REVIEW ARTICLE

High Pressure Liquid Chromatography

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Prior to 1967, liquid column chromatography (LCC) separations were not normally carried out in an optimum manner; for this reason, the technique was viewed as slow and inefficient (1 3). Consequently, other techniques such as thin-layer chromatography (TLC) and gas chromatography (GC) found favor. Within the last 5 years, LCC has undergone dramatic changes and now rivals GC in speed of analysis and column efficiency. The modernized technique is commonly known as high pressure liquid chromatography (HPLC).

Table I roughly compares HPLC with other commonly used chromatographic techniques in terms of support particle size and speed of analysis, N_{eff}/t (see *Measurement of Column Performance*). A maximum N_{eff}/t means the shortest analysis time. Classical LCC and TLC are seen to be slow, although a new form of high speed TLC, thin-film chromatography, was recently described (4). GC and HPLC are seen to be comparable in speed and efficiency. A common misconception in comparing these two techniques is that HPLC is less efficient. Rather, with GC, longer columns and, hence, many more theoretical plates can be used. Because of the much lower permeability of HPLC col-

umns, the apparatus places a limitation on the column length. The lower permeability results from the smaller particle sizes and the higher viscosities of liquids. However, even with relatively short columns, very difficult separations can be accomplished with HPLC due to its greater selectivity. Greater selectivity is achieved through choice of the mobile phase, solvent programming, and variation of the separation mechanism, *i.e.*, adsorption, ion exchange, or partition.

Much early work in HPLC involved the development of high efficiency and high speed systems according to theoretical guidelines. With a knowledge of modern chromatographic theory (5, 6), a better understanding of the basic principles underlying HPLC and the deficiencies of classical LCC techniques is achieved.

THEORY

General Considerations Improvement in LCC has been achieved primarily through the use of more efficient columns and of higher mobile phase velocities. Column efficiency, or band broadening, is described by the height equivalent to a theoretical plate, *H*, concept, which is a measure of solute band broadening per unit length of the column. The height *H* is defined as:

$$H = \frac{\sigma_l^2}{L} = \frac{L}{N} \qquad \text{(Eq. 1)}$$

where σ_l is the standard deviation of the Gaussian function (the solute band profile) in length units, *L* is the column length, and *N* is the number of theoretical plates. The latter is given by:

$$N = 16(L/W)^2 \qquad \text{(Eq. 2)}$$

Table I—Comparison of Chromatographic Performance

Technique	Particle Diameter (d_p), μ	N_{eff}/t^a	Reference
TLC	2–20	<0.08	3
Classical LCC	150	<0.05	3
HPLC			
Silica gel	20	2	3
Porous layer beads			
Zipax	30	8	114
Corasil	28–37	2	114
Silica microspheres	5	23	51
GC			
Classical packed	130	10	150
Porous layer beads	90	50	150

^{a} Effective plates per second, where $N_{eff} = N[k'/(1 + k')]^2$, $N =$ number of theoretical plates, and $k' =$ capacity factor.

where W is the peak width determined by tangents to the Gaussian profile. In the construction and operation of columns, it is desirable that H be as small as possible.

Until recently, the Van Deemter equation:

$$H = A + B/V + CV \qquad \text{(Eq. 3)}$$

had been used to represent the chromatographic band broadening mechanism. In Eq. 3, the A term arises from complex flow processes (eddy diffusion), the B term describes solute dispersion by longitudinal molecular diffusion, and the C term relates to nonequilibrium resulting from resistance to mass transfer in the stationary and mobile phases. There is now considerable evidence to show that Eq. 3 is generally inadequate (3, 5–7).

Huber and Hulsman (8) and Huber (9) developed an equation, in which H is the sum of four terms, which takes into account four independent processes: mixing by longitudinal molecular diffusion, H_{MD}; mixing by convection, H_{MC}; resistance to mass transfer in the stationary phase, H_{ES}; and resistance to mass transfer in the moving phase, H_{EM}. Hence:

$$H = H_{MD} + H_{MC} + H_{ES} + H_{EM} \qquad \text{(Eq. 4)}$$

The longitudinal molecular diffusion, H_{MD}, is given by:

$$H_{MD} = 2D_m/T_mV \qquad \text{(Eq. 5)}$$

Since H_{MD} is inversely proportional to mobile phase velocity, V, and since the diffusion coefficients, D_m, in liquids are 10^4–10^5 smaller than those in gases, this term contributes significantly only at very low velocities in LCC. Figure 1 illustrates this point and compares H versus V curves of LCC and GC. Consequently, at the velocities employed in HPLC, this term is negligible (5, 6).

The most complex dispersion mechanism is convective mixing in the mobile phase. This term is related to point-to-point velocity inequalities within the packed bed (eddy diffusion) and the compensating effects of transverse molecular diffusion and stream splitting (5, 6). Giddings (5, 10) stressed the fact that these terms are not independent but are coupled. An expression similar to the theoretically derived equation (5, 10) was developed (8, 9). The mobile phase mixing term, H_{MC}, is given by:

$$H_{MC} = \frac{2\lambda_1 d_p}{1 + \lambda_2(D_m/Vd_p)^{1/2}} \qquad \text{(Eq. 6)}$$

At high velocities, H_{MC} approaches a value equal to $2\lambda_1 d_p$ (classical eddy diffusion term). Thus, at the higher velocities, lateral diffusion has a smaller effect in decreasing the magnitude of this term. In addition, this term is independent of the capacity factor, k'.

The magnitude of the H_{MC} term is dependent upon the bed structure, the column-to-particle diameter ratio (d_c/d_p), the particle shape, and the packing technique (11, 12). Columns of uniform, dense packing structure give the best column efficiencies (6, 13, 14a). Such columns are most readily prepared from dense, spherical particles (13). In addition, Eq. 6 predicts that decreasing the particle diameter should increase efficiency. This effect is experimentally found to be true for particles less than 100 μ only if the correct packing technique is employed (4, 9, 14a). Furthermore, theory predicts that increasing the column-to-particle diameter ratio will increase band spreading due to the increasing importance of transcolumn flow inhomogeneities (5). However, the optimum column diameter was found to be between 2 and 4 mm. i.d. for regular packed columns $(d_c/d_p > 10)$ (13, 14b). Below 2 mm., efficiencies are significantly poorer (13, 15). Apparently, this discrepancy is associated with the difficulty of packing small particles homogeneously into narrow bore tubes. With columns greater than 4 mm., distorted and doublet peaks have been noted (15). This distortion is probably associated with the different packing structure in the vicinity of the column wall (wall effects) (5), where the velocity is greater than the average. Since solute diffusion is slow in liquids, the solute in the vicinity of the walls does not have enough time to diffuse back to the center before elution; hence, bands elute unsymmetrically. However, Knox and Parcher (16) proposed a theory where slow diffusion leads to improved chromatographic performance. These authors calculated that, with a 5-mm. i.d. column of length <0.33 m. containing particles with a diameter of 30 μ, the solute never reaches the wall area before elution. Consequently, the band is not distorted by wall effects. This type of column is known as the "infinite diameter" column and was successfully demonstrated with columns of 7.94 and 10.9 mm. i.d. (15). A column is judged to be of infinite diameter if the column diameter, d_c, is greater than $(2.4Ld_p)^{1}$.

At velocities above the minimum in H versus V curves (Fig. 1) for GC or LCC, H_{ES} and H_{EM} terms become increasingly important as the velocity increases. These terms relate to nonequilibrium resulting from resistance to mass transfer in the stationary phase, H_{ES}, and the mobile phase, H_{EM} (5, 6). The extent of broadening from nonequilibrium is a function of the rate of flow of the mobile phase relative to the rate of mass transfer of the solute between the mobile and stationary phases. Rate-limiting processes that contribute to this type of broadening in LCC include desorption kinetics and diffusion in the mobile phase which is stagnant in pores of the support[1]. General

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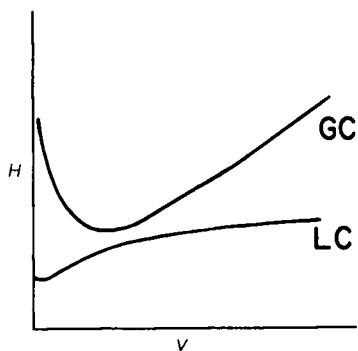[1] For a detailed discussion of these terms, see Reference 5.

