 1,1,1,2,2,3,3-Heptafluoro-7,7-dimethyl-4,6-octanedione	H(hpm)	n-heptafluoro	tert-butyl

7. Pivaloylacetone	H(pacs)	tert-butyl	methyl
8. Thenoyltrifluoroacetone	H(tta)	2-thienyl	trifluoromethyl
9. Furoyltrifluoroacetone	H(fta)	2-furyl	trifluoromethyl
10. Benzoylacetone	H(bza)	phenyl	methyl
11. Benzoyltrifluoroacetone	H(bzta)	phenyl	trifluoromethyl

Huber et al. (6) are generally credited as the first investigators to report on the separation of metal chelates by HPLC. They showed that the metal chelates of Be(II), Cu(II), Al(III), Cr(III), Ru(III) and Co(III) with H(acac) were eluted in the order given in under 25 minutes as a series of symmetrical and well resolved peaks. The elution of the chelates was monitored photometrically at 310 nm using a homemade flow-through photometric cell with a pathlength of 10 mm and a volume of 7.5 μl. A thick-walled glass column (2.7 mm i.d. and 10- or 25- cm in length) packed with diatomaceous earth with particle sizes in the ranges 5-10 μm and 10-20 μm was used. The stationary phase, consisting of the water-rich phase of a ternary two-phase system of water-2,2,4-trimethylpentane-ethanol, was supported on the diatomaceous earth. The mobile phase was the organic-rich phase which was pumped through the column at a constant flow rate of 1.8 mm sec^{-1} with a pressure drop of 17 bars (246 psi). A pre-column was employed to keep the mobile phase saturated with respect to the stationary phase and samples were injected onto the column through a rubber septum.

Although the apparatus used by these authors was somewhat primative compared to modern instrumentation and the non-bonded stationary phases posed some problems, they did report 2,200 theoretical plates for the Co(III) complex and a calculated peak capacity of their column of 14 peaks in 20 minutes. This is quite similar to what has been reported to date for metal coordination compounds by other investigators using more

sophisticated apparatus and modern columns. Several prescient comments were made by these authors. They attributed asymmetric peaks (tailing) for the Ni(II), Al(III) and Fe(III) complexes to hydrolysis reactions and found more symmetric peaks were obtained by including small amounts of the ligand in the mobile phase. Up to three peaks were observed for the Al(III) complex which had been allowed to stand for two weeks before it was injected into the chromatograph. These were attributed to hydrolysis products of the complex and it was suggested mixed hydroxy-acetylacetonato complexes could be separated by this technique. They also reported that the selectivity factor is determined by the metal ion and is nearly independent of the ligand for $Co(acac)_3$ and $Cr(acac)_3$, and $Co(tfa)_3$ and $Cr(tfa)_3$.

Tollinche and Risby (12) later reported on the separation of metal chelates by HPLC with acetylacetone; trifluoroacetyacetone; dipivaloylmethane, H(dpm); and 1,1,1,2,2,3,3-heptafluoro-4,6-octanedione, H(fod). The H(dpm) ligand was incorrectly named as a 2,2,7,7-complex in this paper and it might be noted that other authors have used H(fod) for the compound with R_1 as in Table 1 but R_2 = tert-butyl. The use of alumina, silica gel, polyurethane, and bonded phase column packings was studied and silica gel columns were found to give the best separations. The elution order was $Be(acac)_2$, $Ru(acac)_3$, $Cr(acac)_3$, $Al(acac)_3$ and $Co(acac)_3$ on a 10-µm Partisil column with a 95% CH_2Cl_2 and 5% CH_3CN mobile phase. The separation of cis(or fac) and trans(or mer) geometrical isomers of the unsymmetrical chelates of $Cr(tfa)_3$, $Co(tfa)_3$, $Ru(tfa)_3$, $Rh(tfa)_3$, $Co(fod)_3$ and $Cr(fod)_3$ on 5-µm and 10-µm Partisil and HIEF MicroPart silica columns were reported with various mobile phases including; n-heptane-isopropanol, n-hexane-benzene, toluene,

n-heptane-dichloromethane, n-hexane, and n-pentane. The trans (or mer) isomer always eluted first as expected due to less interaction with the polar silica stationary phase. Some separation of metal-acac complexes was reported on bonded phase columns (C-8 and C-18) but no retention of the metal-dpm or metal-hpm complexes for any mobile phase studied.

O'Brien (13) studied the separation of Ni(II), Fe(II), Cu(II), Mn(II) and Co(II) complexes with H(hfa), H(tfa) and H(hpm) as well as adducts of these species with di-n-butylsulfoxide (DBSO) on columns packed with alumina, Silica Gel H, Partisil A, Kel-F impregnated with DBSO, Corasil, and Corasil-C18 with various mobile phases including toluene, chloroform, ethyl acetate, and methanol-water. He obtained sharp peaks for the Ni(II), Mn(II) and Co(II) complexes with H(hfa) with the Corasil-C18-ethyl acetate system but no separation. A good separation of the $Ni(hfa)_2$ and $Mn(hfa)_2$ complexes was obtained on a Porasil A column with ethyl acetate as the mobile phase. Separation of the $Cu(hfa)_2$-2DBSO, $Ni(hfa)_2 \cdot 2DBSO$ and $Mn(hfa)_2 \cdot 2DBSO$ complexes was observed on the Corasil-C18 column with toluene as the eluent but the column was very inefficient and the peaks were very broad. Well shaped peaks but little separation was found for the Fe(II), Co(II), Ni(II), Mn(II) and Cu(II) complexes with H(hfa) on a 35-50 μm Porasil A column with ethyl acetate as the mobile phase. Linear calibration plots were obtained over the range 0.5-600 ng of metal injected with photometric detection at 300 nm when the chelates were injected one at a time.

Uden et al. (14) reported the mer and fac complexes of Co(III) and Cr(III) with various unsymmetrical β-diketones including H(tfa), H(bza), H(pac) could be separated by HPLC. The mer complexes of $Co(bza)_3$ and of $Cr(pac)_3$ eluted from a 10-μm Partisil column with

dichloromethane-acetonitrile as the mobile phase before the fac complexes. These authors used atomic emission spectroscopy with a DC plasma source to selectively detect metal containing species eluted from the column as well as photometric detection at 254 nm. The mer and fac species were collected and further characterized by mass spectrometry. The separation of geometrical isomers of the mixed ligand species of $Cr(tfa)(hfa)_2$ and $Co(tfa)(hfa)_2$ was also reported.

Schwedt (15) reported a detection limit of 150 pg of beryllium based on the elution of $Be(acac)_2$ from a 7-μm silica column with photometric detection at 254 mm. Willett and Knight (16) determined chromium in orchard leaves by HPLC based on the elution and photometric detection of $Cr(acac)_3$ and claimed a detection limit of 1 ng. These authors reported that $Cr(acac)_3$ was irreversibly bound to active silanol groups on a μ-Porasil column but that reproducible results were obtained on a μ-Bondapak C18 column with 66% water- 36% acetonitrile as the mobile phase. Numerous other well defined peaks in the chromatograms obtained were attributed to other metal acetylacetonates but these were not identified. The identify of the chromium complex was confirmed by mass spectrometry.

Gurira and Carr (17) recently reported on the liquid chromatographic separation of a number of kinetically inert metal acetylacetonates and benzoylacetonates on bonded-phase columns (25-cmx4.6mm i.d., 5-μm Ultrasphere-ODS and a 15-cmx4-6 mm i.d., 5μm-Supelco-C18) with acetonitrile-water and methanol-water as mobile phases. The separation of seven complexes in less than 12 minutes is shown in Figure 1. The resolution was found to increase as the acetonitrile to water ratio decreased and the iridium and ruthenium acac complexes, which overlapped under the conditions shown in Figure 1,

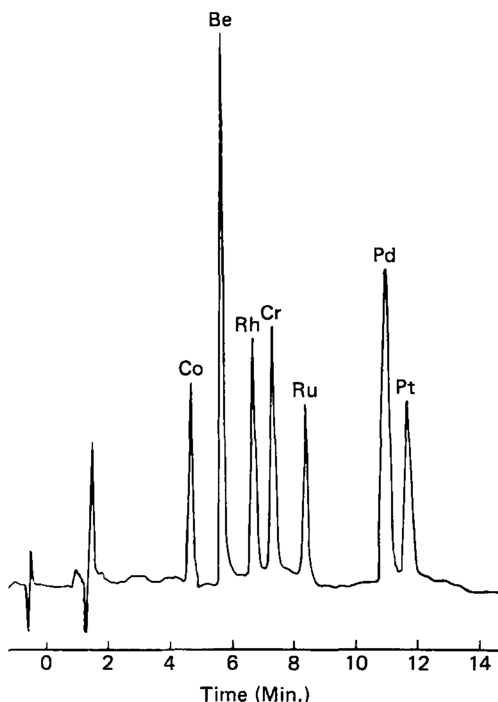


Fig. 1. HPLC separation of ACAC metal complexes on an Ultrasphere C_{18} column, 25-cm x 4.6-mm i.d., 5-μm particle; solvent, 40% acetonitrile/water; flow rate, 2 ml/min; detection at 254nm; 0.04 AUFS; sample, 20 μl of 1 x 10^{-5} in chelates. Reprinted by permission from Reference 17 (Preston Public.).

could be partially resolved with a 30-70 acetonitrile-water mobile phase. Mixtures of $Cr(acac)_3$ and $Cr(bza)_3$ gave two well defined peaks with the acac complex eluting first. Linear calibration curves were obtained over two orders of magnitude for the $Cr(acac)_3$ and $Co(acac)_3$ complexes with the curves passing through the origin indicating no decomposition or irreversible sorption at low

concentrations. Detection limits of less than 1 ng for both complexes were reported.

Yammamota et al. (18) reported on the gel chromatography of $Cr(acac)_3$ and $Co(acac)_3$ on styrene-divinylbenzene copolymer columns (TSK G-1000H, pore size 10^1A). Calibration curves of molar volume versus K_D were prepared based on the elution of Polystyrene 4000 (K_d taken as = 0 for this species) n-docosane, n-hexadecane, n-decane and n-pentane (K_d = 1). The authors noted that Irving (19) found partial molar volumes of $Cr(acac)_3$ of; 266.7 ml in benzene; 269.3 ml in tolune; 256.8 ml in carbon tetrachloride; and 269.5 ml in chlorofrom which were not too different than the molar volume of 273.3 for $Cr(acac)_3$ in the solid state. The elution of $Cr(acac)_3$ and $Co(acac)_3$ from the above column, however, gave effective molar volumes far different in many cases than those reported by Irving. Values obtained for the $Cr(acac)_3$ and $Co(acac)_3$ molar volumes, respectively, were, 250 and 240 ml in benzene; 160 and 140 ml in toluene; 120 and 110 ml in carbon tetrachloride; 350 and 340 ml in chloroform; and 160 and 140 ml in p-xylene.

The variation in K_d (or effective molar volume) of these chelates on gel chromatography with these different solvents was attributed to sorption effects on the column. An attempt was made to rationalize the variation in K_d with solubility parameters of the solvents, the complexes, and the gel and to rationalize the effective molar volumes found with solvation of the complexes in some solvents. The lower plate counts obtained for the complexes (N=230 to 3,000) than with n-alkanes ($N \sim 7,000$) together with the molar volume data do indicate adsorption effects are important with the non-polar solvents and are probably the reason for the larger differences in K_d values observed for the complexes with p-xylene.

Noda et al., (20) used gel chromatography on a polystyrene-divinylbenzene polymer (Shodex 801 gel, 10-15 μm) and an ethyl acetate mobile phase to isolate the mixed ligand complex Be(acac)(tta) from $Be(acac)_2$ and $Be(tta)_2$. Three well resolved peaks were obtained using UV detection at 308 nm and $Be(tta)_2$, Be(tta)(acac) and $Be(acac)_2$ eluted in the order given. The mixed ligand complex was eluted without dissociation and was characterized by its UV and H-NMR spectra.

Saitoh and Suzuki (with others) (21-24) had previously published a series of papers on the gel chromatography of β-diketones and their metal complexes. They found that $Cr(acac)_3$ and H(acac) were eluted from a Merckogel OR-2000 column with chloroform as the mobile phase as two well separated peaks (21). Retention behavior was reported to be due entirely to the sieve effect and K_d values were independent of concentration and column temperature with no evidence of adsorption on the stationary phase. In a later paper (22) these authors extended this study to other metal complexes: $Co(acac)_3$, $Fe(acac)_3$, $Cr(acac)_3$, $Al(acac)_3$, $Cu(acac)_2$, $Ni(acac)_2$ and $Be(acac)_2$. A general correlation of increasing retention with decreasing molar volume was found for H(acac) and the metal complexes on a Merckogel OR-2000 column with tetrahydrofuran as the mobile phase but these species fell on a different curve than the n-alkanes. Skewed peaks were observed for Cu(II) and Ni(II) complexes indicating on-column decomposition or adsorption. In a later paper (23), other mobile phases were studied. Effective molar volumes greater than reported by other methods were observed for the trivalent metal complexes in chlorofrom except for $Fe(acac)_3$. Other solvents studied included 1,4-dioxane, benzene, toluene, tetrahydrofuran, ethylacetate, acetone, ethylmethyketone,

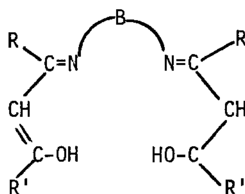
butylacetate, and methanol. Effective molar volumes for the trivalent chelates and $Be(acac)_2$ generally decreased in the order given for these solvents with K_d values greater than one for the last four solvents. Appreciable interaction of the metal chelates with the stationary phase is indicated which appeared to be quite dependent on the solvent. The same authors (with Shibukawa) (24) studied the gel chromatography of seven different β-ketones including; H(acac), H(tta), H(tfa), H(fta), H(bza), H(bzta), and H(dpm), and the Cr(III) chelates of all these species on two polyvinylacetate gels (Merckogel OR-PVA 500 and Merckogel OR-PVA 2000) and two polystrene gels (Bio-Beads S-X8 and Poragel 60A) with p-dioxane as the mobile phase. K_{av} values based on the relationship,

$$K_{av} = (V_e - V_o)/V_x$$

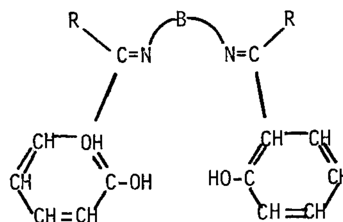
were found for all species studied. V_e is the elution volume of the solute, $V_x = V_t - V_o$ is the volume of the swollen gel phase, V_t the total column volume and V_o the retention volume of polystyrene (200,000 mol. wt.). The K_{av} values were found to depend strongly on the gels and could not be correlated with the molecular weights of the compounds. The dependance of the β-diketones and the metal chelates on the gel was different than for the n-alkanes as found in an earlier paper (22).

2.2 β-Ketoamines

General Structures



I



II

Name	Abbreviation	Structure
1. bis(acetylacetone)ethylene-diimine	H_2(bae) (Ref 25,26; H_2enAA_2) (Ref 27; H_2enAA)	I. R=R'=CH_3, B=ethylene
2. bis(salicylaldehyde)ethylene-diimine	H_2(salen) (Ref 25,26; H_2enSal_2)	II. R=H
3. bis(acetylacetone)propylene-diimine	H_2(bap) (Ref 26; H_2pmAA_2) (Ref 27; H_2(tm)AA)	I. R=R'=CH_3, B=propylene
4. bis(acetylacetone)butylene-diimine	H_2(bab) (Ref 26; H_2bmAA_2)	I. R=R'=CH_3 B=butylene
5. bis(phenylacetylacetone)-ethylenediimine	H_2(bpae) (Ref 27; H_2(en)BA)	I. R=CH_3 R'=phenyl B=ethylene
6. bis(trifluoroacetylacetone)-propylenediimine	H_2(btfap) (Ref 12; H_2(pntfa)	I. R=CH_3,R'=CF_3 B=propylene
7. bis(trifluoroacetylacetone)-ethylenediimine	H_2(btfae) (Ref 12,26; H_2(entfa))	I. R=CH_3,R'=CF_3 B=ethylene
8. bis(trifluoroacetylacetone)-butylenediimine	H_2(btfab)	I. R=CH_3-R'=CF_3- B=butylene

The ligands called β-ketoamines (7,12,25,26) are Schiff bases formed by the condensation of ethylenediamine, propylenediamine or butylenediamine with acetylacetone, trifluoroacetylacetone, phenylacetylacetone or salicyaldehyde. The separation of metal chelates with these ligands by HPLC was studied by several groups.

Uden and Walters (25) studied the separation of Ni(bae), Cu(bae), Ni(salen) and Cu(salen) on 10-μm Partisil columns using 80%

methylenedichloride-20% acetonitrile as the mobile phase. The Ni(bae) and Cu(bae) were eluted in the order given and well resolved. The Ni(salen) and Cu(salen) were also eluted in the same order with greater retention and resolution. The nature of the species eluted was confirmed by mass spectroscopy, ultraviolet spectroscopy and the Cu(salen) species by electron spin resonance spectroscopy. Peak areas were proportional to amount of chelate injected over the range 25 ng to 240 μg of chelate based on photometric detection at 254 nm. The peaks were rather broad and the Cu(salen) peak tailed. Plate counts with N ranging from 309-493 were reported.

The above authors (with Parees) (26) reported on the chromatography of Cu(II), Ni(II) and Pd(II) complexes of H_2(bae), H_2(salen), H_2(bap), H_2(bab) and H_2(btfae) using a 10-μm Bondapak-C18 column with methanol-water-acetonitrile mobile phases. The Ni(II) complexes with H_2(bae), H_2(bap) and H_2(bab) were well resolved using a 55% methanol-45% water mobile phase and eluted in the order given showing greater retention as methylene groups were added to the bridging group B in structure I. The same order was observed for the Cu(II) chelates with the latter complexes all having longer elution times than their Ni(II) analogs. The Pd(II) complex with H_2(bae) generally eluted between the Ni(II) and Cu(II) complexes but its elution behavior relative to these complexes varied with mobile phase composition. With 50% methanol-50% water Pd(bae) eluted with the Ni(bae) complex while with 20% acetonitrile-80% water it eluted after the Cu(bae) species. The elution order of the Cu(btfae), Cu(btfap) and Cu(btfab) complexes increased in the order given with a 50% acetonitrile-50% water mobile phase and the Ni(II) complexes with the same ligands followed a similar pattern. The Ni(II) species all eluted after the Cu(II) analogs which

is the reverse of what was observed for the H_2(bae), H_2(bap) and H_2(bab) complexes. The Cu(salen), Ni(salen) and Pd(salen) complexes eluted in the order given with a 20% acetonitrile-80% water mobile phase. A successively higher water content was required to resolve the metal complexes of H_2(bae), H_2(btfae), and H_2(salen). It was concluded that the H_2(bae) complexes showed an anomalous elution behavior in the reversed phase mode in that the elution order was the same (Ni(bae) before Cu(bae)) as on a silica column. The elution order of the H_2(btfae) and H_2(salen) metal complexes were in reverse order from those of the H_2(bae) metal complexes on the C18 column.

Gaetani et al. (27) reported on the chromatography of Co(II), Ni(II), Cu(II) and Pb(II) complexes with H_2(bae), H_2(bap) and H_2(bpae) on both C18 (10 μm-Micropak CH) and a 3- aminopropyltri-ethoxysilane bonded-phase column (10 μm-NH_2). The elution order of H_2(bae), Co(bae), Ni(bae) and Cu(bae) was reported for the C18 column with a 65% methanol-35% water mobile phase buffered at pH 7.8 with a phosphate buffer. The Pd(bae) peak overlapped the Ni(bae) peak. The elution of Ni(bae) before Cu(bae) was the same (anomalous?) order observed by Uden et al. (26) on a C18 column. The Cu(II) complexes with H_2(bae), H_2(bap) and H_2(bpae) eluted in the order given with a 50% methanol-50% water mobile phase buffered at pH = 7.8. The retention volume for the H_2(bpae) complex with Cu (II) was considerably greater (26.5 ml) compared to the other ligands due to the effect of the phenyl ring. The Cu(bae) and Ni(bae) peaks overlapped on the C18 column and could not be resolved but were well separated on the -NH_2 column and eluted in the order Cu(bae) before Ni(bae) with a 40% methanol-60% water mobile phase buffered at pH 7.8. The areas of the Ni(bae) and Cu(bae) complexes (injected separately) increased in a linear manner

with concentration and detection limits of 0.2 and 0.5 ng of metal injected for Ni and Cu, respectively, were reported. Plate counts reported varied from 346 to 940 and the peaks appeared symmetric.

Tollinche and Risby (12) also studied the separation of Ni(II) and Cu(II) complexes with H_2(btfae) and H_2(btfap) on a silica column (5 µm-HIEFF Micropart) using mobile phases of methylenedichloride with 20% n-heptane, with 10% n-heptane and with 1% acetonitrile. In all cases the elution order was Ni(btfae) before Cu(btfae) and Ni(btfap) before Cu(btfap).

Walters (28) reported on the use of multiple linear regression analysis to predict retention behavior as a function of solvent compositions for Cu(II), Ni(II) and Pd(II) complexes with H_2(bae) and H_2(bap) on µ-Bondapak C18, Partisil-ODS, and Alltech RP-8 columns (all 10-µm particle size). The retention data were fit to the equation,

$$t = a + bx + cy,$$

for ternary mobile phases consisting of methanol-water-acetonitrile. The retention time for the peak is given by t; a, b, and c are the experimentally determined multiple linear regression coefficients, x is the percentage methanol and y is the percentage acetonitrile. An abnormally high coefficient, b, for the Pd(bae) complex on the µ-BondapakC18 column accounts for the observed behavior of Pd(bae) in binary systems where it elutes with Ni(bae) with a water-methanol mobile phase and with Cu(bae) with a water-acetonitrile mobile phase. Use of the above equation permits calculation of the ternary solvent composition 21% methanol-21% acetonitrile-58% water for the elution of Pd(bae) halfway between the Ni(bae) and Cu(bae) complexes.

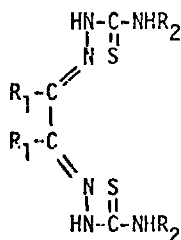
Calculations show that Ni(bae) and Cu(bae) can reverse their order of elution with certain compositions of the mobile phase consisting of

methanol-water-acetonitrile but there is no possibility of reversal on the RP-8 column in the system methanol-water-tetrahydrofuran. This approach was extended to the quarternary system methanol-water-acetonitrile and tetrahydrofuran. Modifier strength decreases in the order tetrahydrofuran, acetonitrile to methanol.

Calligares et al. (29) have reviewed the structural aspects of metal complexes with H_2(bae) and H_2(salen). Both tetradendate complexes tend to have the four donor atoms coplanar with only small deviations toward tetrahedral geometry. Axial coordination positions in the solid complexes for the Cu(bae) and Cu(salen) complexes are filled with water or another Lewis base. The (salen) ligand also gives dimeric species which have not been observed with (bae).

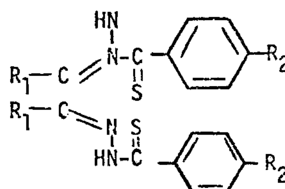
2.3 Thiosemicarbazones, Thiobenzhydrazones, Hydrazones and Dithizone

General Structures



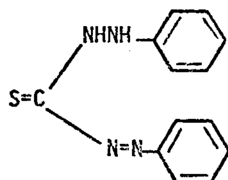
1,2-diketobisthiosemicarbazones

I

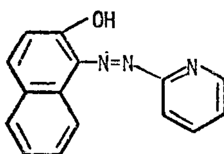


1,2-diketobisthiobenzhydrazones

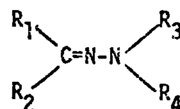
II



III
dithizone



IV
pan



V
substituted hydrazone

Name	Abbreviation	Structure
diacetylbis(thiobenzhydrazone)	$H_2(tbh)$	(II) R_1 = methyl R_2 = H
glyoxalbis(2,2,3,3-tetramethyl butyl)-thiosemicarbazone	$H_2(gbbtc)$	(I) R_1 = H R_2 = 2,2,3,3-tetra methylbuty
diacetylbis(cyclohexyl) thiosemicarbazone	$H_2(dahtc)$	(I) R_1 = methyl R_2 = cyclohexyl
dithizone	H(dz)	(III)
1-(2-pyridylazo)-2-napthol	pan	(IV)
pyridene-2-aldehyde-2-quinolylhydrazone	pac	(V) R_1=2-pyridyl, R_2=R_3=H, R_4=2-quinolyl
substituted hydrazone	L^1	R_1=R_2=methyl, R_3=methyl, R_4=phenyl
substituted hydrazone	L^2	R_1=R_2=ethyl, R_3=methyl, R_4=phenyl
substituted hydrazone	L^3	R_1=methyl R_2=isopropyl, R_3=methyl R_4=phenyl
substituted hydrazone	L^4	R_1=R_2=R_3=R_4=methyl
substituted hydrazone	L^5	R_1=R_2=R_3=methyl, R_4=phenyl
substituted hydrazone	L^6	R_1=H, R_2=R_3=methyl R_4=phenyl

Heizman and Ballschmiter (30) separated Hg(tbh) and Cu(tbh) on a 20-μm Merckosorb SI 60 column with a resolution, Rs, of 1.5 using benzene as the mobile phase and could determine 2×10^{-9}g of Hg and 0.5×10^{-9} g of Cu with photometric detection. With gradient elution the $H_2(tbh)$ chelates of Cu(II), Hg(II), Pb(II) and Zn(II) could be eluted in a reasonable time. In a later paper (31), the chelates of Hg(II), Ni(II), Cu(II), and Pb(II) were found to elute from a 30- μm LiChrosorb SI 60 column in the order given with various

n-heptane-benzene mobile phases. The Zn(II) complex could not be eluted under isocratic conditions. Resolution of various solute pairs varied from 0.5 to 3.2 with different n-heptane-benzene ratios with increasing resolution and longer retention volumes as the benzene concentration decreased. Gradient elution using n-heptane-chloroform as the mobile phase permited the elution of the all five chelates and well resolved peaks were shown for the Hg(II), Ni(II), Cu(II) and Pb(II) chelates. In the same paper (31), the above authors reported on the use of glyoxalbis(2,2,3,3-tetramethylbutyl)thiosemicarbazone), H_2(gbbtc), and diacetylbis(cyclohexylthiosemicarbazone), H_2(dahtc), as ligands. The Hg(II), Cu(II) and Ni(II) chelates with H_2(gbbtc) were eluted in the order given from an alumina (30 μm Alox T) column with benzene as the mobile phase. The Pb(II) chelate decomposed on the stationary phase and the Cd(II), Zn(II), and Co(III) chelates were strongly adsorbed. The Hg(II) and Cu(II) chelates with H_2(dahtc) were separated on both a Li Chrosorb SI 60 and an AloxT column using benzene-3% tetrahydrofuran as the mobile phase. The Pb(II) chelate with H_2(dahtc) also decomposed on the stationary phase and the Zn(II), Ni(II), Co(III), and Cd(II) chelates were very strongly adsorbed.

Gasparrini et al. (32) reported the separation of trans-[PdL_2Cl_2] complexes with various substituted hydrazones, L^1 through L^6, using a bonded phase 10-μm LiChrosorb DIOL column and 88% n-hexane-12% dichloromethane as the mobile phase. These complexes decomposed on unmodified silica columns. Chelates with all six ligands, L^1 through L^6, were separated in a single run and mixed chelates such as $Pd(L^2)(L^5)Cl_2$ could be separated as well. Both isocratic and gradient elution modes were studied. The capacity factor, k, was found to decrease with the chain length of R_1 or R_2 and also depended on the substituent, R_3, on the sp^3-nitrogen.

Schwedt and Budde (33) reported the Cu(II), Ni(II) and Co(III) complexes with pan could be extracted into chloroform, dissolved in acetonitrile after evaporating the chloroform and separated on a RP-2 column (LiChrosorb RP-2) using acetonitrile-water-citrate buffer (80:18:2) at pH=5 as the mobile phase. A Cu(II), Fe(III), Co(III) mixture could be treated in the same way and separated. Apparently Ni(II) and Fe(III) co-eluted. The elution of the chelates was monitored at 565 nm.

Lohmuller et al. (34) reported the elution of the Hg(II), Ni(II), Co(II) and Pb(II) chelates with dithizone on a LiChrosorb SI 60 column using benzene as the mobile phase. The Cu(II), Ni(II) and Zn(II) chelates were not resolved under these conditions but could be eluted individually. The lead peak tailed significantly and the cadmium chelate was strongly absorbed and could only be eluted by using a more polar eluent and still tailed badly. The elution of the peaks was monitored at 525 nm. O'Laughlin and O'Brien (35) obtained similar results on a Corasil and a μ-Porasil column using toluene as the mobile phase. The cobalt complex, assumed to be a Co(III) complex eluted after the lead complex and was fairly symmetrical. The lead complex tailed and apparently decomposed based on the slope of plots of peak area versus amount injected. Poor calibration plots were also obtained for Cu(II) and Hg(II). The elution of the peaks was monitored at 270 nm.