Figure 1—*Typical shapes of* H *(height)* versus V *(velocity) curves for GC and LC.*

equations (8, 9) that describe these processes are:

$$H_{EM} = \frac{1}{5.7} \frac{\epsilon_\alpha - \epsilon_m + K\epsilon_\beta}{\epsilon_\alpha + K\epsilon_\beta} \frac{\epsilon_m^{1/3} d_p^{2/3} \eta^{1/4} V^{1/2}}{(1 - \epsilon_m) D_m^{1/2}} \qquad \text{(Eq. 7)}$$

and:

$$H_{ES} = \frac{1}{30} \frac{\epsilon_\alpha - \epsilon_m + K\epsilon_\beta}{(\epsilon_\alpha + K\epsilon_\beta)^2} \frac{\epsilon_m(1 - \epsilon_m)}{\epsilon_s} \frac{T_s d_p V}{D_{is}} \qquad \text{(Eq. 8)}$$

H_{ES} is more strongly dependent than H_{EM} upon the mobile phase velocity, V, and the particle diameter, d_p. However, both terms are minimized by decreasing d_p. Many workers have noted, however, that for $d_p < 100$ μ, H increases (4, 17, 18). This discrepancy from theory is associated with the difficulty of packing homogeneously small particles, especially those of a low density, nonspherical type (15, 18). However, with special packing techniques, the use of small diameter particles was impressively demonstrated by Hamilton (19), Scott *et al.* (20), and Burtis (21) using roughly 10–20-μ particle diameter ion-exchange resins. Piel (22) successfully used a variety of supports in the submicron region, and Huber and Hulsman (8) and Huber (9) packed efficient columns with 20–30-μ diatomaceous earth. The successful use of small diameter particles to reduce mass transfer effects requires dense uniform packing, *i.e.*, minimal contribution from H_{MC}. Recently, Kirkland (23) demonstrated the superiority of small particles with 5-μ porous silica microspheres (Table I).

Equations 7 and 8 indicate that the mass transfer term can also be reduced by maximizing solute diffusion coefficients, D_m and D_{is}, in the mobile and stationary phases, respectively. Since the liquid diffusion coefficient is inversely proportional to liquid viscosity, the stationary and mobile phases with the lowest possible viscosities should be chosen (2, 24, 25).

Resistance to mass transfer from diffusional processes in the stationary phase can also be reduced by minimizing the diffusion path length (*i.e.*, minimizing stationary film thickness) (5, 11). In addition, a stagnant mobile phase in the pores of the support further contributes to slow mass transfer (5, 6, 11). These effects have been decreased by the reduction in particle size, since specific pore volume decreases with decreasing particle size (8, 9), and by the development of porous layer beads, which will be described.

Equation 8 can be simplified if it is assumed that the pores of the support are completely filled with β (sta-

tionary phase). In such a case:

$$H_{ES} = \frac{1}{30} \frac{k'}{(1 + k')^2} \frac{T_s d_p^2 V}{D_s} \qquad \text{(Eq. 9)}$$

where k' is the capacity factor equal to $K(\epsilon_\beta/\epsilon_\alpha)$.

The capacity factor function reaches a maximum at $k' = 1$ and then decreases strongly with increasing k'. If mobile phase effects predominate over stationary phase effects, H is essentially independent of k' (6). However, since the magnitude of k' is related also to sample resolution and since low k' values are desirable for fast analysis, there is an optimum k' value (6). Snyder (3) calculated the optimum value of k' to be 5 for a given column length and any desired column pressure and 2.9 for a given column pressure and any column length.

Speed and Optimization in Liquid Chromatography— A number of recent papers dealt with the theoretical treatment of the optimization of chromatographic parameters for fast separations (1, 8, 17, 22, 26–28). Since the time of separation is a function of a number of parameters, mathematical optimization with respect to all relevant parameters is virtually impossible (28). The problem is simplified by holding a number of parameters fixed (26, 28). However, all treatments of the resolution of a two-component system start with the following equation:

$$t_r = (1 + k') \frac{L}{V} = N(1 + k') \frac{H}{V} \qquad \text{(Eq. 10)}$$

Resolution is related to N, the number of theoretical plates necessary to achieve the separation. The difficulty of the optimization problem arises from the complexity of the H function, which is in itself a function of the other parameters in Eq. 10. Equation 10 does, however, show that the retention time, t_r, can be reduced by minimizing the H/V ratio. It follows that the optimum conditions for fast analysis correspond to the minimization of the H/V factor. However, the H/V factor initially decreases sharply with increasing V but then decreases slowly at higher velocities (6, 9, 26). The point at which the slope of the H/V *versus* V plot stops changing significantly can be considered as the optimum velocity, especially where pressure drop is a limitation. The optimum velocity for 2–3-mm. i.d. columns is about 2–3 cm./sec. (6). Beyond the optimum velocity, a decrease in analysis time is afforded only by a large increase in pressure drop, ΔP, in the column. Pressure drop is related to velocity through the column permeability, K^0:

$$K^0 = \frac{V\eta Lf}{\Delta P} \qquad \text{(Eq. 11)}$$

The importance of pressure drop and permeability in regard to analysis time can be seen by substituting the expression for V from Eq. 11 into Eq. 10:

$$t_r = \frac{(1 + k')\eta L^2 f}{K^0 \Delta P} \qquad \text{(Eq. 12)}$$

For constant values of k', η, L, f, and ΔP, the more permeable the column the faster is the analysis time. Equation 12 also indicates that the analysis time can be

decreased by lowering the solvent viscosity, η, and the column length, L.

Equation 10 indicates that t_r can be reduced by optimizing factors that minimize H. These factors were discussed previously, but, in the main, a decrease in particle size (8, 9, 26) and the development of support materials that give flat H versus V curves will give the fastest analysis time.

The equations used previously to describe analysis time neglected the effects of extracolumn band broadening. The shorter the column length, the smaller must be the contributions from injection and detection (26, 28). Smuts and Pretorius (28) showed that, for simple separations, the minimum separation time is limited by extracolumn effects.

COLUMN DESIGN

Column Packings—The use of the relevant theories has led to the development of two methods to achieve high speed analysis by LCC: the development of porous layer beads of 30–50-μ diameter and the use of supports with particle diameters less than 10 μ. Because both supports have shallow pore depths, slow mass transfer in the stagnant mobile phase held within the pores is reduced.

A porous layer bead has a hard, nonporous core surrounded by a thin, porous shell. Golay (29) and Bohemen and Purnell (30) first suggested, from a theoretical standpoint, the advantages of porous layer beads for fast analysis. Other authors (31, 32) suggested the use of such supports for LCC. Rapid mass transfer is possible, since diffusion occurs only in a thin, porous layer. As a result, flat H versus V curves are produced; consequently, high carrier velocities can be used with small loss in efficiency. The various types of porous layer beads were described (6, 33, 34). Two commercially available supports, Zipax[2] (26, 35) and Corasil I and II[3] (36), have siliceous, porous layers of roughly 1-μ thickness. Zipax is a relatively inert support (35) and is used primarily in liquid–liquid chromatography (LLC). The Corasils are much more active and, therefore, can be used for both LLC and liquid–solid chromatography (LSC) (36). Supports with a porous layer of alumina have also been prepared (11, 37). A new type of support, the surface-textured bead having 30-40-μ diameter and surface-etched pores with 2–4-μ depths, has potential in high speed LLC (24). However, surface-textured beads have even a lower sample capacity than the porous layer beads. Porous layer adsorbents also have been developed for ion-exchange chromatography. A pellicular ion-exchange bead[4] in which the ion-exchange resin is polymerized on the surface of a spherical, solid glass bead has been introduced (11, 38a). Kirkland (39, 40) developed similar materials.

In addition to the fast mass transfer properties of porous layer beads, their spherical shape, high density, and rigid structure yield dense, regular packed beads which are stable at high pressures. However, because

of their low surface areas, small sample sizes and very sensitive detectors must be used.

A possible solution to the capacity problem is to use porous supports of small particle diameters (<10 μ). As already noted, the use of supports with such small particle sizes has been successfully demonstrated. Supports that have been used are diatomaceous earth (8, 9, 41), silica gel (4, 22), alumina (22), carbon black (22), silica microspheres (23), and ion-exchange resins (20, 42). Huber and Hulsman (8), Huber (9), and Deelder et al. (41) used diatomaceous earth of 28–32 and 9 μ. The efficiency of these columns for retained or unretained solutes was comparable to efficiencies of porous layer beads. However, with the exception of ion-exchange resins, for which special packing techniques have been developed (43, 44), practical difficulties arise in preparing homogeneous beds of these small particles (4). Very narrow size fractions are a definite must in preparing satisfactory columns (8). Batch separation into narrow size fractions by elutrification was described for diatomaceous earth (41) and ion-exchange resins (42). With the development of reproducible packing techniques, 2-5-μ porous particles will become commonplace in the future. Some supports available for HPLC are shown in Table II.

Column Tubing and Shape—Kirkland (39) found significantly poorer efficiencies for solutes chromatographed in columns of seamless stainless steel relative to precision glass[5] and precision bore stainless steel. Karger et al. (24) also found precision glass tubing to give better efficiency than seamless stainless steel; however, the difference between the two column tubings was not as great as was found by Kirkland (39). Karger et al. (24) recommended that the seamless stainless steel be washed as follows: chloroform, acetone, water, 50% phosphoric acid, 10% nitric acid, water (until washings are neutral), acetone, and chloroform. Kirkland (45) recommended scrubbing the interior with laboratory detergent solution.

Columns filled with packings and then bent have shown a significant decrease in efficiency in comparison to straight columns (39, 46). Straight columns 50 or 100 cm. in length are generally used. To obtain more theoretical plates, columns can be connected in series with low dead volume connectors. Kirkland (33) obtained a 5-6% loss in efficiency for two connected columns and a 10% loss for three connected columns.

Column Packing Techniques—Column packing is one of the most difficult and most important experimental operations in high efficiency LCC. The various packing techniques were reviewed (4, 47). Columns are packed by either dry-fill (14, 45, 48) or wet-fill (14, 44, 49) techniques.

The standard dry-fill technique involves small incremental additions of dry packing, with horizontal tapping of the column at the level of the packing and with simultaneous vertical tapping of the column on the floor or benchtop. Sie and van den Hood (14a) found H to decrease by a factor of 5 or more for relatively large column diameters (d_c = 1.0 cm.) when a special dis-

Table II—Supports for High Pressure Liquid Chromatography

Type	Particle Size, μ	Use
Porous Layer		
Silica (active)	37–50[a]	LLC, LSC
	37–44[b]	LLC, LSC
	30–40[c]	LLC, LSC
	30–44[d]	LLC, LSC
Silica (inactive)	44–53[e]	LLC
	25–37[f]	LLC
Alumina	37–44[g]	LLC, LSC
Porous		
Silica	20–44[h]	LSC, LLC
	37–75[i]	LSC, LLC
	<40[j]	LSC, LLC
	36–45[k]	LSC
	10,20,30[l]	LSC, LLC
Alumina	18–30[m]	LSC, LLC
	<74[n]	LSC, LLC

[a] Corasil I and II (Waters Associates). [b] Pellosil (Northgate Laboratories). [c] Perisorb A [Varian, E. Merck (Germany), E. M. Laboratories]. [d] Vydac (Applied Science Laboratories, Separations Group). [e] Liqua-Chrom (Applied Science Laboratories). [f] Zipax (Dupont Instruments). [g] Pellumina (Northgate Laboratories). [h] BioSil (Varian, Bio Rad Laboratories). [i] Porasil (Waters Associates). [j] Spherosil [Pechiney-Saint-Gobain (France)]. [k] Sil-X (Nester-Faust). [l] Li Chrosorb [Varian, E. Merck (Germany), E. M. Laboratories]. [m] Woelm (Waters Associates). [n] Bio Rad AG (Bio Rad Laboratories).

tributor head for feeding the adsorbent evenly into the column, plus rapid rotation and tapping of the column, is used. These workers also found that smooth particles gave consistently better columns by the dry-fill technique. They found H to decrease in the following order: silica beads > silica gel > alumina > sil-o-cel. In addition to vibration, Stewart *et al.* (50) recommended gentle tamping (weight of rod on top of the packed adsorbent) for the dry fill of silica gel. The efficiencies of tamped columns improved with decreasing particle size down to about d_p equal to 50 μ. The loss in efficiency for smaller particle sizes is probably due to the difficulty of obtaining dense, regular bed structure with irregularly shaped particles of alumina or silica gel (4). Conversely, the dense, spherical porous layer beads can be efficiently packed with the standard dry-fill techniques for particle sizes down to 20–30 μ (33). Kirkland (45) described an optimum procedure for packing Zipax. Column efficiency was found to improve with a decrease in particle size down to at least 37 μ. For 1% β,β'-oxydipropionitrile on Zipax (<37 μ), plate heights of 0.23 mm. ($k' = 2$) were found at 1 cm./sec. mobile phase velocity. Although other porous layer packings were not included in this study, similar results are expected.

Other workers (8, 9, 41) were able to pack good liquid partition columns with diatomaceous earth with d_p down to 10 μ. Columns were packed by small incremental additions of the support, with tamping between increments using a closely fitting rod. However, such packings have the disadvantages of bed compression and lower permeability at pressures exceeding 1 atm./cm. (41).

For supports of particle sizes less than about 20 μ, the slurry packing technique is often used (14, 44, 49). The balanced slurry technique (49), where the support particles are suspended in a solvent of equal density, is probably the best method. Sie and van den Hood (14a)

recommended the use of calcium sulfate hemihydrate as a binder in the slurry. Silica[6] with a mean particle size of 10 μ, when packed by the balanced slurry technique, gave columns of high efficiency (51). On the other hand, Stahl *et al.* (52) found poorer efficiencies by the slurry technique (probably not balanced slurry) than by a dry-fill technique for silica gel particles of less than 15 μ. More recently, Kirkland (23) introduced silica microspheres (5–6-μ particle diameter) and generated 23 effective plates/sec. (see *Measurement of Column Performance*) with these particles packed by a slurry technique in a column 35 × 0.32 cm. at 600 p.s.i. pressure drop.

INSTRUMENTATION

Many papers have been written on the overall design of LCC systems (53–61). Scheme I shows a block diagram of a simplified liquid chromatograph. Although many manufacturers supply complete systems, a liquid chromatograph can be built easily from individual components which are readily available. In general, all components should be constructed of corrosion-resistant materials such as stainless steel, glass, or Teflon.

Flow System

Pumps can be classified into two major types: mechanical and gas displacement. Mechanical pumps are constant flow rate devices such as the screw-driven syringe pump and the reciprocating piston or diaphragm pumps. The former are pulse free, whereas the latter type requires pulsation dampening for detector stability. Several methods for pulsation dampening have been described (57, 60, 61). Karger and Berry (62) constructed a simple device for producing pulseless flow with inexpensive reciprocating piston pumps. Mechanical pumps can be purchased which operate to pressures of about 5000 p.s.i.

Of the gas-displacement pumps, the gas pressurized reservoir (2, 54) is the least expensive and is capable of pulseless flow. The reservoir can be a stainless steel container or a coil of stainless steel tubing, pressurized by nitrogen from a gas cylinder. A second type of gas-displacement pump is the gas-driven piston pump (55), which amplifies the gas pressure by utilizing a large area gas piston pushing against a smaller area liquid piston. The prime disadvantage of gas-displacement pumps is that the flow rate changes with varying back pressure.

The design of the injection port[7] is critical to minimize band spreading, especially for solutes of low k'. Inlet bandwidths must be restricted to submillimeter values. The major factors that describe band spreading in the injection port are: (a) distance between syringe needle and the column inlet, (b) mobile phase linear velocity, (c) sample volume, and (d) rapidity of radial mass transfer (63). Direct on-column injection gives minimum band spreading (63). Mobile phase swept injection ports (including sampling valves) are satisfactory for solutes with $k' > 1$ (53). Smuts *et al.* (63) designed
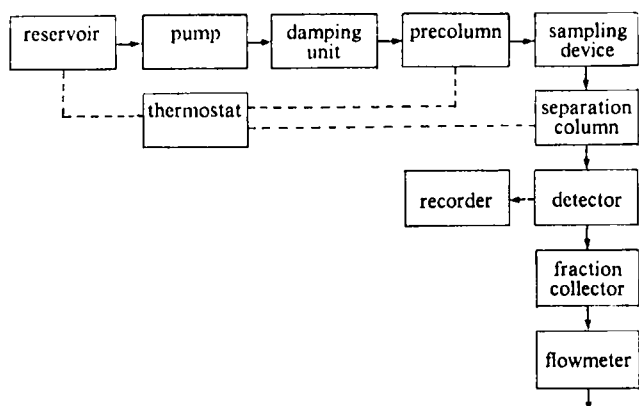
Scheme I—Diagram of a liquid chromatograph

a unique solvent swept inlet system giving bandwidths as small as 0.3 mm.

Detectors

A number of excellent papers have been published on the various detectors available for LCC (64–66). Munk (67) and Huber (68) compared the most commonly used detectors. LCC detectors can be categorized as solute sensitive or bulk sensitive.

Solute-Sensitive Detectors—*UV and Visible Absorption*—The UV photometer is most commonly used since it is relatively insensitive to variations in flow rate or temperature and is adaptable to solvent programming. A number of workers (69–71) described the construction of low dead volume UV detectors. These detectors and the commercially available detectors are single wavelength units (254 or 280 nm.). Such detectors are capable of sensing less than 10^{-9} g./ml. (55). Commercial spectrophotometers have also been used successfully as high performance detectors (68, 72–74). The obvious advantage is wavelength selectivity. In addition, in some cases the number of solvents to choose from is increased. In cases where solutes have little or no UV absorption, the use of color-forming reactions can be of great value for increasing sensitivity and selectivity. Stahl *et al.* (52) described the construction of a high performance system for the analysis of carboxylic acids in the nano-equivalent scale, using the sodium salt of *o*-nitrophenol as the indicator. The authors suggested the use of umbelliferone, whose protonated form is highly fluorescent, as an alternative indicator. Such a technique can be of extreme value in trace analysis and metabolism studies.