Henderson et al. (36) obtained similar results on the separation of the metal dithizonates to those reported above using aliphatic solvents with polar modifiers as the mobile phase. Results obtained using acetic acid as a modifier were claimed to be comparable to those obtained using toluene or benzene but except for a symmetrical elution peak for the

Pb(II) complex from a Spherisorb GP silica column using 10% hexane-0.2% acetic acid-0.1% butylamine in methylene dichloride as the mobile phase, the results shown do not support this conclusion. Although the authors apparently successfully eluted the $Ni(dz)_2$, $Zn(dz)_2$, $Cd(dz)_2$ and $Cu(dz)_2$ complexes, these peaks were not well separated and it is stated no metal separations were obtained except for that of $Co(dz)_2$ from all the above complexes. These authors also reported the $Hg(dz)_2$ complex decomposed and a peak near the column volume was due in part to a decomposition product of the $Hg(dz)_2$ complex. This is somewhat unexpected in view of the stability of the Hg(II) reported by other

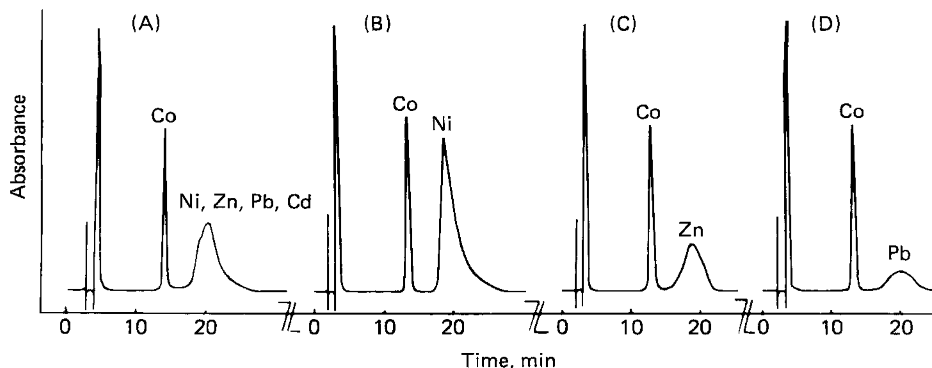


Fig. 2. Chromatograms of the mixture of some metal-dithizonates. (A) 5.2 ng $Co(dz)_3$, 2.50 ng $Ni(dz)_2$, 18.8 ng $Zn(dz)_2$, 52.6 ng $Pb(dz)_2$ and 5.2 ng $Cd(dz)_2$; (B) 5.2 ng $Co(dz)_3$ and 62.0 ng $Ni(dz)_2$; (C) 5.2 ng $Co(dz)_3$ and 47.0 ng $An(dz)_2$; (D) 5.2 ng $Co(dz)_3$ and 131.0 ng $Pb(dz)_2$. Mobile phase: 85%(V/v) methanol-water; Flow rate: 1.0 ml/min; Detection: 550 nm. Column: Shimadzu Zorbax ODS (4.6 mm x 250 mm). Reprinted by permission from Ref. 39 (Japan Soc. for Analytical Chemistry).

authors (37,38) and the symnmetrical peaks observed for the $Hg(dz)_2$ complex by Lohmuller et al. (34) and O'Laughlin and O'Brien (35).

Ohashi et al. (39) reported that Co(II) could be determined by RP-HPLC in the 5.2 to 30 ng range. The Co(II) together with Ni(II), Zn(II), Pd(II) and Cd(II) were extracted into a chloroform solution of dithizone at pH 8.5 in the presence of disodium tartrate. Co(II) was assumed to form the $Co(dz)_3$ complex on extraction while the other metals formed the complexes $Pd(dz)_2$, $Zn(dz)_2$, $Ni(dz)_2$ and $Cd(dz)_2$. The $Co(dz)_3$ complex eluted from a Zorbax ODS column with 85% methanol-15% water as a sharp peak and was well separated from the peaks due to the other complexes which all eluted considerably later and gave broad peaks which tailed to some extent. A typical separation is shown in Figure 2. The significant difference in the elution behavior of the cobalt dithizone complex from those of the other divalent metals shown in Fig. 2 is the best chromatographic evidence for formulating the complex as $Co(dz)_3$.

2.4 Disubstituted Dithiocarbamates

General Structure

R_1, R_2 N-C S SH

B N-C S SH

I	II
Dialkyldithiocarbamic acid	1-Pyrrolidine carbodithioic acid ($B=(CH_2)_4$)

Name	Abbreviation	Structure	
Di-n-ethyldithiocarbamic acid	H(DDTC)	(I)	$R_1=R_2$=ethyl

Benzylmethyl-dithiocarbamic acid	H(BMDTC)	(I)	R_1=methyl R_2=benzyl
Diethoxyethyl-dithiocarbamaic acid	H(DEDTC)	(I)	R_1 = R_2 = ethoxyethyl
Dihexyldithio-carbamic acid	H(DHDTC)	(I)	R_1=R_2=hexyl
bis(n-butyl-2-naphthyl-methyldithiocarbamate	H(BNMDTC)	(I)	R_1=n-butyl R_2=2-napthylmethyl
1-Pyrrolidinecarbo-dithioic acid (tetramethylenedithio-carbamic acid)	H(TMDTC)		(II)

Schwedt (40) reported the elution of Se(IV), Pb(II), Ni(II), and Cu(II) diethyldithiocarbamates in the order shown from 10-μm Nucleosil 10-C18 column with a 65% acetonitrile-35% water mobile phase. The elution of the peaks was monitored at 254 nm and picogram amounts of the metals detected. In a later paper Schwedt (41) showed that Se(IV), Cr(III), Ni(II), Co(III), Pb(II) (or Cu(II)), and Hg(II) diethyldithiocarbamates were well separated on a LiChrosorb RP-8 column with a 70% methanol-30% water mobile phase. The Pb(II) and Cu(II) chelates apparently co-eluted under these conditions. In a subsequent paper (42) the elution order of Pb(II), Ni(II), Co(III), Cu(II) and Hg(II) for the diethyldithiocarbamate metal complexes was reported on a 10-μm LiChrosorb RP-18 column with 65% acetonitrile -35% water as the mobile phase. The tetramethylenedithiocarbamate complexes were also studied and the observed elution order on a 10 μm-Chromosorb RP8 column was Cd(II), Pb(II), Ni(II), Co(III), Zn(II), Cu(II) and Hg(II).

Heizmann and Ballschmiter (31) reported on the normal phase chromatographic separation of metal substituted dithiocarbamates. The Cu(II), Hg(II), Ni(II) and Co(III) diethyldithiocarbamates were reported to elute in the order given from a 30-μm LiChrosorb SI 60

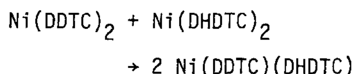
column with benzene as the mobile phase. The elution of the complexes was monitored at 330 nm and glass columns were employed to eliminate any contact between the chelates and metal parts. A flow-program was used to elute the Zn(II), Ni(II) and Co(III) benzylmethyldithiocarbomates (in that order) from a 40-μm LiChosorb SI 60 column with 75% benzene-25% cyclohexane as the mobile phase. The flow rate was increased from 8 ml/hr to 20 ml/hr after the elution of Ni(II). The same flow-program and column were used for the separation of Cu(II), Ni(II), Co(III) and As(III) diethoxyethyldithiocarbamates using 4% acetonitrile in carbon tetrachloride as the mobile phase. The As(III) peak was eluted last in just under 40 minutes and was very symmetrical. Elution of the peaks was monitored at 360 nm. No data on the plate counts were given but a value for N of about 500 based on the As(III) peak in Figure 11 in Reference 31 can be estimated. The observed retention times for Co(III), Cd(II), Ni(II), Cu(II) and Zn(II) decreased in the same order as R_F values for the benzylmethyldithiocarbamates increased on a thin layer plate (DC-Alufolie SiO_2) with 75% benzene-25% cyclohexane as the mobile phase.

Uden and Bigley (43) reported the separation of Cu(II), Ni(II) and Co(III) diethyldithiocarbamates by normal phase chromatography on a 4-mm x 25-cm stainless steel column packed with 8-μm Spherisorb SGP. The best separation was observed using 5% acetonitrile-15% diethylether-80% Skelly B as the mobile phase. The $Cu(DDTC)_2$, $Ni(DDTC)_2$ and $Co(DDTC)_3$ complexes eluted in the order given and a plate count of 1550 was reported for the $Co(DDTC)_3$ peak. A d.c. argon plasma emission spectrometer system in series with the photometric detector was used as a metal specific detector to confirm the peaks eluted were due to the metal complexes and not degradation products. Quantitative

studies based on the response of the photometric detector at 254 nm showed a linear reponse for $Co(DDTC)_3$ and $Ni(DDTC)_3$ from 5-500 ng of metal and for $Cu(DDTC)_2$ from 10-500 ng of the metal. On-column degradation was avoided by pretreatment of the column with pyridine. It was stated that solvent extraction of the metal complexes into chlorofrom would serve to separate the complexes from the reagent and preclude possible reactions of the chelating agent with the column but it was not clear whether this was done. It appears that the chelates studied were prepared and characterized as the pure solids and solutions of these were injected.

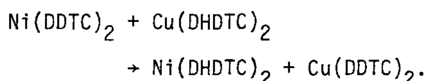
Liska et al. (44) noted mixed ligand complexes formed when mixtures such as $Ni(DDTC)_2$ and $Ni(DHDTC)_2$ were separated on a 10-μm Li-Chrosorb SI 60 column (stainless steel) with various combinations of chloroform, carbon tetrachloride, dichloromethane, cyclohexane, n-hexane and n-heptane as the mobile phases and UV detection at 325 nm. A third peak, apparently due to Ni(DDTC)(DHDTC), was observed that eluted between the peaks for the symmetrical chelates. Similar results were observed for other pairs of dialkyldithiocarbamates including diethyl with-dipropyl,-dibutyl,-dipentyl, and-diheptyl as well as other possible pair combinations. Capacity ratios, k, were given for the Ni(II) chelates of all the ligands mentioned above and for the mixed chelate species, L_1-Ni-L_2, as a function of the eluotropic strength, $\varepsilon°$, of the mobile phase. Liska et al. (45) unsuccessfully attempted to isolate the mixed ligand complex of Ni(DDTC)(DHDTC) by classical column preparative chromatography on glass columns packed with silica or alumina. Three colored zones were observed but they were not well separated and the middle zone, corresponding to the mixed ligand chelate, disappeared very quickly. It did prove possible to separate

and indentify all three species by two-dimensional thin layer chromatography using Silufol silica gel TLC plates. The molecular weight of both the symmetrical and mixed ligand Ni(II) chelates were determined in chloroform and the species were found to be monomeric. In a later paper in this series, Lehotay et al. (46) studied the elution behavior of various dialkyldithiocarbamate metal complexes and obtained well defined peaks for both the symmetrical and mixed ligand complexes. This work was done using a commercial HPLC unit with photometric detection at 254 and 280 nm. A metal column packed with 10-μm LiChromsorb SI 60 was used and various combinations of organic solvents used as the mobile phases. Mixtures of chloroform and dichloromethane with a less polar solvent (n-pentane, carbon tetrachloride or cyclohexane) gave the best results. Peaks resulting in exchange reactions such as



$$Ni(DDTC)_2 + Ni(DHDTC)_2 \rightarrow 2\ Ni(DDTC)(DHDTC)$$

were observed for all metal chelate pairs but no exchange reaction was observed for complexes of different metals with different ligands that led to mixed ligand complexes. The only reaction observed, for example, in the latter case was complete exchange,



$$Ni(DDTC)_2 + Cu(DHDTC)_2 \rightarrow Ni(DHDTC)_2 + Cu(DDTC)_2.$$

Liska et al. (47) showed that the diethyldithiocarbamate complexes of Zn(II), Cu(II), Mn(II), Ni(II), Pb(II), Cr(III), Co(II), Cd (II) and Fe(II) eluted in the order given from a stainless-steel column packed

with 10 μm-LiChrosorb SI 60 with 10% chloroform in cyclohexane as the mobile phase. The elution was monitored at 254 nm and well defined peaks attributed to each of the above species were generated in a single run on injection of 5 μl of a synthetic mixture of all the above complexes in chloroform ($5.6x10^{-7}$ M for each compound).

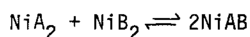
O'Laughlin and O'Brien(35) reported an elution order of Cu(II), Ni(II), Hg(II), Zn(II), Cd(II), Co(III) and Pb(II) for the normal phase chromatographic separation of the diethyldithiocarbamate complexes on a 37-50-μm Corasil column with toluene as the mobile phase. The $Ni(DDTC)_2$-$Co(DDTC)_3$ and $Hg(DDTC)_2$-$Pb(DDTC)_2$ couples were separated but peaks due to Cu(II), Ni(II), Hg(II) and Zn(II) were not resolved on this rather inefficient column. The $Pb(DDTC)_2$ peak tailed badly and a large peak near the column void volume was observed. Elution of the peaks was monitored at 270 nm. A good separation of a mixture of $Co(DDTC)_3$ and $Co(dz)_3$ was observed on a μ-Porasil A column with no evidence of any mixed chelate with this system. The dithizone complex was assumed to be $Co(dz)_3$. The composition of cobalt dithizonate is still an unresolved question but recent evidence tends to support the $Co(dz)_3$ formula (39,48) although in the absence of oxygen and with a large excess of reagent as in extraction studies $Co(dz)_2$ may be present. Budesinsky and Sagat (37) reported a β_n value for $Co(dz)_2$.

Mangia et al (49) reported the separation of Cu(II), Ni(II), Mn(II) and Co(II) as the diethyldithiocarbamate complexes on a 10-μm silica column with 20% methylene chloride-80% hexane as the mobile phase. The response of the photometric detector at 254 nm was determined for different amounts of Co(II). The cobalt complex was prepared by extracting a solution containing 0.05-5 ppm of Co(II) and NaDDTC with

carbon tetrachloride. An aliquot of the organic phase was injected and a linear calibration curve over the region 1-100 mg of metal injected was obtained. It seems likely the complex was $Co(DDTC)_3$ and not $Co(DDTC)_2$ as reported by the authors. In a subsequent paper (50) with Gaetani and Laureri this complex is formulated as $Co(DDTC)_3$. In this paper, an excellent separation of Cu(II), Ni(II), Mn(III) and Co(III) complexes with diethyldithiocarbamate was reported on a 10-μm MicroPak-CN column with 85% hexane-15% dichloromethane as the mobile phase. The peaks were monitored at 254 nm and a plate count for the Co(III) peak of 1040 was reported. Although the authors prepared the $Mn(DDTC)_3$ and $Co(DDTC)_3$ complexes by extraction of the divalent cations into carbon tetrachloride, they observed a change in the shape of the chromatographic peaks with time. They attributed this to oxidation to the Mn(III) and Co(III) complexes and confirmed the fact that these two metals eluted as $Mn(DDTC)_3$ and $Co(DDTC)_3$ by mass spectroscopy. It is not clear how mass spectrometry can be used to confirm the oxidation state and more likely it only indicated the approximate stoichiometry. In this same paper the separation of $As(DDTC)_3$, $Sb(DDTC)_3$ and $Bi(DDTC)_3$ was reported on a 10-μm MicroPak CN column with 90% hexane-8% dichloromethane-2% acetonitrile as the mobile phase. An interesting separation of $Ni(DDTC)_2$ and $Pd(DDTC)_2$,which are isostructural in the solid phase, was achieved using toluene as the mobile phase in which a large separation factor, $[Pd(DDTC)_2]/[Ni(DDTC)_2]$ = 9, was observed with detection at 320 nm. A separation factor for the $Zn(DDTC)_2/Cd(DDTC)_2$ pair, also isostructural in the solid state, was 2.1 with 80% toluene-20% dichloromethane as the mobile phase. Linear calibration plots were obtained over the range 1.0-25 ng of metal injected with detection

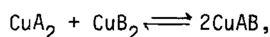
limits for Cu, Ni, Pd and Co between 0.1 and 0.3 ng of metal. The quantitative studies were all done on solutions of the metals in carbon tetrachloride obtained by solvent extraction of the complexes from an aqueous solution of the metal containing a 10-100-fold excess of Na(DDTC).