Fluorescence—Although no work has been published using a fluorescence detector in HPLC, a high performance, dual-beam fluoro monitor[8] is on the market. In addition, filter fluorometers[9] can be converted with suitable flowthrough cells (75). The technique has the advantages of specificity and high sensitivity (about 10^{-9} g./ml.). In addition, fluorescent derivatives can be prepared from many compounds lacking fluorescent capabilities.

Flame Ionization—To obtain a suitable response in a flame-ionization detector, the solvent must be removed. This is done by coating part of the column effluent on a moving wire which then passes through a heated chamber. Currently, two general methods of getting the sample into the flame are used: direct combustion in the flame (76–81) and pyrolysis of the residue on the wire prior to detection in the flame (82–85). The former technique is noisier due to the movement of the wire through the flame and the intermittent misalignment of the wire in the flame. Scott and Lawrence (83) modified the pyrolysis detector in such a way as to reduce significantly the noise level. Instead of pyrolyzing the solute, combustion of the solute in an oxygen atmosphere to carbon dioxide is used. The carbon dioxide is then hydrogenated catalytically over nickel to methane which is then passed into the flame-ionization detector. This detector's response is directly proportional to the carbon content, and the sensitivity is about 2 mcg./ml. of mobile phase. The moving wire detector can be used with all modes of column chromatography including gradient elution. However, it has the disadvantages of: (a) poor sensitivity since only part of the effluent is detected, (b) markedly reduced sensitivity as solute volatility increases, and (c) imprecise quantitation if the sample is not homogeneously dispersed over the column cross section (68).

Polarographic—The polarographic detector is specific and highly sensitive. The detector is sensitive to solutes that can be oxidized or reduced at the mercury electrode. Additional specificity results from varying the half-wave potential. The only other requirement is that the mobile phase must have sufficient conductivity.

A detector based upon the dropping mercury electrode (DME) suitable for high speed LCC was described by Koen *et al.* (86). The cell volume was only a few cubic millimeters. The detector was successfully applied to the chromatographic analysis of methyl parathion and parathion at concentrations down to 10^{-8} mole/l., with a standard deviation less than 2%. The major problem of this design is dissolved oxygen in the mobile phase and the sample solution, and damping is required to reduce the noise associated with the DME. The sensitivity of the detector depends upon the electrochemical valency, the solute diffusion coefficient, the flow rate and drop time of the mercury, and the flow rate of the eluant.

Joynes and Maggs (87) described a simpler system based upon a carbon-impregnated silicone membrane as the electrode. The advantages over the DME are low residual currents, decreased proneness to mercury oxide films, simple construction, low noise level (no damping required), no atmospheric exposure of mercury, and 100 times lower interference from oxygen in the mobile phase. The linear dynamic range is of the order 1×10^5, with a detection limit of about 2×10^{-9} mole/l. (organics), and the reproducibility is about 0.25%.

Radioactivity—This detector is extremely useful for metabolic studies because of its specificity and high sensitivity. The utilization of β-radiation using suspended scintillators in continuous-flow systems was described (88). A detector suitable for high speed LCC

was also described (89). The detector cell consists of a Teflon tube, 36 mm. in length $\times$ 0.38 mm. i.d., in a continuous-flow Geiger-Muller counter.

The following expression for detector sensitivity was derived:

$$\text{peak area} = \frac{S_A V_D}{\mu} A \qquad \text{(Eq. 13)}$$

where S_A is the sensitivity of the detector, V_D is the volume of the detector cell, μ is the volume flow rate, and A is the total activity of the sample component. The sensitivity is seen to be inversely proportional to the flow rate but directly proportional to the cell volume. A compromise must be made between the cell volume size and the tolerable band spreading.

Davies and Mercer (90) described an automated system for the analysis of labeled, unsaponifiable lipids. The scintillator flowthrough cell was packed with europium-activated calcium fluoride crystals.

Bulk-Sensitive Detectors—*Refractive Index*—The refractive index detector monitors the difference in refractive index between the reference and analytical column effluents, and sensitivity is determined by this difference. This detector is close to being a universal detector, the exception being when the solute and solvent have the same refractive index. Two types are commercially available: the deflection type[3] and the reflection type[10] (91). The sensitivity is about 10^{-7} refractive index unit full scale. However, to achieve such sensitivity, the temperature and flow rate must be controlled precisely (64, 67). The temperature coefficient is about 10^{-4} refractive index unit/°C. Heat exchanger tubing is commonly used. To minimize band spreading and to improve temperature stability in the heat exchanger, Deininger and Halasz (61) recommended that the free cross section of the heat exchanger be squeezed alternately at 90° angles. Thus, high radial mass transport in the heat exchanger tube is achieved through turbulent flow. The peak broadening is minimized to that of the high efficiency UV detectors, and the noise levels are reduced to less than 3×10^{-8} refractive index unit.

Solvent programming is not feasible unless solvents of the same refractive index are used (92). A new detector (93) based upon continuous evaporation of the eluate and redissolution of the residue in a fixed solvent gives the maximum response in a refractive index detector.

Heat of Adsorption—The heat of adsorption detector or microadsorption detector (94–100) is a universal detector which measures minute temperature changes accompanying the sorption of the eluent and the solute on an active solid surface, usually the support held in the chromatographic column. The detector can be used with all modes of chromatography. However, this detector is extremely flow and temperature sensitive. In addition, it requires frequent calibration, and the peak shape (97, 100) is unusual in that a positive component and negative component result. The real value of this detector is in qualitatively visualizing the elution pattern (64).

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[10] Laboratory Data Control.

Other Detectors—The conductivity detector is sold by a number of manufacturers and is commonly used in amino acid analyzers and in gel permeation techniques (65). Other techniques that have been applied to HPLC are the gas density balance (101), the mass (102), the electron-capture (103), the interferometer (104), and the capacitance (105–107) detectors.

LIQUID CHROMATOGRAPHY METHODS

Selectivity—The ability of any chromatographic technique to perform separations depends upon the efficiency and selectivity of the chromatographic system. The former was discussed previously while the latter is expressed in terms of the relative retention, α, that is equal to the ratio of retention volumes of two solutes. Selectivity is determined by solute–solvent or solute–adsorbate interactions and, to a lesser extent, by their molecular size and shape (108).

Although chromatographic systems are primarily chosen by trial and error, some theoretical and empirical treatments have been developed which can be of aid. Snyder (109) discussed the role of the mobile phase in LCC. Solvent polarities are measured in terms of the Hildebrand solubility parameter, δ, and the solvent strength parameter, ϵ^0. In addition, Keller and Snyder (110) discussed the relationship between δ and ϵ^0.

Martire and Locke (111) considered the thermodynamic basis for selectivity in terms of both the athermal (size) and the thermal (energy) factors in LLC. Locke (112) measured the infinite dilution activity coefficients of hydrocarbons in the acetonitrile–squalene system.

Huber (113) reviewed the methods for prediction of partition coefficients in liquid–liquid systems. Recently, Huber et al. (114) developed a new method of predicting partition coefficients. The partition coefficient (K_i) is described as a function of P parameters, each having a solute factor (A_{ip}) and a liquid–liquid system factor (X_{kp}). The equation has the form:

$$\log K_i = \sum_P A_{ip} X_{kp} \qquad \text{(Eq. 14)}$$

The method was tested for 28 steroids in six ternary liquid–liquid systems composed of water, ethanol, and isooctane. For $P = 3$, a precision of about 4% was obtained.

Liquid–Liquid Partition—*Liquid Liquid Systems* The types of supports available for partition chromatography are shown in Table II. The various porous layer beads were evaluated by Karger et al. (115), and a similar comparison was made by Kirkland (33) and Majors (116). On the basis of the data given, Zipax appears to be the best porous layer support for liquid–liquid chromatography. The Corasils are much more active than Zipax since Corasil I, when packed dry, adsorbs 1.1% (w/w) β,β'-oxydipropionitrile from eluent saturated with β,β'-oxydipropionitrile. On the other hand, Zipax adsorbs only 0.25% (115). Majors (116) recommended that Corasil be deactivated by heating to 350° for LLC. Tailing was partially eliminated upon deactivation. Huber et al. (117, 118) successfully used small particles of ground Porasil (2–5 μ) and diatomaceous earth (5–10 μ, 20–30 μ) as LLC supports.

To produce columns of high capacity, Halasz et al. (119) proposed the use of heavily loaded columns. Columns packed with Porasil A (38–53 μ) coated with 50% (w/w) β,β'-oxydipropionitrile exhibited high capacity factors which were independent of carrier linear velocity. Such columns have low bleed rate and large sample capacity (at least 1 mg. in 2-mm. i.d. column). Heavily loaded columns should perform well in preparative applications, providing the stationary phase can be separated easily from the solutes. Kirkland (23) recently introduced the use of 5 6-μ silica microspheres. This packing is not yet commercially available.

A fluorocarbon support[11] coated with 23% Amberlite LA-1 gave excellent separation of steroids by reverse phase chromatography (120). Zipax and diatomaceous earth supports were also used in this study.

Karger et al. (24) introduced surface-textured beads, small particle diameter fractions of GLC-100 support[12]. Although very efficient columns (5500 plates/m., 1 cm./ sec.) were produced, the capacity of the columns was poor.

Kirkland (33, 48) reviewed the various packed columns and the experimental techniques of LLC.

The choice of stationary phase in LLC is, at this stage, largely trial and error. Commonly, binary two-phase systems are used. For polar solutes, polar mobile phases such as β,β'-oxydipropionitrile, 1,3-pro-panediol, ethylene glycol, and Carbowax are used with nonpolar carriers saturated with stationary phase. To decrease retention times, polar modifiers such as chloroform, tetrahydrofuran, or alcohol can be added to the carrier up to about 10% (v/v). Binary, ternary, or quaternary two-phase systems are also useful.

Huber et al. (114, 118) described six ternary two-phase systems of water, ethanol, and isooctane for the analysis of steroids. With such systems, the relative polarities of the two phases can be varied to achieve optimum k' and α values. Other possible ternary systems are chloroform cyclohexane nitromethane, benzene–water–alcohol, and water ethyl acetate-n-butanol. Commonly, the polar phase is used as the stationary phase; however, for reverse phase applications, the less polar phase can be coated onto surface-deactivated supports. Commonly used nonpolar, stationary phases are squalene (112), hydrocarbon, or cyano ethyl silicone polymers (121). A series of liquid–liquid partition systems which may find use in LLC was described by Metzsch (122).

Experimental techniques necessary to obtain precise quantitative analysis with high speed LLC were described by Leitch (123).

Permanently Bonded Stationary Phases—LLC has such disadvantages as the tendency of the stationary phase to bleed and the inability to solvent program. To overcome these disadvantages, column packings with chemically bonded, organic, stationary phases have been developed. Two different types of chemically bonded phases are commercially available.

1. Esterified Siliceous Supports (Brushes)—Esteri-

fication of surface-active silanol groups (Si—O—H) of silica with alcohols creates the Si—O—C bond at the silica surface. Halasz and Sebastian (124) bonded 3-hydroxypropionitrile to the surface of Porasil C and demonstrated the usefulness of "brushes" for fast gas and liquid chromatographic separations. Durapaks[3] are commercially available silicate ester packings. A more efficient packing is made by the esterification of Corasil. The disadvantage of these packings is that the Si—O—C bond is not hydrolytically stable. In addition, extreme caution is necessary if the eluant contains proton donor liquids such as isopropanol (125). With such solvents, a good proportion of the bonded material elutes (126).

A more promising brush-type bonded phase is based upon the much more hydrolytically and thermally stable Si—C bond. Locke et al. (127) produced naphthyl-Porasil C by chlorinating the surface of Porasil with silicon tetrachloride or titanium tetrachloride followed by coupling with naphthylmagnesium bromide. Organic contents of 4.0 ± 0.5% were found. The reactive, bonded naphthalene ring can undergo such reactions as bromination or sulfonation. Repeated bromination of naphthyl-Porasil C and combination with naphthylmagnesium bromide provided a polynaphthyl-Porasil containing as much as 24% by weight of organic material. Both cation and anion exchangers were prepared from these substituted Porasils.

2. Chemically Bonded Silicone Polymers—Bonded silicone polymers are prepared by the reaction of organochlorosilanes with the active surface hydroxyls to form silicone polymers of the type Si—O (R$_2$ Si—O -)$_n$, where R is an organic radical bonded by carbon to the siloxane chain. Aue and Hastings (128) described the means of preparation and chromatographic applications of surface-bonded silicones to diatomaceous earths, silica gels, silica beads, and glass beads. These materials were thermally and hydrolytically stable.

Similarly, Kirkland (49) and Kirkland and De Stefano (129) introduced two permanently bonded silicone polymer phases, where R = C$_6$H$_{12}$O$_2$, R' = O—(ether bonded phase) and R = C$_2$H$_1$CN, R'' = CH$_3$ or -O— (nitrile bonded phase), for HPLC. Schmit et al. (130) introduced a bonded phase where R = C$_{18}$H$_{37}$ (octadecyl silicone) for reverse phase chromatography. Both the ether and nitrile bonded phases show selectivity toward proton donors (129). The octadecyl phase is selective for samples that are sparingly soluble in water and fairly soluble in alcohols and/or alkanes (130). The retention mechanism on these columns is not clearly understood; however, there is evidence that the mechanism is a combination of both partition and adsorption (33, 49, 130). Unlike conventional liquid–liquid systems, the temperature, the modifier, or the percent modifier can be varied at will without any column deterioration. Undoubtedly, many future developments in LCC will involve synthesis of many new bonded phases of varied polarities.

Liquid–Solid Chromatography For reviews and for detailed discussion of the experimental technique and selectivity in liquid–solid chromatography (LSC), consult the works of Snyder (1 3, 109, 110, 131–133).

[11] Plaskon CTFE-2300, Allied Chemical.
[12] Corning Glass.

LSC is best suited to samples of intermediate molecular weight (<1000), particularly those that are oil soluble (131). Any sample type that has been successfully chromatographed by TLC can be separated by LSC. Separations are usually carried out on polar adsorbents such as silica, alumina, or other inorganic solids[13].

Both porous layer materials and porous supports are used in high performance LSC. The porous layer materials (alumina and silica) produce faster and more efficient separations than porous support materials. Little et al. (36) found pellicular silica (Corasil I and II) to give substantially less peak broadening than conventional porous silica beads (Porasil) of the same particle diameter (37–50 μ). However, the disadvantage of porous layer beads is their low sample capacity. Recently, Majors (134) showed that columns packed with 10-μ diameter silica gel[14], when packed by the balanced slurry technique, can give 10^4 theoretical plates/m. Sample sizes of 1 mg./g. of adsorbent can be injected on such columns.

Snyder (2, 3) reviewed the factors affecting column efficiency in LSC.

Stewart et al. (50) compared high pressure LSC with TLC and found comparable separations; however, LSC produced better resolution and shorter analysis times.

Lebedeva et al. (135) studied the effect of the geometrical structure and the surface chemistry of silica gels on separations in LSC. They concluded that, for analytical purposes, it is preferable to use macroporous silica gels with specific surface areas of 80–300 m.^{2}/g. and an average pore radius greater than 80 Å.

The need to control carefully the water content of the adsorbent and the mobile phase has been emphasized (131, 132). The addition of water to the adsorbent increases linear sample capacity and efficiency. For porous silica or alumina, 0.02–0.04 g. of water should be added for each 100 m.^{2} of surface area (131).

The use of adsorbents impregnated with complexing agents can greatly improve separations of isomers and compounds within a homologous series. On the other hand, classical LSC is most selective for compound classes. Snyder (132) reviewed the use of complexing agents in LSC. For example, adsorbents impregnated with silver nitrate are selective for olefins. Recently, Vivilecchia et al. (136) used Zipax impregnated with silver oxide as a specific adsorbent for the rapid separation of polynuclear aza aromatic hydrocarbons. Selectivity is based upon complexation between the aromatic nitrogen atom and the silver ions.

Ion-Exchange Chromatography—The speed of ion-exchange chromatography was significantly improved by the development of pellicular ion exchangers by Horvath and coworkers (38). Pellicular ion exchangers consist of a nonporous, central core and a thin, porous outer shell of the ion-exchange resin. Similar materials were introduced by Kirkland (39, 40). Both anion and cation exchangers are available commercially[15]. These

materials have small ion-exchange capacities in the order of 8–10 μeq./g. Dupont's cation exchanger exhibits an exchange capacity of 3.5 μeq./g. (40). Although sample capacity is low, these exchangers exhibit high efficiency.

Spherical, porous ion-exchange resins of small particle sizes in the range of 10 μ can be used as a high efficiency, high capacity packing (20, 43). A tabulation of the various ion exchangers for HPLC was compiled (134).

The performance of ion exchange is a complex function of many variables, including temperature, pH, buffer type, and ionic strength. Increasing temperature generally reduces retention volumes and improves column efficiency (38a, 40). Column performance improves with increasing temperature due to increased solute diffusivity in both phases. At present, no good guide exists for the optimization of the mobile phase. However, resolution is primarily affected by the pH and ionic strength. Temperature is of less importance in this respect.

To achieve optimum resolution of adenine and its metabolites, Gere et al. (137) reported that it is best to keep the temperature as high as possible while varying the pH and ionic strength. Since columns packed with pellicular ion-exchange resins are characterized by a large ratio of mobile phase to stationary phase, eluent strengths of the mobile phase can be much lower than in conventional ion-exchange columns.

Gel Chromatography—Steric-exclusion chromatography is most commonly used for the analysis of biological macromolecules and synthetic polymers. The large number of publications describing the principles and applications of gel chromatography are far beyond the limits of this paper. General reviews (138–140) and a monograph (141) were published on this subject.

Programming—The broad variance in band migration rates of a multicomponent sample under fixed experimental conditions (normal elution) often leads to poor separation of the first eluted components and/or excessive separation times with concomitantly poor detectability. This "general elution problem" can be handled by several techniques: flow or pressure programming, solvent programming, temperature programming, or coupled column operation (stationary phase programming).