Moriyasu and Hashimoto (51) used adsorption chromatography on deactivated silica columns (10-µm LiChrosorb SI 100 or SI 60) to separate and determine Hg(II), Cu(II), Cd(II), Pb(II), Cr(VI), Ni(II), Bi(II), and Co(III) as the diethyldithiocarbamate complexes. Cr(VI) is reduced to Cr(III) by the reagent and the peak observed was likely due to the Cr(III) complex. These chelates eluted in the order shown with 98% hexane (H_2O saturated) -2% ethylacetate as the mobile phase. Linear calibration plots were reported for the Hg(II), Cu(II) and Ni(II) complexes but not for the Pb(II), Bi(III) or Cd(II) complexes. In a subsequent paper, the same authors (52), noted that mixed ligand complexes similar to those reported by Liska et al. (44) formed when two different disubstituted dithiocarbamate complexes of Ni(II) or Cu(II) were mixed. The mixed ligand species was separated on a deactivated silica column (Shodex Silipak) with a 83.3% hexane-16.7% ethylacetate mobile phase which was saturated with water. Rate constants for the reactions,



$$NiA_2 + NiB_2 \rightleftharpoons 2NiAB$$

and



$$CuA_2 + CuB_2 \rightleftharpoons 2CuAB,$$

were reported for ligands where A=diethyldithiocarbamate and the structure of B was I with $R_1 = R_2 = CH_3$, I with $R_1 = R_2 = CH_3CH_2CH_2$-; I with $R_1 = R_2$ = benzyl; II with B = $(CH_2)_4$; II with B = $(CH_2)_5$; and II with B =

$-CH_2CH_2OCH_2CH_2^-$. Rate constants for Ni(II) were in the range 10^1-$10^2M^{-1}s^{-1}$ and for the more labile Cu(II) complexes the constants were on the order of $10^3M^{-1}s^{-1}$.

Moriyasu et al. (53) also reported that the nickel chelate of dithiocarbamate derived from (+)-or(-)-ephedrine gave a single peak while that from (±)-ephedrine gave two peaks and that optically active and racemic ephedrines were determined.

Schwedt (54) determined Cr(III) and Cr(VI) in the presence of each other by separation of the different reaction products with ammonium pyrrolidinedithiocarbamate (II with B = $(CH_2)_4$). Cr(VI) reacted at room temperature and Cr(III) only at 60°C. The reaction products were separated on 5-μm LiChrosorb RP-18 (RP-8) columns with 80% (70%) acetonitrile -20%(30%) water as the mobile phases. The reaction products were detected at 254 nm and both Cr(III) and Cr(VI) were determined in waste water. Tande et al. (55) reported Cr(III) formed the $Cr(DDTC)_3$ chelate at pH 5.8 with NaDDTC in aqueous solution but not at pH 4. Cr(VI), added as $K_2Cr_2O_7$, reacted with NaDDTC at both pH4 and pH 5.6 to give three peaks attributed to an unidentified disulfide, a species formulated as $Cr(S_2CN(C_2H_5)_2)_2(OS_2CN(C_2H_5)_2)$ and $Cr(S_2CN(C_2H_5)_2)_3$. The ratio of the last two peaks was found to be constant at different Cr(VI) concentrations and by preparing standard curves for both peaks 2 and 3, originating from Cr(VI) and reacted at pH 5.6, and a third calibration curve for $Cr(S_2CN(C_2H_5)_2)_3$ when Cr(III) was reacted with the ligand at pH 5.6, it was possible to determine Cr(III) and Cr(VI) simultaneously in an unknown water sample.

Häring et al. (56) determined Co, Cu and Ni in the 0.2-10 μg/L range by preconcentration of the diethyldithiocarbamate complexes on a

LC 5-Phenyl reversed-phase precolumn from water and the elution of these chelates from a LC 5-phenyl reversed phase analytical column with 75% methanol-25% water. They found it necessary to use glass or Teflon to construct the analytical system to avoid ligand exchange, memory and contamination phenomena.

Edward-Inatimi and Dalziel (57) reported that copper, nickel, mercury, lead, cobalt, manganese, and bismuth diethyldithiocarbamates (neither the oxidation state of the metal or stoichiometry of the complexes are given in this paper) could be extracted from a pH 8.5 aqueous buffer into 5 ml of an organic solvent. A 5-µl portion of the organic extract was injected into the HPLC and the metal chelates separated on a 5-µm Hypersil column using benzene as the mobile phase. The elution of the peaks, in the order given above, was monitored at 280 nm. Linear calibration plots based on peak height were reported for all six metals and limits of detection based on metal concentration in original aqueous sample from 50-500 ppb was reported. The peak for lead was poorly defined and the calibration plot for lead had the smallest slope.

Bond et al. (58) showed that the Cu(II) diethyldithiocarbamate or the Cu(II) 1-pyrrolidenecarbodithioate chelates could be detected electrochemically in the 10^{-6} to 10^{-7} M concentration range. The $Cu(DDTC)_2$ was found to undergo one-electron reduction and oxidation steps at a platinum, gold, or glassy carbon electrode. Copper could be detected down to levels of 1 ng with no interference from a 10-fold excess of 20 selected ions. Peaks were also observed using electrochemical detection for the chelates of Cd(II), Pb(II), Co(III) and Fe(III) and it was assumed this method would be suited for multi-element analysis. Excellent agreement was reported for copper in

tap water when determined by HPLC with electrochemical detection and when determined by atomic absorption methods. In one variation of the above procedure, the reagent was included in the mobile phase and aqueous solutions of Cu(II) were injected with on-column formation of the complex.

Smith et al. (59) have also reported that Cu(II), Ni(II), Co(II), Pb(II) and Fe(III) could be determined by reversed-phase liquid chromatography on Hypersil-ODS by direct injection of aqueous solutions of these ions. The mobile phase included Na(DDTC) and the complexes were formed directly (on-column), separated and the peaks detected at 350 nm. A mixture containing 100 ppm Cu (II), 5 ppm Co(II) 110 ppm Pb(II) and 100 ppm Cd(II) gave four well defined peaks eluting in the order Cd(II), Pb(II), Co(III), Cu(II) using 75% methanol-35% water containing 0.05% Na(DDTC) as the mobile phase. The Co(II) eluted as the Co(III) complex according to these authors. It was found that Hg(I) and Hg(II) mixture gave two peaks and Zn(II) gave one peak but in these cases the retention times were not reproducible. Smith et al. (60,61) also have reported on the use of transition metal cations as "ion-pair" reagents in the HPLC separation of dithiocarbamates. This appears to involve the formation of metal chelates and is not ion-pair chromatography in the sense that this term is now used.

Sheh and Carr (62) have proposed a new ligand, bis(n-butyl-2-napthylmethyldithiocarbamate)zinc II, $(Zn(BNMDTC)_2)$ for multi-element trace metal analysis. These authors note that it was reported that metal-DDTC complexes dissociate at low concentrations (51), have maximum absorption bands at very different wavelengths precluding optimization of response without multi-channel spectrophotometric capabilities, and in some cases tend to react on the

column with metal components. The napthyl group in the proposed reagent absorbs strongly at 221 nm and the bulky side groups are claimed to stabilize metal complexes with this ligand. They use two pre-columns, the first as silica column to saturate the mobile phase with silica and a second, a silica column derivatized with diaminosilane, to remove trace metals from the mobile phase. The analytical column used in this work was a 10-μm Waters RCM-100 C18 which was used to minimize generation of interferences from metal frits in conventional columns. The metal complexes were prepared by reacting metal salts with an excess of $Zn(BNMDTC)_2$ dissolved in the mobile phase buffered at pH 8.25 with Tris. The pH was adjusted to 8.25 prior to the addition of methanol with phosphoric acid. A typical chromatogram is shown in Fig. 3. The best results were obtained using methanol-water mobile phases although other mobile phases were studied. Retention values, k, on other columns varied dramatically even though the same mobile phase was employed (although the elution order remained the same). Columns studied included a 5-μm Supelcosil LC-8 and a 10 μm-Waters μ-Bondapak C18 in addition to the RCM-100 column. Linear calibration plots with a zero intercept were obtained for Hg(II), Cu(II) and Fe(III) using the RCM-100 column.

Hutchins et al. (63) reported that Co(III), Cr(III), Cu(II), Hg(II), Ni(II), Pb(II), Se(IV), and Te(IV) diethyldithiocarbamates could be separated on a Waters Radial Pak C18 column using a ternary mobile phase consisting of 40% methanol-35% acetonitrile-25% water provided the column was conditioned by prior injection of a concentrated mixture of the complexes or with 0.005 M EDTA. The Pb(II), Cd(II) and Fe(III) complexes gave poor peak shapes attributed to substitution reactions with nickel from metal components in the chromatographic system. A

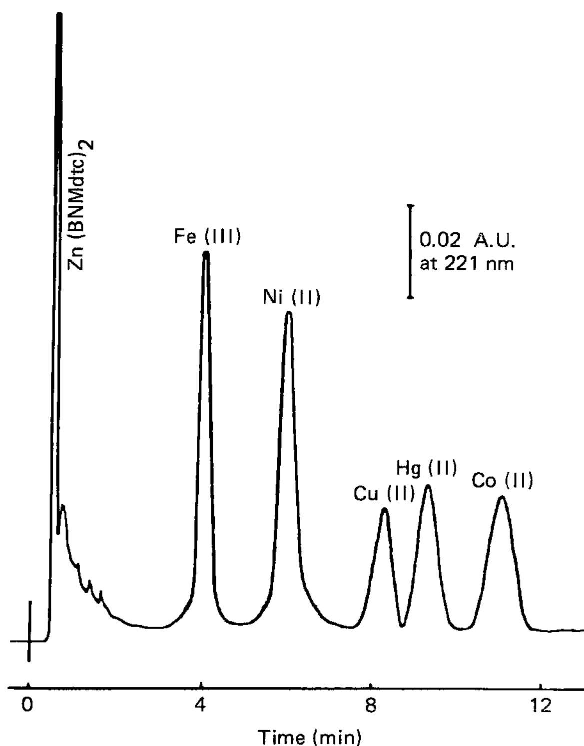


Fig. 3. Chromatogram of the metal $(BNMDTC)_n$ complexes. Sample was 20 μl of a synthetic mixture which was 1×10^{-4}M in each complex. Flow rate 2 ml min^{-1}; pressure drop less than 1500 psi. Waters Radial Pak C_{18} column. Mobile phase 95% methanol-5% water and 1.0mM in Tris. pH=8.25. Reprinted by permission from Ref. 62 (Elsevier).

typical separation is shown in Figure 4. The first peak eluted has been attributed by other investigations as a disulfide and was identified by Hutchins et al (63) as bis(diethylthiocarbonyl)disulfide (disulfiram) produced by the oxidation of diethyldithiocarbamic acid. The lack of any peak for Zn(II) in Figure 4 was attributed to the fact this species

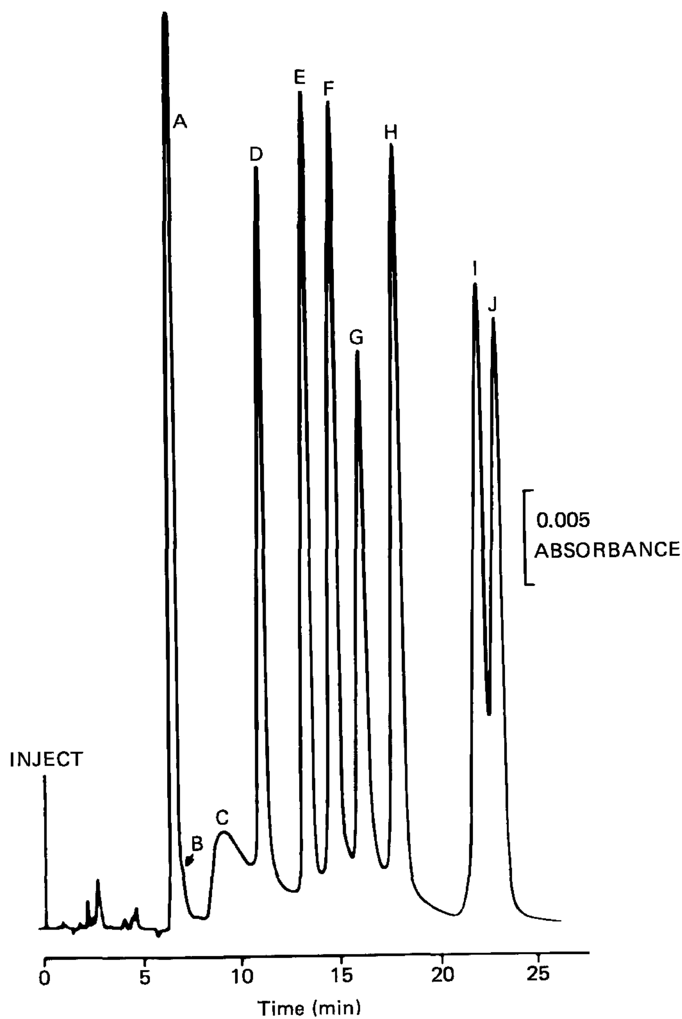


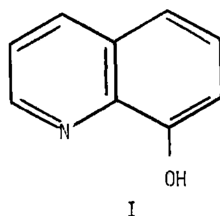
Fig. 4. Separation of a mixture of diethyldithiocarbamate complexes on a Waters Radial Pack C_{18} column. Conditions: mobile phase methanol-acetonitrile-water (40:35:25) flow rate 2.0 ml/min, detector wavelength 254 nm; detector sensitivity 0.05 a.u.f.s; injection volume 10 µl. A = disulfiram; B = Cd(II), 0.12 µg; C = Pb(II), 0.11 µg; D = Ni(II), 0.14 µg; E = Co(III), 0.07 µg; F = Cr(III), 0.11 µg; G = Se(IV), 0.32 µg; H = Cu(II), 0.18 µg; I = Hg(II), 0.51 µg; J = Te(IV), 0.17 µg. Reprinted by permission from Ref. 63 (Elsevier).

is unstable and reacted with the stainless steel components in the system (presumably the stainless steel spreader plates on the column or in the U6K injector). Applications of the above method to the determination of metals and organic brightening agents in electroplating solutions are discussed.