Flow programming (142, 143) involves the continuous increase of mobile phase velocity to increase band migration rates of the later eluting peaks. In addition, slow mobile phase velocity at the beginning of elution can lead to increased resolution for the weakly retained components due to an increase in column efficiency at lower flow rates. However, resolution at the back end of the chromatogram is sacrificed (higher mobile phase velocity) as compared to normal elution (143).

The other techniques involve changes in capacity factors (k' programming). Of these techniques, solvent programming (gradient elution) is the most common. Snyder (144) reviewed gradient elution, and Henry (53) described several commercial gradient devices. Other gradient devices were recently described (92, 145, 146). Snyder and Saunders (147) theoretically derived optimum conditions for solvent programming

[13] See Reference 134 for a complete compilation of LSC adsorbents.
[14] Available from E. M. Laboratories and Varian.
[15] Northgate Laboratories and Dupont Instruments.

with LSC. Under optimum conditions, gradient elution can provide marked improvement in resolution at all points in the chromatogram, particularly at the front end (143).

Temperature programming in LCC has not found much use. Two types of temperature programming have been used: "normal" programming where the temperature is raised during the separation (135) and "inverse" programming where the temperature is lowered (142, 148). Inverse programming is restricted to LSC with mobile phases containing a "moderator," a very strongly adsorbing compound at low concentration (e.g., 0.15% isopropanol in n-heptane). As the temperature is lowered, the moderator covers more of the adsorbent active sites, thereby reducing the k' values of the solutes. The major problem with this technique is the long times required to reequilibrate the column to the starting condition (142).

Coupled-column operation involves the use of two or more columns, each with a different stationary phase or with the same stationary phase but different column lengths (143). The latter does not involve change in k' values. Snyder (143) described an LSC system where adsorbent strength is varied by water deactivation. Recently, Scott et al. (20) coupled anion- and cation-exchange columns for the analysis of urine constituents. With parallel gradient elution of the columns after injection, 16 new peaks (total >100) were resolved and analysis time was reduced from 24 to 14 hr.

Snyder (143) compared normal elution, coupled columns, solvent programming, flow programming, and temperature programming under time normalization. He found that resolution decreased in the order solvent programming > coupled columns > temperature programming > flow programming > normal elution. Solvent programming is especially useful for unknown samples but of less value for routine or repetitive samples since reequilibration time is long. Temperature programming has the same disadvantage. Coupled columns and flow programming are more amenable to repetitive analysis.

MEASUREMENT OF COLUMN PERFORMANCE

Since capacity factors (k') in HPLC are generally small, the use of the number of theoretical plates (N) as a measure of the separating power of a column is unsatisfactory since resolution is also a function of k'. A more suitable parameter is the number of effective plates, N_{eff} (6, 149), where:

$$N_{eff} = \left(\frac{k'}{1 + k'}\right)^2 N = 16\left(\frac{t_r - t_0}{w}\right)^2 \qquad \text{(Eq. 15)}$$

Effective plates is related to resolution by:

$$R_S = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)N_{eff}^{1/2} \qquad \text{(Eq. 16)}$$

The number of effective plates per unit time, N_{eff}/t, can tell directly how long a separation must take; N_{eff}/t is related to k' by the following expression:

$$N_{eff}/t = \frac{V}{H}\frac{k'^2}{(1 + k')^3} \qquad \text{(Eq. 17)}$$

If H is assumed to be independent of k' (which is often true in HPLC), the optimum k' for fast separations is about 2 (6).

Some workers prefer the reduced parameters (5) for comparing column efficiency of different chromatographic systems as a function of mobile phase velocity. Reduced plate height (h), equal to H/d_p, and the reduced velocity (v), equal to Vd_p/D_m, are used instead of H and V. The reduced plate height normalizes H for particle diameter, and the reduced velocity normalizes for diffusion over a particle diameter distance. Halasz (125) and Snyder (3) pointed out the dangers of using reduced parameters for comparing different HPLC columns.

A semiquantitative method for comparing H versus V curves of different columns was described (3, 143); H versus V curves were found to obey the empirical relationship:

$$H = DV^n \qquad \text{(Eq. 18)}$$

where D is a constant for a given column and set of experimental conditions (e.g., type of column packing, particle size, and packing technique), and n is 0.4 for LSC columns. This equation affords a simple basis for comparing column efficiencies obtained by different workers, using any set of experimental conditions.

The value of D for silica is found to decrease with decreasing d_p (4). Kirkland (45) found D to decrease with decreasing d_p down to at least 37 μ with Zipax coated with 1% stationary liquid.

The importance of extracolumn band broadening from the injector and detector should not be neglected in the determination of column performance since the variance of the peak measured by the detector is a summation of all the variances in the chromatographic system. Deininger and Halasz (150) considered the effect of the equipment on measured column efficiency and resolution.

PHARMACEUTICAL APPLICATIONS

To date, a relatively limited number of practical applications of HPLC has been reported in the literature. This paucity of publications can be expected to change very rapidly with the ever increasing availability of commercial instrumentation, with improved column technology, and with the realization that many separations, which had heretofore been both tedious and complicated, may now be effected quite readily by means of this powerful analytical technique. Many applications that have been developed are illustrative of this method's immense value for pharmacological studies of drug substances and their metabolites and for clinical investigations of metabolic disorders.

Schmit et al. (130) described the use of high speed reverse phase liquid chromatography for the separation of natural products and also biochemicals. The biologically active constituents of hashish were isolated and chromatographed by gradient elution (water to methanol) on an octadecylsilane permaphase column. Both water-soluble and oil-soluble vitamins have been separated on an octadecylsilane permaphase column, using a water to methanol gradient.

General—In a brief survey of the applicability of high speed liquid chromatography (HSLC) to pharmaceutical analysis, Williams *et al.* (151) described procedures for the rapid determination of different classes of drugs.

Using a Zipax controlled-surface porosity support coated with a 1% polyamide stationary phase, the separation of such antibiotics as penicillin and its derivatives, myosins, and rifampin was achieved. A suitable mobile phase was hexane–ethanol (75:25), which was also capable of separating impurities from these antibiotics.

Steroids and a variety of their derivatives have been successfully chromatographed from a number of matrixes using reverse phase chromatography on a hydrocarbon column, with a water–methanol solution as the eluant. Efficient separations of some steroids have also been obtained by normal phase chromatography on an ethylene glycol column, using hexane–chloroform as the eluting solvent.

A single-tablet analysis of thiabutazine was presented as an example of a rapid separation for a diuretic. The analysis was performed on an oxypropionitrile column, using *n*-hexane for the elution. The total time for elution was 4 min., with the minimum detectable limit being 10 ng. of the diuretic.

The antihistamine chlorpheniramine maleate was chromatographed in 2 min. on a trimethylene glycol column using 10% chloroform–*n*-hexane. Analyses were carried out on single-component and multicomponent tablets with no interferences from excipients or the other actives.

Barbiturates and Alkaloids—The applicability of high speed chromatography for the analysis of barbiturates, diphenylhydantoin, and their hydroxylated metabolites was reported (152). Separations were performed without the necessity of any derivatization, using a pellicular anion-exchange column and eluting with either a nongradient or gradient mode of operation. Column inlet pressures ranged from 700 to 1600 psig., and flow rates ranged from 26 to 36 ml./hr. The eluting peaks were detected at the microgram level with the aid of a fixed wavelength (254 nm.) UV detector.

Optimal conditions for the separation of diphenylhydantoin and its phenolic metabolite were established to be analogous to those conditions necessary for the separation of phenobarbital and the hydroxy derivative of phenobarbital. A nongradient mode of elution, performed at 80° using a 20.0 m*M* phosphate solution, buffered at pH 3.5, produced the most rapid and satisfactory resolution for these compounds. These conditions were unsatisfactory for other barbiturates, however. Successful separation of these parent compounds and their metabolites required the use of a linear sodium chloride gradient at 80°. The sensitivity of the method could be increased by working with neutral solutions due to the increased absorptivity of the barbiturates in more alkaline media.

Alkaloids in plant extracts have been separated on oxypropionitrile and cyanoethyl silicone columns, using either *n*-heptane or *n*-heptane with a polar modifier as the eluant (121). The choice of eluant depended upon how strongly the alkaloids were retained on the columns.

Urine and Blood Analysis—Burtis *et al.* (153) reported a high pressure anion-exchange chromatographic technique for the separation and identification of two metabolites of phenacetin in urine. These metabolites were ultimately shown to be 4-hydroxyacetanilide and 3-methoxy-4-hydroxyacetanilide. The system employed was a UV analyzer[16]; this system consisted of a 200-cm. small bore column filled with Dowex 1-X8 resin and a spectrophotometer, which was used to monitor the column effluents at various wavelengths. The mobile phase was an ammonium acetate–acetic acid buffer.

The UV analyzer (154–158) was able to separate 140 UV-absorbing compounds in urine and should prove to be invaluable in many pharmacological studies. In the present example, the two metabolites of phenacetin were separated on the anion-exchange column and subsequently identified by their UV, mass spectrometric, and IR spectra.

Recently, Scott *et al.* (20) described modifications of their original system to increase resolution and also to decrease analysis time. By using coupled anion- and cation-exchange chromatography and gradient elution (in which the concentration of the acetate ion in an ammonium acetate–acetic acid solution buffered at pH 4.4 was gradually increased from 0.015 to 6 *M*), 16 additional peaks were separated from urine samples. The anion-exchange resin used was Aminex A-27, with quaternary ammonium active sites, while the cation-exchange resin employed was Aminex A-6, with sulfonic acid active sites. The eluant was pumped at a flow rate of about 10.5 ml./hr. through the 150-cm. long column at pressures up to 3500 p.s.i., and the eluting peaks were detected at two wavelengths in the UV (254 and 280 nm.). With this system, the time of analysis was decreased from 24 to 14 hr.

Although the UV analyzer is capable of resolving a large number of components in complex biological mixtures, the qualitative identification of the eluting peaks, for the most part, has been extremely difficult. By using the chromatographic technique proposed by Scott *et al.* (154), Burtis (21) reported that reproducible chromatograms of urinary samples could be accomplished on a liquid chromatograph[17] by high pressure anion-exchange chromatography. Detection of nanogram amounts of substances was possible using an efficient microvolume UV photometer. It was proposed that the resultant chromatograms or "urinary profiles" could be used to great advantage in the diagnosis of certain abnormal metabolic or pathological conditions.

At present, only 50 of the molecular constituents that have been reported have been identified by a combination of analytical techniques (159).

Scott *et al.* (157) also reported the development of a companion carbohydrate analyzer, which has successfully separated and detected 48 different carbohydrates in urine and blood serum. This system also utilizes a high pressure anion-exchange column. Qualitative and

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quantitative detection of peaks was accomplished by use of a colorimeter, the color being produced through reaction of the eluting carbohydrates with a phenol-sulfuric acid reagent. Sodium borate-boric acid buffer concentration gradient solutions served as the eluant. The most effective anion-exchange resin was reported to be a Type 1 resin of small particle size, with a final cross-linkage of 7-10%.

The use of the carbohydrate analyzer in the clinical laboratory in the investigation of various carbohydrate metabolism disorders was proposed (160). The detection of excreted sugars and some of their derivatives was possible without interferences from other eluting substances such as vitamins, aliphatic and aromatic hydroxy and keto acids, and polysaccharides.

The automation of a system was described for rapid separations of saccharides by high pressure ion-exchange chromatography, using a neutral pH boric acid-2,3-butanediol buffer solution to minimize alkaline rearrangement reactions (161).

An improved automated system with more rapid analysis times was developed by Floridi (162). This system utilizes from one to three different borate buffers adjusted to pH 8.40 or 8.80. The analysis of the eluting mono-, di-, and trisaccharides was accomplished by the orcinol colorimetric method[18]. The one-buffer elution system allowed rapid and effective separations of monosaccharides, the two-buffer elution system produced high resolution of di- and trisaccharides, while the three-buffer solution effected a separation of 17 different saccharides in 10 hr. In all cases, the analyses were carried out on Dowex ion-exchange resin at a column temperature of 55°. Flow rates were varied according to the particular system employed.

A high speed ion-exchange chromatographic method was devised for the direct analysis of glucuronide and sulfate conjugates of drug substances in biological samples (163, 164). The analyses were carried out at 80° in narrow bore stainless steel columns, 250 cm. in length, containing a pellicular anion-exchange resin. Column inlet pressures were maintained between 800 and 1000 psig., giving rise to a flow rate of 0.5 ml./min. A fixed-wavelength (254 nm.) UV detector was employed to monitor the column effluent.

Both nongradient and gradient elution methods were required. The use of pH 3.0 formic acid solutions of high ionic strength (1.0 M potassium chloride) and a linear gradient solution of potassium chloride permitted the successful separation and quantitative determination of sulfate and glucuronide conjugates in urine in less than 40 min.

Benzodiazepines—To avoid the undesirable task of acid hydrolysis or derivatization, which is necessary for the GC determination of benzodiazepines, Scott and Bommer (126) utilized HSLC for the separation of these pharmacologically important compounds. The stationary phase was Durapak "OPN"—the oxypropionitrile group chemically bonded to porous glass beads—which was dry packed in a narrow bore stainless steel column 1 m. in length. Elution of the column was effected at a solvent flow rate of 1 ml./min. using

either: (a) a constant composition hexane-isopropanol mixture or (b) an initial elution with 100% hexane followed by a stepwise change to a 70:30 mixture of hexane-isopropanol. The latter solvent system served to sharpen the elution pattern of the slowest eluting component without sacrificing the resolution of the more rapidly eluting substances. A fixed-wavelength (254 nm.) UV microvolume photometric cell detected the eluting substances at the microgram level.

This procedure not only effectively separated mixtures of benzodiazepines but also was useful for the separation and quantitative determination of the parent compounds and their metabolites in a pharmacological study of urine samples.

Steroids—High resolution liquid chromatography (HRLC) at moderate pressures, using small bore borosilicate glass columns, 50 cm. in length, was shown to be effective for the rapid analysis of steroid hormones (120). A representative number of polar and moderately polar steroids was separated by reverse phase chromatography, and separation conditions for the various classes of steroid hormones were described.

Three types of solid supports were investigated: (a) Anakrom AB diatomaceous earth, (b) Plaskon CTFE-2330 terpolymer, and (c) Zipax controlled-surface porosity silica beads. In all cases, Amberlite LA-1, a water-insoluble amine, was used as the stationary phase; each support was coated at a level giving optimal results for that material. Water or a water-methanol mixture was used as the eluant.

The separation patterns observed for the four classes of steroids studied (adrenal corticosteroids, androgens, progestins, and estrogens) indicated that the main factor governing the chromatography of these compounds was actually a competition between liquid-liquid partitioning with the stationary liquid phase and liquid-solid adsorption with the solid support. It was further demonstrated that the relative effect of each separation mechanism was dependent upon the percentage of liquid loading used. Therefore, optimal resolutions for various pairs of compounds could be obtained by merely varying the level of liquid loading.

Henry et al. (165) surveyed numerous applications of HSLC to the analysis of steroids and steroid conjugates. The column effluent was continually monitored with both a fixed-wavelength (254 nm.) UV photometer and a refractive index detector. The advantages and disadvantages of each detector were noted. For UV-absorbing steroids, the UV detector had a lower detection limit of about 10 ng., while the refractive index detector had a 1-ng. lower detection limit whether or not the steroid absorbed in the UV. The formation of a strongly absorbing 2,4-dinitrophenylhydrazine derivative of the nonabsorbing carbonyl-containing steroids was proposed as a useful and attractive technique for increasing sensitivities for these compounds.

Examples of separations of all major types of steroids (including estrogens, androgens, progestogens, and adrenocortical hormones) by conventional liquid-liquid partition chromatography, reverse phase chromatography, and ion-exchange chromatography were presented, and useful column packing materials, stationary phases and mobile phases were described.

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[18] Performed with a Technicon Auto-Analyzer.

Mollica *et al.* (166) described a suitable stability-indicating assay for the steroid flumethasone pivalate. A quantitative determination could be effected by using a controlled-surface porosity support coated with β,β'-oxydipropionitrile as the stationary phase and eluting with a solvent system of 5% ethyl acetate and 0.2% acetonitrile in hexane. Other chromatographic studies were reported for the steroids dexamethasone, prednisolone, and hydrocortisone.

A rapid and precise method for the quantitative determination of estrogens in urine was developed and applied to the determination of estriol in pregnancy urine (118). Detection of the estrogens was accomplished with a UV spectrophotometer operated at a fixed wavelength of 281 nm., corresponding to the absorption maximum of this class of compounds. Separation was effected with the use of two coexistent phases of a water–ethanol–2,2,4-trimethylpentane system (molar ratios 0.229:0.680:0.09 and 0.019:0.177:0.804) as the stationary and mobile phases, respectively. The water-rich phase was coated on diatomaceous earth (28–32 μ in diameter), which was then packed into a 2.7-mm. i.d. borosilicate glass column (50 cm. in length) through which the water-poor phase eluant was pumped. All analyses were performed at a constant temperature of 22°.

Analgesics—Henry and Schmit (167) described the analyses of the active ingredients in analgesics. The analyses were performed on Zipax controlled-surface porosity supports coated with an anion-exchange resin, using small bore stainless steel columns 1 m. in length. The mobile phase was distilled water buffered at pH 9.2. To lower the retention times of the more highly retained components, the ionic strength of the mobile phase was increased by the addition of ammonium nitrate. Detection of the eluting peaks at the nanogram level was accomplished by means of a fixed-wavelength (254 nm.) UV photometer. By using benzoic acid as the internal standard, both single- and multicomponent tablets were determined quantitatively without any interference from excipients. A routine time of analysis was 10 min., using a flow rate of 1.5 ml./min. at a column inlet pressure of 1200 psig. Since all separations were performed at ambient temperature, no problems of thermal degradation occurred, as had been encountered in GC. In addition, by merely increasing the ionic strength of the mobile phase, the determination of the decomposition product, free salicylic acid, could be performed quantitatively, with pyromellitic acid employed as the internal standard.