Bannister et al. (64) reported that Na(DDTC) reacted with cis-dichlorodiamminoplatinum(II) in urine to form $Pt(DDTC)_2$. The latter chelate was extracted into chloroform and determined by HPLC. The elution of the $Pt(DDTC)_2$ from a µ-Bondapak CN (C-18 or alkylphenyl columns were unsatisfactory) with 82% heptane-18% isopropanol was monitored at 254 nm. Platinum in urine at the 25 ng/ml level could be determined with a relative precision of 2.5% and accuracy of 4%.

2.5 8-Hydroxyquinoline

General Structure



I

Name	Abbreviation	Structure
8-hydroxy-quinoline(oxine)	H(Ox)	I

Bethod et al. (65) used reversed-phase chromatography on a RP-8 column to separate the Cu(II), Co(II), Ni(II), Hg(II) and Fe(II) chelates of 8-hydroxyquinoline. Solutions of Cu(II), Co(II), Ni(II) and Hg(II) were injected into a column already holding 8-hydroxyquinoline.

The chelates were then separated using a 55% methanol-45% water mobile phase which was also $5x10^{-3}M$ in oxine. Electrochemical, UV absorption and atomic absorption methods were used to detect the peaks eluted. Hambali and Haddad (66) reported the separation of Al(III) and Co(III) chelates with 8-hydroxyquinoline on a 10-μm LiChrosorb SI 60 column using a 5% methanol-95% chloroform mobile phase. The elution of the peaks was monitored at 254 nm and linear calibration curves of peak area versus amount of metal ion injected over the ranges 0-0.5 μg for Co(III) and 0-0.20 μg for Al(III) were obtained. Detection limits of 0.9 ng and 17ng for Co(III) and Al(III) were reported. These authors were unable to separate Co(II) by TLC as the oxinate due to oxidation to Co(III) and the formation of two spots on the TLC plate and Co(II) was not studied in the column work. Wenclawiak (67) reported the separation of the V, Mo, W, Co and Cr chelates with 8-hydroxyquinoline on a SI 60 silica column with a 60% tetrahydrofuran-40% chloroform mobile phase. The oxine complexes were formed prior to injection on the column. The peaks eluted in the order given and detection limits based on absorption at 254 nm of <1 ng for V, Mo, and Cr, 0.5 ng for Co, and 1.5 ng for W were reported. Hoffman and Schwedt (68) compared the pre-column derivatization method proposed by Wenclawiak (67) and the on-column injection method of Bethod (65) for the possible separation of Mn(II) and Mn(III) in natural waters. They found that Co(II), Mo(VI), Mn(II) and Mn(III) could not be separated by the pre-column derivatization method and the separation of chelates by this method was not reproducible. The on-column derivatization method, however, was successful and peaks (with retention time in minutes) for Cr(VI), (2.45), Co(II) (4.01 and 4.09), Mn(II) (4.60), Zn(II) (4.88), Cu(II) (12.29), Al(III) (19.26), and Mn(III) (25.77) were obtained on a RP-8

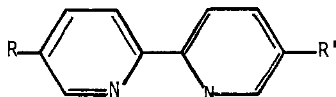
column with a 60% methanol-40% water mobile phase which was 10^{-3}M in oxine. The best results were obtained on a 5-μm RP-18 column according to the authors but no data was given for this column. It was claimed that Mn(II) and Mn(III) could be determined in river water and fertile soils. The authors note that many metals change their oxidation state by reaction with air during the derivatization step and that two peaks were observed for Co(II) if injected immediately after derivatization (with the pre-column derivatization method) but only one peak if allowed to stand) for several hours prior to injection. They made the rather strange observation that "If Al(III) is present with Mn(III), several peaks with t_R values differing from those of the pure oxinates will result". Al(III) thus appears to interfer with the Mn(III) determination which makes it difficult to see how the method could be used to determine Mn II/Mn(III) ratios in "fertile soils".

Watanabe et al. (69) reported trace elements in sea water could be preconcentrated by formation of the 8-hydroxyquinoline complexes and adsorption of these metal complexes on a C18 column (Bondapak Porasil B). Quantitative recovery of copper and manganese spikes (10 and 9 μg/L, respectively) was reported on elution of the complexes from the column with methanol.

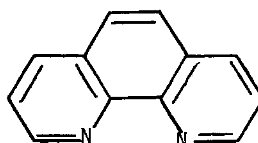
It is somewhat surprising that relatively few papers have been published on the separation of metal oxinates in view of the large number of metal ions known to form stable chelates with this ligand and the fluorescent nature of many of these chelates. It is well known that divalent metals tend to form complexes in which additional molecules of neutral oxine apparently displace coordinated water. This might explain the greater reproducibility of the direct injection method reported by Hoffman and Schwedt (68). It appears additional research is needed in this area.

2.6 Bipyridine and 1,10-Phenanthroline

General Structure



I. 2,2'-Bipyridine



II. 1,10-Phenanthroline

Name	Abbreviation	Structure
2,2'-bipyridine	bpy	I. R=R'=H
2,2'-bipyridine dicarboxylate	bpy-1	I. R=R'= $-COO^-$
2,2'-bipyridine monocarboxylate-monoester	bpy-2	I. R= $-COO^-$ R'= $-COOC_2H_5$
"	bpy-3	I. R = $-COO^-$ R'= $-COOC_{18}H_{37}$
2,2'-bipyridine diester	bpy-4	I. R=R'= $-COOC_2H_5$
"	bpy-5	I. R=R'= $-COOC_{18}H_{37}$
"	bpy-6	I. R= $-COOC_{18}H_{37}$ R'= $-COOC_{16}H_{33}$
"	bpy-7	I. R= $-COOC_{18}H_{37}$ R'= $-COOC_{20}H_{47}$
1,10-phenanthroline	phen	II.

Valenty and Behnken (70) were the first to report on the use of reversed-phase paired-ion HPLC to separate ionic metal chelates. They studied the separation and quantitation of $Ru(bpy)_3^{+2}$ derivatives with the general structure, $(bpy)_2Ru(L)$, where the ligand L was bpy-1 through bpy-7. The charge on the metal chelate thus varied from a net zero for $Ru(bpy)_2(bpy\text{-}1)$, the dicarboxylate species, to plus one for

the $Ru(bpy)_2$(bpy-2) and $Ru(bpy)_2$(bpy-3) the mono-ester species, to plus two for the diester species $Ru(bpy)_2(bpy\text{-}4)^{+2}$, $Ru(bpy)_2(bpy\text{-}5)^{+2}$, $Ru(bpy)_2(bpy\text{-}6)^{+2}$ and $Ru(bpy)_2(bpy\text{-}7)^{+2}$. The observed elution order for the Ru(II) tris chelates was $Ru(bpy)_2$(bpy-1), $Ru(bpy)(bpy\text{-}3)^{+1}$, $Ru(bpy)_2$(bpy-6), $Ru(bpy)_2$(bpy-5), and $Ru(byp)_2$(byp-7) with retention times of 1.1, 2.4, 6.8, 7.3 and 8.0 min., respectively, on a μ-Bondapak-C18 column using a 20-minute linear solvent gradient from 50% THF-50% H_2O to 100% THF. The flow rate was 2.0 ml/min and elution of the species was monitored photometrically at 254 nn and 280 nm. The mobile phase was kept 0.015M in methanesulfonic acid and 0.5% in acetic acid. The species $Ru(bpy)_2$(bpy-1), $Ru(bpy)_2$(bpy-2), and $Ru(bpy)_2$(bpy-4) eluted in the order given on the same column and under the same conditions as above with a 10-min linear solvent gradient from 10% THF-90% H_2O to 40% THF-60% H_2O (both solvents 0.005M in n-heptanesulfonic acid and at pH=3.5). Observed retention times were 4.9, 7.7 and 10.4 minutes respectively. The peaks were sufficiently sharp that the diester species $Ru(bpy)_2(bpy\text{-}5)^{+2}$, $Ru(bpy)_2(bpy\text{-}6)^{+2}$ and $Ru(bpy)(Bpy\text{-}7)^{+2}$ which differ by only two methylene units in the hydrocarbon tails on the ester could be separated. The neutral dicarboxylate species, $Ru(bpy)_2$(bpy-1), eluted first followed by the monoester species with a charge of plus one. Because a different gradient program was used as well as a different counter ion (n-heptane sulfonate for the bpy-1, bpy-2 and bpy-4 species), it is not possible to compare retention times for the monoester species with $R=COOC_2H_5$ (bpy-4) and $R = -COOC_{18}H_{37}$ (bpy-5) or the retention of the dicarboxylate species (bpy-1) with the two counter ions. Although the retention time was shorter for the

dioctadecyl ester than the diethyl ester it should be noted that the 7.7 min retention time for the former species was obtained for a gradient program going up to 100% THF and the 10.4 min retention time for the diethyl ester involved a gradient program going only to a maximum concentration of 40% THF in water after ten minutes. Under isocratic condtions it would be expected that the diethylester would elute first. These authors reported a detection limit for the species, $Ru(bpy)_2(bpy\text{-}5)$, of $1.x10^{-12}$ mol at a S/N ratio of 2 at 280 nm and $e^{280} = 5x10^4$ L/mole cm. The detector response was linear over the range $5x10^{-12}$ to $2x10^{-8}$ mol/L of the above compounds with precision of ±2% at the $2.5x10^{-9}$ molar level. The authors concluded that monitoring hydrolysis reactions of the above diester compounds by HPLC was superior to photometric absorption or fluorimetric methods.

O'Laughlin and Hanson (71) reported that the kinetically inert tris(1,10-phenanthroline)iron(II) and ruthenium(II) chelates could be separated by paired-ion chromatography on a μ-Bondapak CN column (10-μm particle size) using methanol-water or acetonitrile-water mobile phases which were also 0.015 M in methanesulfonic acid and 0.5% in acetic acid. The retention volumes for both chelates (and the resolution) decreased with increasing concentration of the organic component and a 20% methanol-80% water composition permitted the separation of the two species in under ten minutes with baseline resolution.

The effect of pH and pairing ion concentration on the resolution was studied. The former appeared to have relatively little effect over the range pH 2.9 to 6.0 on the separation although the retention volumes for both species first decreased to minimum values around pH 4.5 and then increased. Capacity factor values, k, for $Fe(phen)_3^{+2}$ decreased from 2.40 at pH 2.9 to 1.93 at pH 4.5 and then increased to

2.81 at pH=6.0. Both capacity factors and the resolution increased as the concentration of the pairing ion was increased. With n-heptanesulfonate as the pairing ion and using a 40% methanol-60% water mobile phase (also 0.06M in acetic acid), k values for Fe(II) and Ru(II) increased from 1.46 and 1.72, respectively, at 10^{-4}M heptanesulfonate to 2.46 and 2.92 at 10^{-2} M heptanesulfonate. The plate count (based on the iron(II) peak) also increased from 1350 to 1860 and the resolution, R_s, from 0.84 to 1.02.

The elution of the $Fe(phen)_3^{+2}$ and $Ru(phen)_3^{+2}$ species was monitored using a variable wavelength photometric detector at 448 nm and 512 nm in the visible (corresponding to the wavelength for maximum absorbance in the visible for the $Ru(phen)_3^{+2}$ and $Fe(phen)_3^{+2}$ species) and at 265 nm where both species adsorbed strongly. The elution of the $Ru(phen)_3^{+2}$ species was also monitored fluorimetrically at 565 nm. It was found that the kinetically inert $Ni(phen)_3^{+2}$ species eluted with the same retention volume as the $Ru(phen)_3^{+2}$ species and, in the absence of the latter, the elution of the $Fe(phen)_3^{+2}$ and $Ni(phen)_3^{+2}$ species could be monitored at 265 nm. Plots of peak area versus nanograms of metal injected were linear for both complexes. No separation of the above complexes was observed on a μ-Bondapak C18 column with methanesulfonate as the pairing ion at methanol concentrations greater than about 60%. Some separation was evident at 50% methanol-50% water with retention volumes for the $Fe(phen)_3^{+2}$, $Ni(phen)_3^{+2}$ and $Ru(phen)_3^{+2}$ of 9.01, 9.25 and 10.48 ml, respectively. All the peaks tailed badly and could not be resolved. O'Laughlin (72) later reported a better separation of the $Fe(phen)_3^{+2}$ and $Ni(phen)_3^{+2}$ on a μ-Bondapack C18 column was possible using a 30% acetonitrile-70% water mobile phase containing 2.0g

$LiClO_4$/L. Some tailing was still evident although baseline separation of the peaks was possible with retention volumes of 21.2 and 25.0 ml, respectively, for the Fe(II) and Ni(II) species. No peaks were observed on either column for the kinetically labile Co(II), Zn(II) or Cd(II) complexes although the possibility that these complexes could be separated if the ligand was added to the mobile phase was suggested.

O'Laughlin (73) in a subsequent paper showed that well resolved peaks for the labile Zn(II) and Cd(II) complexes with 1,10-phenanthroline were obtained on a μ-Partisil SCX column using an 80% acetonitrile -20% water mobile phase which was 0.048M in $LiClO_4$ and 10^{-4}M in 1,10-phenanthroline. The elution of the peaks was monitored at 265 nm. Separate peaks for the inert $Ni(phen)_3^{+2}$, $Ru(phen)_3^{+2}$ and $Fe(phen)_3^{+2}$ were obtained on the μ-Partisil SCX column using a 80% acetonitrile -20% water mobile phase which was 0.06M in $HClO_4$. The elution order did not change for the $Fe(phen)_3^{+2}$ and $Ni(phen)_3^{+2}$ species as the acetonitrile concentration was decreased to 50% but the retention volumes and the resolution of the two peaks increased in a uniform manner. The retention volumes for these two complexes varied inversely with the square of the perchlorate ion concentration but did not appear to be a function of the hydrogen ion concentration. It was shown (72) that the retention volumes for the kinetically inert complexes were independant of the 1,10-phenanthroline concentration in the mobile phase but those for the Co(II), Zn(II) and Cd(II) complexes varied with the 1,10-phenanthroline concentration. A typical separation of several metal complexes at one ligand concentration is shown in Figure 5. When the 1,10-phenanthroline concentration was 10^{-4}M the Cd(II) species eluted last.

O'Laughlin also reported (72) that the neutral bis-(cyano)bis-(1,10-phenanthroline)iron(II) complex,

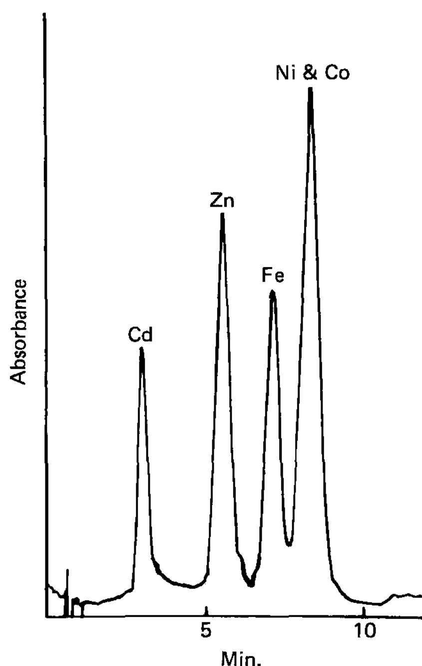


Fig. 5. Separation of $M(phen)_3^{+2}$ complexes on a PRP-1 column. Mobile phase 35% CH_3CN-65% H_2O with 4g $LiClO_4$ per L and 3.5 x 10^{-5}M in phen. From Ref. 72.

$Fe(CN)_2(phen)_2$, could be separated from the $Fe(phen)_3^{+2}$ species on both a µ-Bondapak C18 and a PRP-1 (polystyrene-divinylbenzene) column. Rentention of the $Fe(CN)_2(phen)_2$ species on the µ-Bondapak C18 column decreased as the acetonitrile content of an acetonitrile-water mobile phase decreased from 90% to 70% and then increased slightly. If the mobile phase contained 2.0g of $LiClO_4$/L the $Fe(phen)_3^{+2}$ species eluted before the $Fe(CN)_2(phen)_2^{+2}$ species when the acetonitrile concentration was over 70% and after the

$Fe(CN)_2(phen)_2$ species at lower acetonitrile concentrations. This was suggested as a method for the determination of the cyanide ion and linear calibration plots of peak area of the $Fe(CN)_2(phen)_2$ peak versus amount injected were obtained when the peak was monitored at either 540 nm or at 242 nm.

It is interesting to note that the elution order of the inert chelates was $Fe(phen)_3$, $Ru(phen)_3^{+2}$ and $Ni(phen)_3^{+2}$ on the μ-Bondapak CN, μ-Bondapak C18 and PRP-1 columns but in the inverse order on the μ-Partisil SCX column regardless of the organic to water ratio in the mobile phase with either ClO_4^- or CH_3SO_3- as of the pairing ion. Although the concentration of the pairing ion and ratio of organic solvent to water in the mobile phase had the largest effect on resolution and retention volumes it appears the cation exchange sites on the μ-Partisil SCX, do have an effect on elution order and selectivity.

Yoneda (74) has noted an inversion in the elution order for the $Co(phen)_3^{+3}$ and $Fe(phen)_3^{+2}$ species on an SP-Sephadex cation-exchange column as the KBr concentration in the mobile phase was increased. At low KBr concentrations the $Co(phen)_3^{+3}$ was more strongly retained than the $Fe(phen)_3^{+2}$ but at higher KBr concentrations the order was inverted with the inversion occuring at 0.3 mol dm^{-3}. No inversion was noted for the corresponding ethylenediamine complexes $Co(en)_3^{+3}$ and $Fe(en)_3^{+2}$ even up to 1 mol dm^{-3} KBr. On the contrary, when K_2SO_4 was used in the mobile phase the $Co(en)_3^{+3}$ - $Fe(en)_3^{+2}$ did show an inversion in elution at 0.18 mol dm^{-3} but not up to 0.2 mol dm^{-3} for the phen complex pair. This was interpreted in terms of ion association of the hydrophobic $Fe(phen)_3^{+2}$ and $Co(phen)_3^{+2}$ with the large and relatively poorly hydrated Br^- ion. On the other hand there is little

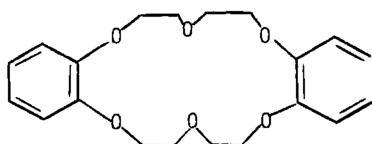
tendency toward association of the hydrophillic SO_4^{-2} ion and the phen species. The opposite is expected for the en species. This suggests that not only the nature but the concentration of the pairing ion might be critical with regard to selectivity in ion-pair chromatography of charged metal chelates.

Lundgren and Schilt (75) studied the adsorption of metal ions on Amberlite XAD-2 styrene-divinylbenzene resins coated with the ferroin type ligand 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triamine (PDT) as a function of pH and anion. They attributed the adsorption of this ligand and metal complexes to π-electron overlap between the styrene moieties on the resin and the adsorbate molecules. The order of increasing distribution coefficients for divalent metals perchlorates on XAD-2 resin columns coated with PDT was substantially different than the elution order observed by O'Laughlin (73) for the divalent metal phenanthroline complexes on the PRP-1 column.

Mangia and Lugari (76) studied the separation of the tris(2,2'-bipyridine) nickel(II) and-iron(II) complexes by ion-pair chromatography. Three well separated peaks, for the 2,2'-bipyridene ligand, the Ni(II) chelate and the Fe(II) chelate were found to elute in that order from a 10-μm -Bondapak-CN column with a 60% methanol-40% water mobile phase which was 0.02 M in KNCS. The retention volume for the Ni(II) complex was found to decrease in an exponential manner with increasing concentration of the pairing ion, NCS^-. The data were fitted to a linear equation, $1/V_R = 1.241 + 0.511 \log [NCS^-]$, with a coefficient of correlation of 0.9948 and a standard deviation of the slope of 2.1%. It was noted this elution order and pairing ion dependence was similar to that found by O'Laughlin (73) for the 1,10-phenanthroline complexes on a Partisil SCX column. It should be

pointed out, however, that O'Laughlin and Hanson (71) observed the opposite elution order ($Fe(phen)_3^{+2}$ before the $Ni(phen)_3^{+2}$ complex) on a μ-Bondapak CN column and an increase in V_R with pairing ion (heptanesulfonate) concentration.

2.7 Crown Ethers



Dibenzo-18-crown-6

The crown ethers, such as dibenzo-18-crown-6, are capable of forming cationic complexes with many metals. Kolthoff (77) has reviewed the the use of crown ethers, aza-crown compounds, and related compounds such as the cryptands. The use of crown ethers as anchor groups on ion-exchange resins is also reviewed as well as the use of crown ethers in extraction chromatography.

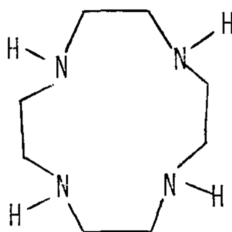
Mangia et al. (78) studied the HPLC separation of dibenzo-18-crown-6, DBC, complexes of Cd(II), Pb(II), Ag(I) and Hg(II) halides on a 10-μm Micropak CH column (octadecyl) with a methanol-aqueous phosphate or borate buffer. Only one peak due to the free ligand was observed for the first three complexes at any pH of the mobile phase. Peaks attributed to $HgCl_2$-DBC, $HgBr_2$-DBC and HgClBr-DBC were observed for the Hg(II) halides in addition to a peak for the free ligand. The mixed halide peak was obtained as well as the $HgCl_2$-DBC and $HgBr_2$-DBC peaks when an alkaline solution containing Br^- and Cl^- was treated with Hg(II) acetate and extracted with a

solution of DBC in methylene dichloride. The plate count reported for the $HgBr_2$-DBC peak of only 290 was very low and three peaks attributed to $HgCl_2$-DBC, HgClBr-DBC and $HgBr_2$-DBC were only partially resolved.

Kimura and Shono (79) have recently reviewed work on the application of crown ethers in liquid chromatography. The separation of LiCl, NaCl, CsCl, RbCl and KCl in that order on a crown-ether modified silica, the synthesis of which was previously reported by Kimura et al. (80), is reported. On a different crown-ether modified silica the KCl eluted between RbCl and CsCl and the alkaline earth chlorides $MgCl_2$, $CaCl_2$, $SrCl_2$ and $BaCl_2$ eluted in the order shown. Peak widths tended to be very broad and the peaks tailed significantly. Resolution of the peaks in the alkaline earth series was poor.

It would appear that the crown ethers should have great potential in HPLC, especially using ion pair HPLC. Although some interesting separations such as that of the alkali metals using extraction chromatography with DBC have been reported (81), it seems that the usefulness of these interesting ligands in HPLC has yet to be fully exploited.

2.8 Macrocyclic Amines



Cyclam

Macrocyclic amines such as the compound above given the trivial name cyclam by Troutner et al. (82), are formal analogs of the crown ethers with the imino group replacing the oxygen atoms. Troutner et al. (82) reported cyclam was an efficient complexing agent for Tc-99m and has potential use as a radiopharmaceutical. Hoffman et al. (83) have studied the separation of the Tc-cyclam complex by HPLC.

Royer et al. (84) studied the separation of optical isomers of Co(III) derivatives of macrocyclic amines similar to cyclam but with six nitrogen atoms in the ring which they call 21N6. The $Co(21N6)^{+3}$ species was resolved by eluting from a SP-Sephadex C-25 column (180x2 cm) in the sodium form. The separation of geometric isomers of Co(III) complexes with unsymmetric ligands similar to 21NG was also reported.

2.9 Porphyrins

General Structure

I

II

Metalloporphyrins occur commonly in nature. Richter and Rienits (85) reported on the separation of zinc protoporphyrin (IX) and magnesium protoporphyrin (IX) as the neutral dimethylesters (protoporphyrin has the general structure of I with $R_1=R_3=R_5=R_8=CH_3$, $R_2=R_4=CHOHCH_3$ and $R_6=R_7=CH_3CH_2COOH$). These authors used a 10-µm LiChrosorb silica column with 15% acetone-85% hexane as the mobile phase. The formation of the magnesium chelate is regarded as an

essential step in the biosynthesis of chlorophyll and these authors note this is one of the least well understood steps due partially to inadequate methods for routine assay of the magnesium chelate. HPLC seems to offer a viable solution.

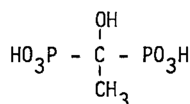
Hajibrahim et al. (86) showed that the Ni(II) complex of etioporphyrin (structure I with $R_1=R_3=R_5=R_7=CH_3$ and $R_2=R_4=R_6=R_8=CH_2CH_3$) and octaethylprophyrin (structure II with $R=C_2H_3$) could be separated from the more polar vanadyl desoxophyhloerythroetioporhyrin on a 10-μm Sphersorb silica column using a linear solvent gradient program from 2% to 50% chloroform in hexane in 20 min. with photometric detection at 400 nm. They also reported nickel and vanadyl petroporphyrins extracted from Boscan crude oil were separated using a concave solvent gradient from 10% toluene-90% hexane to 50% toluene-50% chloroform over 25 min with the nickel fraction detected at 395 nm and the vanadyl complexes at 572 nm. Spencer et al. (87) used the basic scheme suggested by Hajibrahim et al. (86) to separate vanadium containing compounds into two groups, porphyrin and non-porphyrin. The latter was partially separted using gradient elution programs and was a highly complex mixture with many vanadium containing complexes. These authors used flame emission and furnace atomic absorption to follow the elution of the vanadium complexes. On-line flame emission could not be successfully used to monitor the peaks from the HPLC according to these authors due to the lack of sensitivity, solvent effects (especially with a gradient program) and poor compatability of the nebulizer uptake ratio and column flow rate. The furnace AA methods involved discrete sampling and it was thought some vanadium species could be lost in the dry ashing step. These authors (87) claim a simpler and faster procedure was developed for the isolation of the vanadium porphyrin fraction nearly

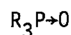
free of vanadium non-porphyrins and nickel porphrins than procedures used previously (86).

Although metalloporphyrins are generally considered to be "non-chromotographable" by gas chromatorgraphy, Marriott (88) has recently successfully separated a number of metalloporphyrins on a capillary column with Kovats retention index values over 5,000. There was no evidence of metal exchange among the different porphyrins. Considering the difficulty with the GLC separation of these chelates, the stability of the porphyrin chelates with many metals, and the ease of detection with photometric detector, it is surprising to this reviwer that more work has not been reported on the HPLC of different metal porphyrin complexes. Hui et al. (89) have studied the TLC behavior of the tetraphenylporphyrin chelates of manganese, iron, cobalt, nickel, copper, zinc, rhodium, cadmium, mercury and lead on silica gel and alumina. A number of good separations can be deduced from the published R_F data and sharp separations by HPLC on silica columns should be possible. The intense colors of these chelates should permit selective detection in the visible region of the spectrum.

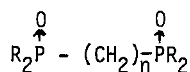
2.10 Organophosphorus Reagents

General Formulas





I II



III

Name	Abreviation	Structure
Hydroxyethylidene-diphosphonate	HEDP	I
Tri-n-butylphosphate	TBP	II $R=-OC_4H_9$
Methylenebis(di-n-hexyl)phosphine oxide	MHDPO	III n=1 $R=C_6H_{13}$

Pinkerton et al. (90) reported on the separation of Tc-99m complexes with HEDP. These complexes are used as imaging agents in nuclear medicine. The chemistry of formulation of the Tc-99m complex involves the reduction of a mixture of $^{99m}TcO_4^-$ and the daughter product $^{99}TcO_4^-$ with a reducing agent ($NaBH_4$) and a stabilizer (ascorbic acid). The resulting mixture is somewhat complex and contains several complexes containing Tc-99 or Tc-99m. These authors were able to separate a number of these species on a 13-μm Aminex A-27 anion-exchange column. An aqueous sodium acetate solution at pH=8.4 was used as the mobile phase. Detection of the species eluted was based on photometric detection at 250 nm and 405 nm and radiometric detection. The efficiency of the column as determined by the number of plates generated was found to increase sharply around 50°C from around N=400 at temperatures below 40° to over 4,000 at 60°C based on the peak width of the most prominent peak. Wong (11) has reviewed application of HPLC for the separation of Tc-99m complexes with pryophosphates, methylene diphosphonates and other ligands.

O'Laughlin and Jensen (91) reported on the use of MHDPO and the related MEHDPO (structure III - R=ethylhexyl and n=1) as stationary phases for the separation of the lanthanide elements by extraction chromatography using 9M nitric acid as the stationary phase. Although this work and similar studies by Siekierski and Fidelis (92) with TBP as

the stationary phase was done using classical column techniques, the very sharp separations obtained and low plate heights suggest some of these systems might be of interest to people using modern HPLC equipment. Extensive reviews on extraction chromatography of inorganic species have been published by Cerrai and Ghersini (4) and Braun and Ghersini (5). Bushee et al. (93) noted the lack of any research using modern HPLC equipment and extraction reagents previously found useful in extraction chromatography. The separation of Cd(II), Zn(II) and Hg(II) was reported by these authors on a C18 column (several different types were studied) using an aqueous mobile phase which was 2M in LiCl and saturated with TBP. The elution of the metals was monitored using a refractive index detector and by atomic emission spectroscopy with an ICP inductively coupled plasma source. The results obtained were encouraging and suggest that with modifications in the equipment (glass lined columns, Teflon valves) the method has considerable potential.

3. DETECTION SYSTEMS

Most metal chelates have strong molecular absorption bands in the ultraviolet and many have useful absorption bands in the visible region of the spectrum. Consequently, simple ultraviolet detectors or variable wavelength photometric detectors were employed in most of the published work on the HPLC of metal chelates. In some cases, the less sensitive refractive index detector or the more sensitive and selective fluorimetric detectors were employed. All three are commonly used with HPLC equipment and in terms of cost, sensitivity, and convenience the photometric (and fluorimetric where it can be used) detectors are hard to match. Except for the fluorimetric detector and, to a lesser degree, the variable wavelength photometric detector they lack specificity.

Detectors which respond to specific metals or metal chelates are very useful to development of HPLC methods for metal chelate separations and are of great interest in many potential applications such as speciation studies. Some of these more specific detection systems which have been used in the HPLC of metal containing species are covered in more detail in the following sections.

3.1 Atomic absorption, AA, and Flame Emission.

Manahan and Jones (94) passed the effuent from a Chelex ion-exchange resin in the Cu(II) form directly to a Perkin-Elmer Model 403 AA unit. Samples containing EDTA (ethylenediaminetetraacetic acid) or NTA (nitrilotriacetic acid) resulted in copper being stripped from the column and the elution of the Cu(II) chelate was monitored using flame atomization by the atomic absorption due to copper. A detection limit of 5×10^{-7} millimoles of EDTA or NTA (and presumably for Cu(II), was reported. O'Laughlin and Hansen (71) used the same technique to confirm the elution of $Ni(phen)_3^{+2}$ (Section 2.6) but noted the poor sensitivity of AA as compared to photometric detection. Jones and Manahan (95) calculated minimum detectable amounts of copper which can be detected by flame AA when eluted as the Cu-NTA and Cu-EDTA complexes from a Chelex ion exchange column as 3.13 and 6.72 ng but were only able to detect 10.7 and 23.6 ng of Cu as these complexes experimentaly.

Freed (96) directly coupled a HPLC unit to a Beckman Total Consumption burner and used flame emission to monitor the elution of the alkaline earths and the lanthanide elements from a Zipax-SCX cation exchange (pellicular resin) column. With 0.01M nitric acid as the mobile phase and the detection system used in the non-selective mode (no slits), he was able to monitor the elution of Ca, Sr, and Ba. Up to five lanthanides could be eluted as separate peaks from the same column

with 0.2M citric acid as the mobile phase and the elution of the peaks monitored by flame emission in the non-selective mode. Plots of concentration versus response were claimed to be linear in the 2-10 ppm range.

Although flame AA or emission might be useful to confirm the elution of a particular metal, the inherent noise from the flame, background absorption or emission (which might be particularly troublesome with organic solvents or gradient elution methods) and the relatively poor sensitivity of both flame AA and emission methods in this mode of operation limits the usefulness of these methods of detection at trace concentration levels and more attention has been given to furnace AA and flame emission with plasma excitation sources.

Brinkman et al. (97) coupled a commercial graphite furnace AA unit with an HPLC unit for the specific detection of organometallic compounds at the nanogram level. The graphite furnace AA detector (GFAA) was operated either in a rapid sampling mode for achieving complete resolution or in a batch survey mode for maximum sensitivity. This device used an autosampler which was rotated and caught specific sized samples of the column effuent in sampling cups. An aliquot of each cup was automatically pipetted into the graphite furnace and the sample dryed, ashed and atomized in a manner consistent with the particular sample. A "reconstituted" chromatographic peak could be constructed from the individual detector pulses. Applications of the GFAA-HPLC system to a number organometallic compounds including alkyl arsenic, lead, mercury and tin compounds were reported. Parks et al. (98) used a GFAA-HPLC system similar to that just described for the analysis of biocidal organotin moieties and organotin silicates by size exclusion HPLC and reversed phase HPLC.

Vickrey et al. (99) noted a problem with the "pulsed mode" sampling in the work described above (97,98). Sharp peaks could be missed because the sampling rate depends on the rate of furnace analysis and subsequent cool-down time. They developed a method which stores an eluate sample which contains a peak (determined by UV detector response) in a capillary tube. The contents of the tube are later incrementally analyzed off-line by the furnace AA. The entire operation was controlled by a Motorola 6800 based Heathkit microprocessor. The detection limits for $Pb(Rh)_4$ was about 20 pg for solutions of $Pb(Ph)_4$ directly analyzed by GFAA and 480 pg of $Pb(Ph)_4$ for samples subjected to liquid chromatographic separation (LCGFAA) analysis using this equipment. They note that the off-line storage of peaks allows a much higher number of AA data points to be collected per peak. It appears that if the solute did not absorb in the UV its retention time would have to be known.

Van Loon (100) reviewed atomic spectroscopy as applied to the detection of metal containing species separated by chromatography. In an earlier paper Van Loon et al. (101) proposed the use of atomic fluorescence as a metal specific detection system in metal speciation-chromatography studies.

3.2 DC Plasma and Inductively Coupled Plasma Atomic Emission

Uden et al. (102) described a DC argon plasma detector and interface system to a liquid chromatograph. A detection limit of $3.5x10^{-10}$g Cu/s^{-1} was obtained for this detector for the elution of $Cu(en)(acac)_2$ by monitoring the 324.7 nm copper emission line. Calibration plots were linear over the range 30-4,000 ng Cu. The separation of a number of metal (Cu(II), Hg(II), Cr(III)) chelates and

mixed chelates (of en, acac, β-ketoamine, hfa, tfa) by reversed phase HPLC on a C18 column and the detection of these species by atomic emission is reported. The authors note that solvent systems common to reverse-phase and ion-exchange systems cause few problems but that hydrocarbon and halocarbon solvents common to normal phase separations present difficulties. It is stated these can be minimized with a new nebulizer system.

Gast et al. (103) reported on the use of an inductively coupled plasma atomic emission spectrometer, ICP-AE, as a detector in HPLC. The high temperature of the plasma which excites useful emission lines for essentially all the metallic elements and elimination or depression of problems caused by compound formation in flames are claimed to make the ICP very attractive for the continuous monitoring of a large number of elements at very low levels. These authors investigated the usefulness of ICP-AE detection for a number of iron and molybdenum carbonyl complexes, ferrocene, organoarsenic compounds, organolead and organotin compounds. Detection limits were found to be in the nanogram per mL range and a linear response range of four orders of magnitude was reported. Solvents containing up to 100% acetonitrile, ethanol and higher alcohols caused no serious problems. Only xylene and toluene could be used for normal phase LC. Other solvents put out the plasma. Fraley et al. (104) reported on minimum detectable concentrations, MDL, that could be detected by ICP-AE based on the injection of aqueous solutions of twenty-five different metal solutions (one at a time) through a short dummy column. They claimed passage through the dummy column gave idealized Gaussian peaks due to diffusion processes on the column but apparently did not consider peak broadening effects on this very "inefficient column". MDL values from 1.3 (for Mn) to 1800 μg/L

(for Mg) were reported. These were far different than the MDL values found for continuous aspiration of 1 and 15 μg/L, respectively, for Mn and Mg. Bushee et al. (93) reported similar differences in relative response and found that when using ICP peak heights alone, the ratio of direct ICP to ICP/HPLC response was about 18.0.

Hausler and Taylor (105) noted that a number of investigators demonstrated the use of ICP-AE as a sequential multielement detector using a single channel and usually an aqueous solvent. They discussed the use of ICP-AE as a simultaneous multielement detector in gel permeation chromatography. The separation and detection of organically bound metal compounds using toluene as the mobile phase is reported. Multiple metal peaks (for each individual metal) were observed in some cases in the chromatography of a Conoco C-21 standard which contained 21 elements at specified concentration levels. The Conoco C-21 standard does not necessarily have all the metals present in only one form and the multiple peaks for some metals was expected. In a subsequent paper (106), these authors used pyridine as the mobile phase for the SEC separation of the Conaco-21 standard and a simpler mixture of four metal containing compounds including ferrocene, 1,1'-diacetylferrocene, acetylferrocene and bis(tetrapyrazolylborate)iron (II). These four compounds were clearly separated and gave four peaks on elution with pyridine but a much different chromatogram was obtained on elution with toluene. This, plus other evidence, was interpreted in terms of solute interaction with pyridine. Several peaks were observed for the elution of $Cu(acac)_2$ with pyridine on the 100A μ-Sytragel column but the number of peaks and retention times were not reproducible on successive injections. Coal liquefaction process solvents were also examined by this technique for organically bound trace metals. Several metals show

complex chromatograms indicating the metal is present in more than one form. Although interesting data were obtained using the ICP-AE detection system , the multiple peaks observed for some metals in complex samples are difficult to interpret and the multiple peaks found for just one metal compound, $Cu(acac)_2$, with pyridine suggest additional studies on simple systems are needed.

Jinno and Tsuchida (107) have recently described an interface system for coupling a micro high performance liquid chromatograph (0.5 mm i.d. x12cm Teflon column packed with 5μm ODS material, Jasco SC-01 column) to an ICP-AE unit. A flow rate of only 25 μℓ/min was used and peak heights and widths with UV detection and ICP-AE detection were very similar for $Cu(acac)_2$ and $Cu(DDC)_2$ samples. No significant differences were observed with methanol or methanol-water mobile phases and it was conjectured that gradient elution could be used. A mixture of Cu(II), Zn(II), Fe(III) and Co(III) diethyldithiocarbamates was injected and the peaks were resolved when monitored at specific emission wavelengths for each metal but it was not possible to resolve the four peaks with UV detection because of the small differences in the retention times. The authors are clearly using the term "resolution" in two different ways which is unfortunate and could lead to confusion.

3.3 Electrochemical Detection

MacCrehan and Durst (108) used differential pulse voltammetry to detect organomercury species eluted from a 5-μm Spheriosorb ODS column. The organomercury cations were reacted with 2-mercaptoethanol and the neutral complexes eluted with a 40% methanol-60% aqueous phase buffered at pH 5.5 and 0.06 M in ammonium acetate. The advantages of the differential pulse method and the amalgamated gold working electrode are discussed. MacCrehan (109) extended this method to the determination of

organotin compounds and reported that triphenyltin gave a linear response with concentration in the range 10^{-4} to 10^{-6} mol/L. MacCrehan and Durst (110) described a dual electrode system employing two sequential generator/detector electrodes in which analytes are first electrolyzed and then detected electrochemically at the second electrode. They note their system has particular advantages for the detection of species such as the organotin cations and other complexes with relatively high oxidation or low reduction potentials. Lyle and Saleh (111) describe the amperometric detection of Cu^{+2} and Zn^{+2} eluted from an Ionex-SA (15-17 μm) column using a specially designed dropping mercury electrode.

Bond and Wallace (58) discussed the detection of metal diethyldithiocarbomates electrochemically (see Section 2.4). In a later paper, Bond and Wallace (112) described an automated method for the determination of nickel and copper at trace levels in a wide variety of matrices based on the HPLC separation of the diethyldithiocarbamate or pyrrolidine dithiocarbamate chelates of Ni(II) and Cu(II) with both electrochemical and photometric detection of the metal chelates. The entire operation from sample injection to the waveform applied to the electrochemical detector and data reduction is under microprocessor control. The metal complexes are formed _in situ_ and the ligand was made a component of the mobile phase (70% acetonitrile-30% aqueous acetate buffer at pH6 and 10^{-4}M in ligand). Various electrochemical waveforms were studied at a glassy carbon electrode. Detection was based on the oxidation (not reduction) of the complexes which eliminated the serious problem of oxygen interference in the reductive mode. Electrochemical detection limits of 0.1 and 0.2 ng of nickel and copper, respectively, were reported with DC detection at +0.70 volts vs Ag/AgCl as compared to

photometric detection limits of 0.2 ng Ni/L and 1.0 ng Cu. The authors report that this automated system has been applied in industrial situations for 18 months and that the extreme versatility of the system has enabled a system of continuous monitoring to be developed. It appears to the present author that the excellent work described by Bond and Wallace shows the great potential in the area of trace metal analysis for methods based on the HPLC separation of metal chelates.

Bethod et al. (65) discussed the electrochemical detection of metal oxinates (See Section 2.5).

Lewis et al. (113) used electrochemical detection for the determination of the pertechnetate ion after separation from potential interferences by HPLC on a NH_2-bonded phase column. The determination of total TcO_4^- ($^{99m}TcO_4^-$ + $^{99}TcO_4^-$) is an important problem in radiopharmacy. These authors studied the reduction of TcO_4^- at both solid (glassy carbon) and static mercury drop electrodes with various applied waveforms (sampled DC, normal pulse, and differential pulse). The determination of TcO_4^- down to the 10^{-8}M range in $^{99}Mo/^{99m}Tc$ generator effluents was reported.

3.4 Post-Column Reactors

Hirose et al. (114) described a post-column reaction technique for the detection of the lanthanides eluted from an ion exchange column. The lanthanides were reacted with xylenol Orange and the metal complexes detected photometrically at 630 nm. Elchuck and Cassidy (115) describe a post-column reaction system for the determination of metal cations following the separation of these cations on a cation exchange column. The eluate is mixed with a chromogenic reagent, sodium 4-(2-pyridylazo)-resorcinol, and the metal complexes monitored at 530 to 540 nm. These authors applied this technique to the determination of lanthanides (115)

and transition metals (116-118). With an on-column trace enrichment procedure they were able to determine a number of metals in the pg/mL range. Beckett and Nelson (119) separated potentially fluorescent metal chelates of 4-aminophenylethylenediaminetetraacetic acid on a 10-μm Partisil-SAX column and detected the chelates on elution by reaction with fluorescamine and fluorimetric detection. The fluorescamine derivatives of these chelates exhibited a linear fluorescence detector response over the range $5x10^{-7}$ to $5x10^{-11}$ g of metal ion. No change in relative sensitivity was observed with different metal ions.

4. HPLC SEPARATION OF OPTICAL AND GEOMETRIC ISOMERS

Strazza and Polcaro (120) recognized the advantages of a fast separation method and used HPLC to separate the cis- and trans-isomers of labile Co(III) species. Cis-$[Co(en)_2(OCOC_6H_5)_2]$ NO_3 was mixed with NH_4Cl and the formation of the trans-$[Co(en)_2(OCOC_6H_5)_2]^+$ and an intermediate species thought to be trans-$[Co(en)_2(OCOC_6H_5)Cl]^+$ was evidenced by the appearance of three peaks in a chromatogram of the mixture after it was heated for 10 min. at 130°C. These authors used a 10-μm Micropak-SI column and the mobile phase was a mixture of 95% ethanol, 2-propanol, 25% aqueous ammonium acetate, and 1N acetic acid in methanol in the ratio 140:60:2.5:0.15. Isocratic elution, not gradient as suggested in a recent review (7), was used and the benzoato complexes were detected at 580 nm. The cis- and trans-$[Co(en)_2CO_3]^+$ species as well as intermediate species were also separated on the same column with a mobile phase as above but in the ratios, 60:140:2.5:0.15, with detection at 340 nm. This work appears to be either an example of ion exchange chromatography on a silica column or of ion-pair chromatography with the acetate ion as the pairing ion.

The speed and high efficiency of HPLC are attractive features for the separation of both labile geometric isomers and for the frequently much more difficult separation of optical isomers. The column chromatographic separation of optical isomers by derivatization (i.e. as diastereoisomers) has been used for some years. *Direct* chromatographic resolution of enantiomers is a newer approach which has been reviewed by Audebert (121). Yoneda (122) has reviewed the resolution of enantiomers of octahedral metal complexes and discuss the fundamental factors involved in the resolution of optically active species in terms of the "fit" between a pairing ion such as d-tartrate and the Λ[Co(en)$_3$]$^{+3}$ or Δ-[Co(en)$_3$]$^{+3}$ form of the complex. Yukimoto and Yoneda (123) reported on the separation of enantiomers in the series fac-[Co(β-ala)$_n$(α-ala)$_{3-n}$] on an anion exchange column charged with chiral and achiral anions such as d-tartrate, antimony d-tartrate, chloride, or sulfate. Nakazawa et al. (124) extended the above studies to the separation of geometric isomers of monopositive cobalt(III) complexes such as [Co(gly)$_2$en]$^+$. Yamazaki and Yoneda (125) reported on the resolution of racemic cations on an anion exchange column and developed general equations for the retention volumes and selectivity ratios for two enantiomers with monopositive cations such [Co(gly)$_2$en]$^+$, [Co(gly)$_2$(NH$_3$)$_2$]$^+$ and [Co(N$_3$)$_2$trien]$^+$. If a dinegative chiral selector ion such as [Sb$_2$-d-tartrate)$^{-2}$ is used in the mobile phase it is more strongly held on the anion exchange column than the ion-pair 1:1 complex with a net charge of minus one. Thus, only relatively low concentrations of the chiral selector ion are required in the mobile phase to elute the enantiomer pairs unlike the case when a cation exchange column is used. Calculated values for the optimum chiral selector ion concentration are compared with experimental

results and the latter are shown to agree well with the theory developed. Although the above work does not involve the use of HPLC, the demonstration that outer sphere ion pairing results in separation of enantiomers and the discussion in these papers on how the "fit" of ion pairs affects retention volumes should be of great interest to research workers investigating factors which relate to selectivity in ion-pair HPLC.

Buckingham et al. (126) described the separation of a series of amino acid Co(III) bis(ethylenediamine) complexes, $[Co(en)_2AA]X_2$ by ion pair HPLC on a 10-μm Bondapak C18 column. An excellent separation of the $\Delta[Co(en)AA]I_2$ complexes was achieved where AA was glycine, proline, valine, leucine, phenylalanine with retention times of 5.6,6.8,8.8,10.8 and 13.8 min., respectively. A linear gradient from 0 to 100% methanol in 15 minutes was employed with the mobile phase kept 5mM in p-toluene-sulfonate and at pH 3.5. The elution order was the same with 5mM hexanesulfonate as the pairing ion but the percentage methanol had to be increased ten-fold to achieve comparable k values. The similarily in elution order to that of the amino acids on silica was interpreted to mean there was a preferred orientation of the charged complex with regard to the hydrocarbonaceous stationary phase such that the amino acid side chain participates significantly in the retention mechanism. This feature can be exploited for the separation of the Δ-S and Λ-S or Δ-R and Λ-R diastereoisomeric mixtures. The nature of the counter ion I^-, Cl^-, or ClO_4^- did not affect the resolution.
These authors reported an unusual phenomenon which they believed was clearly due to the association of two (or more) different cationic species. At constant pairing-ion concentration when the sample loading of two charged complexes such as Λ-$[Co(en)_2gly)^{+2}$ and

Λ-$[Co(en)_2Pro]^{+2}$ was increased each peak split into two peaks. This peak splitting was also seen at constant loading of sample when the pairing-ion concentration was decreased. It was not due to overloading as shown by varying the concentration of a single species. Achiral complexes, $[Co(NH_3)_5X]^{+2}$, showed similar peak splitting. These experiments show that there must be an interaction between two (or more) complex ions which infuences their respective distribution ratios according to the above authors. They pointed out this effect could easily be avoided for analytical purposes by proper choice of the counter ion concentration.

Minor and Everett (127) reported that the resolution of electrically neutral complexes such as $M(\beta\text{-dik})_3$ was generally difficult but by using chiral β-diketone ligands such as (+)-3-acetylcamphor, atc, four stereoisomers are possible; Δ-cis, Λ-cis, Δ-trans and Λ-trans. The four stereoisomers of $Cr(atc)_3$ were separated on a Corasil II silica column using a 15% THF -85% hexane mobile phase with a flow rate of 0.4 ml/min.

Warner and Legg (128) describe a "bare bones" liquid chromatographic system suitable for the preparative separation by synthetic inorganic chemists of geometrical isomers and diastereomers of metal complexes. A Merck 4x60 cm "Lo-Bar" column was slurry packed with Whatman LP-1 silica and 70/30 IS-TEA (isopropyl alcohol-2M triethylammonium carbonate buffer at pH 9). Analytical and preparative separations of $[Co(en)_2Tyr]^{+2}$ diastereomers were shown with the elution of the species monitored at 510 nm.

5. CHELATES AS MODIFIERS

A number of authors have used metal chelates as a component of the mobile or stationary phase to modify the elution behavior of organic

solutes. Chow and Gruska (129) chemically bonded dithiocarbamate and β-diketone groups by reacting carbon disulfide or ethylbenzoylacetone, respectively, with a previously bonded-amine Partisil-10. They loaded the bonded-phase material with Cu(II) and noted this stationary phase permitted selective separations of aromatic amines and other compounds not attainable without the Cu(II) bonded-phase. Karger et al. (130-132) explored the use of metal chelates in the mobile phase to enhance selectivity in HPLC. They observed that a metal chelate in the mobile phase was equivalent to a counter ion such as a quaternary ammonium ion in ion-pair chromatography. However with aromatic acids ($RCOO^-$ or RSO_3^-) the functional group selectivity and steric or isomeric selectively was much more pronounced with metal chelates such as C_{12}-dien-Zn(II) relative to the usual counter ions. They noted that the loading of the metal chelate from a mobile phase 10^{-3}M in C_{12}-dien-Zn(II) onto a Lichrospher-C8 column was slow and required roughly 50 column volumes of mobile phase to be passed through the column but that the column quickly reached equilibrium if the salt concentration (ammonium acetate) was changed. The selectivity and high performance of these systems was interpreted in terms of ligand exchange reactions in the outer coordination sphere of the chelate with chiral metal chelates in the mobile phase such as L-2-isopropyl-4-octyl-diethylenetriamine-Zn(II) (C_3*-C_8-dien-Zn(II)). These authors (132) showed it was possible to resolve the optical enantionmers of all the common amino acids except D,L-Dns-Proline (Dns, dansyl). The latter was resolved using L-proline-n-octylamide with a stoichiometric amount of Ni(II), instead of C_3*-C_8-dien-Zn(II).

Lochmuller and Hangac (133) described the use of the square planar and coordinately unsaturated complex, bis(2,2,6,6-tetramethylheptane-

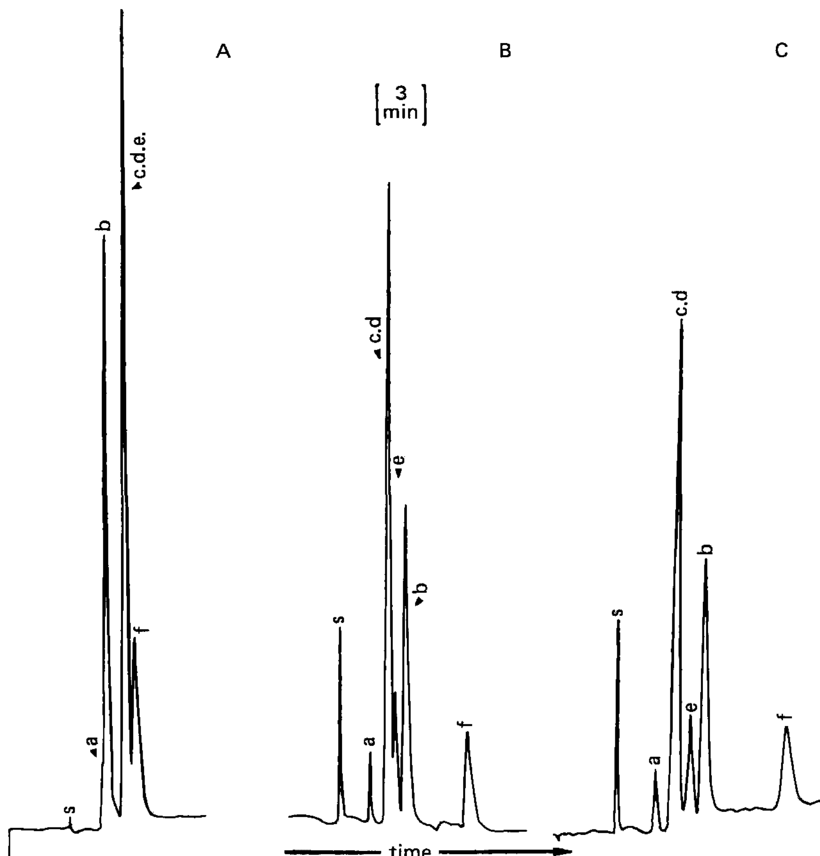


Fig. 6. Chromatograms of amine and pyridine derivatives with and without the Ni(DPM) mobile-phase additive: Column: Whatman ODS-3 (25cm x 4cm); chromatographic conditions: A. 60/40 methanol/water; flow rate: 1 ml/min; temperature: 30°C, B. 1.25 x 10^{-4}M Ni(DPM) - 60/40 methanol/water flow rate: 1 ml/min; temperature: 30°C; C. 4.0 x 10^{-4}M Ni(DPM) - 60/40 methanol/water; flow rate: 1 ml/min; temperature: 30°C Peak a: aniline; b: pyridine; c: o-toluidine; d: 2-picoline; e: p-toluidine; f: 4-picoline. Reprinted by permission from Reference 133 (Preston Public.).

3,5-dionato)nickel(II), (Ni(dpm)$_2$), as a mobile phase modifier. This neutral complex interacts with polar solute molecules and the interplay of factors such as the dipole moment, structure, and solvation effects influence the net interaction. The complex is transparent in the 210-290 nm range permitting UV detection of solutes. The separation of some amine and pyridine derivatives with and without the modifier is shown in Figure 6.

Davankov et al. (134) reported on the resolution of racemic amino acids using ligand exchange on a polystyrene resin containing residues of chiral heterocyclic α-amino acids and charged with Cu(II) ion. Sugden et al. (135) described the resolution of amino acids on a microparticulale silica column holding a chiral Cu(II)-proline complex. Gubitz et al. (136) also used a chiral Cu(II)-proline complex which was chemically bonded to the silica support via 3-glycidoxypropyltri-methoxysilane. Carunchio et al. (137) used a porous silica gel modified with N(aminoethylamino)propyl groups and Λ (+) [Co(en)$_2$(NO$_2$)]Br to separate optical isomers by an outer-sphere complex mechanism. Corradine (138) used a cross-linked dextran gel with amino-Co(III) complex groups to separate some mono-and dinucleotides.

In 1975 Porath et al. (139) introduced metal chelate affinity chromatography for the fractionation of proteins. Hansson and Kagedal (140) coupled iminodiacetic acid to Sepharose activated with 1,4-bis-(2,3-epoxypropoxy)butane. This column was loaded with Zn(II) or Cu(II) and used to separate protein fractions.

The use of metal chelates in trace metal analysis and the use of metal ions and chelates as modifiers in the separation of organic solutes has recently been discussed by Raja (141).

6. CONCLUSIONS

It has been shown by many investigators that HPLC can be used for the separation and determination of metal chelates even at trace concentration levels. With the use of modern microprocessor controlled apparatus, routine monitoring of trace metals by HPLC has become possible. A very nice example of this possibility is the automated HPLC method for the determination of copper and nickel in industrial plant solutions developed by Bond and Wallace (112) and described in Section 3.3. This procedure involves a chemical step (formation of the nickel and copper dithiocarbamate complexes), a separation step (HPLC of chelates), and the detection step (either photometric or electrochemical) with most experimental variables controlled by the microprocessor-based system which also generates the proper electrochemical wave from (for the electrochemical detector), collects, stores, and analyses the data. Other applications of such systems, perhaps with different chelates, seem certain to be forthcoming.

The use of HPLC in speciation studies promises to be a rapidly developing area. The simultaneous determination of Cr(III) and Cr(VI) by HPLC described by Schwedt (54) and of Mn(II) and Mn(III) by Hoffman and Schwedt (68) suggest the usefulness of HPLC in this area. The use of HPLC to separate metal porphyrin species in crude oil described by Hajibrahim et al. (86) and Spencer et al. (87) is another area of great potential in the speciation area. The use of metal specific detector systems such as GFAA and ICP-AE systems coupled with HPLC separation methods will undoubtedly be more widely used in this area. Electrochemical detection systems would also appear to have a bright future in speciation studies.

It seems very likely that HPLC will be much more widely employed by coordination chemists in the near future. The rapid separation of mixed ligand species, geometric and optical isomers of coordination compounds, and the usefulness of HPLC in kinetic studies will certainly be exploited.

The use of metal chelates as modifiers in the mobile phase to increase selectivity in the separation of organic solutes is another area in which rapid growth is already in progress. The relatively few applications referenced in Section 5 should only be viewed as an introduction to this rapidly developing area.

Although much progress has been made in the last decade in the HPLC of metal chelates, some problems have surfaced repeatedly and should be kept in mind. Metal chelates can and do dissociate to some extent during the separation process and tailing peaks and unsatisfactory chromatographic separations will be the result. This may be a very minor or quite insignificant problem for kinetically inert chelates but a major problem for labile chelates even though the thermodymanic formation constants are large. A possible solution in the case of labile chelates is to include the ligand as a component of the mobile phase and to buffer the mobile phase at a pH when the conditional equilibrium constant is sufficiently large. This, of course, raises the possibility of interaction by the chelating reagent with metallic components of the HPLC system. All glass or other metal-free systems can be employed to avoid this problem but this increases the experimental difficulties and may put unacceptably low pressure limits on the system. Commercially available pumps have been used with many mobile phases including chelating reagents with little problem. There is some evidence that suggest the major source of metallic contamination

of the sample occurs is the sample preparation and injection process. It should also be kept in mind that reduction of metal complexes may occur by contact with metallic surfaces and oxidation of Co(II) and Mn(II) in the chelate formation step has frequently been observed.

Peak splitting has been reported by several investigators. Hausler and Taylor (105) reported multiple peaks for $Cu(acac)_2$ with pyridine as the mobile phase (sec. 3.2) and Buckinghan et al. (126) noted peak splitting for both chiral and achiral Co(III) complexes with amino acids (Sec. 4).

While some unresolved problems remain, this reviewer believes that HPLC methodology will be employed much more widely in the near future for the determination of trace metals, in speciation studies, in coordination chemistry, in radiochemistry, in the separation of geometric and optical isomers, and that metal chelates will increasingly be used as modifiers in difficult separation problems.

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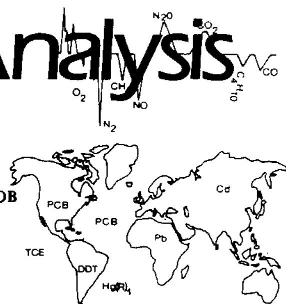

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