In a similar study, the successful quantitative determination of the active ingredients in 12 commercial analgesic tablets was reported, using an anion-exchange column 300 cm. in length (168). The eluant was a 1.0 *M* aqueous solution of tromethamine buffered at pH 9.0. The flow rate was 8.6 ml./hr. at 925–1000-p.s.i. pressure and a temperature of 60°. The eluting peaks were detected with a fixed-wavelength UV detector. This chromatographic system permitted baseline separation of acetaminophen and phenacetin, which had previously eluted at the same time (1); therefore, a qualitative identification of the components in a single tablet could be made by comparison of retention times.

In an extension of this study, Wolford *et al.* (169) reported the separation of oxypurines and the determination of caffeine in pharmaceutical separations. By using 3 *M* NH$_4$OH as the eluant at a flow rate of 3 ml./min., caffeine was eluted as a sharp peak from a 1 × 20-cm. column in less than 10 min.; qualitative results were obtained. Phenacetin, which is strongly basic, appeared between 15 and 30 min. while such compounds as aspirin, salicylamide, tetracycline, and barbiturates were effectively retained.

Amino Acids, Nucleotides, and Nucleic Acid Bases—High speed ion-exchange chromatography on small bore columns has been shown to be amenable to the analysis of amino acids using a modified sequential multisample amino acid analyzer[19] (170).

By eluting the column with citrate buffers at varying pH's, a flow rate of 0.5 ml./min., and a pressure of 600 p.s.i., satisfactory resolutions were obtained for the acid, neutral, and basic amino acids in approximately 60 min. A photometer equipped with a high efficiency flow cell detected the eluting peaks, the areas of which were found to be directly proportional to the amount of amino acid present in the sample.

Numerous authors have used HSLC to great advantage for the separation and identification of nucleotides, nucleosides, and nucleic acid bases.

The ribonucleoside mono-, di-, and triphosphates of cytidylic, uridylic, adenylic, and guanylic acids were separated on anion-active and cation-active resins coated on glass beads, producing pellicular ion exchangers (38a). Rapid separations of nanomole quantities of the substances were achieved by using a linear gradient elution with a phosphate or an ammonium formate buffer solution. The eluting peaks were detected by means of a spectrophotometer at a wavelength of 260 nm. using a microvolume flow cell. Analyses could be accomplished in all cases in less than 90 min.

The four major bases (uracil, guanine, adenine, and cytosine) and nucleosides (uridine, guanosine, adenosine, and cytidine) formed by the hydrolysis of RNA were successfully separated and detected at subnanomole levels using a pellicular cation-exchange resin in small bore stainless steel columns (38b). Acidic potassium or ammonium phosphate solutions served as the mobile phase, and detection at these levels was afforded by a micro-UV detector operating at a fixed wavelength of 254 nm. The elapsed time for an analysis was 6 min. The optimal conditions for these separations were investigated and described. It was found that the pH of the sample and of the eluant had the most pronounced effect on the resolution.

A similar separation of the nucleotides and nucleic acid bases and other less commonly encountered purines and pyrimidines was successfully performed on controlled-surface porosity supports coated with strong cation- and/or anion-exchange resins (40). A 0.01 *N* nitric acid solution was utilized for the elution. The total time of analysis for the major hydrolysates of RNA was 5 min. By employing an ammonium formate solution first at 3000 p.s.i. with a flow rate of 50 ml./hr.

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[19] Technicon.

and then at 4600 p.s.i. with a flow rate of 80 ml./hr., the elution times for these alkaline hydrolysis products were reduced to 4 and 2 min., respectively, by Burtis (171), who also suggested the obvious applicability of the method for the determination of the base composition of DNA following an enzymatic, rather than alkaline, hydrolysis reaction. The elution times and the conditions for many "unusual bases" and common ribo- and deoxynucleosides were also reported. Quantitation and detection at the nanomole to picomole level were possible. These high speed separations, with no derivative formation required, rival those observed for GC separations.

The use of citrate and acetate buffers as effective eluants for the chromatography of these compounds on a cation-exchange resin also was noted (172).

To eliminate the interferences and anomalous peaks caused by UV-absorbing substances present as contaminants in buffer solutions described by Horvath and Lipsky (38b), Shmukler (173) devised a purification system for potassium dihydrogen phosphate. Due to the relatively low solubility of potassium dihydrogen phosphate in water, the non-UV-absorbing salt, potassium chloride, was added to increase the total salt content of the eluant. With this buffer system, the chromatography of adenine nucleotides and ^{32}P-labeled compounds in a deproteinized mitochondrial reaction mixture was studied (174).

Sample preparation methods and elution conditions for the separation of mononucleotides, ribomononucleotides, deoxyribomononucleotides, nucleoside diphosphates, and dinucleotides were reported and their R values were tabulated (175). The chromatographic separation was carried out on an anion-exchange column, maintained at 70°, using a linear or exponential gradient elution technique and "purified" potassium phosphate solutions.

The qualitative and quantitative determination of nucleotide profiles of cell extracts was characterized (176), and optimal conditions for the rapid separation of the mono-, di-, and triphosphates of the naturally occurring ribosides, as well as for the sulfur analogs of some of these compounds, were noted. The method was developed with the aid of a high pressure chromatograph[20] equipped with an anion-exchange column and a double-beam UV detector operated at 254 nm. A potassium phosphate–potassium chloride gradient served as the eluant, and the eluting peaks were identified by one or more of the following methods: (a) spectrophotometric or chemical means, (b) injection of known standards, (c) comparison with chromatograms of known substances, and (d) use of known enzymatic reactions. "Enzymatic peak shifts" not only served to identify peaks but also aided in unmasking a chromatogram and allowing the detection of other smaller peaks to be made. A further use of this system combined with isotopic labeling to measure the radiospecific activities of nucleotides was proposed.

The four common 5'-deoxyribonucleotides were successfully analyzed on a Zipax strong anion-exchange column eluted with 0.008 M phosphate buffer at pH 4.40 (177).

Nucleotides, nucleosides, and nucleic acid bases were also successfully separated by ligand-exchange chromatography using a cation-exchange resin, Chelex 100, impregnated with copper (II) ion (178). The flow rate of the eluting agent (water and/or ammonium hydroxide, depending upon the substances being separated) was maintained at 0.75 ml./min., and the effluent was continually monitored with a UV spectrophotometer. The nucleotides were not retained on the column; the weakly basic nucleosides were separated by elution with water, the more basic nucleosides were resolved with 1 N ammonium hydroxide, and the nucleic acid bases required 2.5 N ammonium hydroxide to be displaced from the column.

The quantitative determination of enzymatically formed adenyl cyclase at the microgram level was reported (179), using high pressure anion-exchange chromatography following an initial zinc–barium precipitation step which removed interfering substances. The elution was effected by a nongradient mode of operation with a pH 2.20 hydrochloric acid solution as the eluant. A flow rate of 12 ml./hr. with an inlet pressure of 450–550 p.s.i. was employed, while the column temperature was controlled at 80°. Detection of the peaks was accomplished with a sensitive microvolume UV flow cell. The total time for an analysis was about 10 min.

Qualitative identification and quantitative detection of adenine and its metabolites in body fluids were described, and an empirical means of optimizing the separation conditions was discussed, presenting a systematic and logical approach to a HSLC problem (137).

Aza Aromatic Hydrocarbons—Snyder (133) developed a rapid liquid–solid chromatographic method for the analysis of hydrogenated quinoline mixtures, using alumina as the adsorbent and a 60% water-saturated 35% dichloromethane–pentane mixture as the eluting solvent. Qualitative and quantitative analyses could be performed over a long period with no changes in the efficiency of the column being observed, provided that the water level of the alumina was maintained at a constant level and the column was regenerated occasionally to remove strongly adsorbed components. Routine analyses of quinoline mixtures could be performed in less than 30 min., with the detection of the eluting substances being realized with a UV spectrophotometer equipped with a microvolume flow cell. Problems inherent in such procedures and methods of solving them were also presented.

A high speed liquid–solid column chromatographic procedure for the separation of polynuclear aza heterocyclic compounds was reported (136). The adsorbent was Zipax, a high efficiency chromatographic support, the surface of which was impregnated with silver ions. Separation was achieved by means of formation of donor–acceptor complexes between the silver ion and the heterocyclic nitrogen. The order of elution was found to be dependent upon both the basicity of the nitrogen and the steric accessibility of the lone pair of electrons.

A 1% (v/v) acetonitrile in n-hexane solution produced

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[20] Picker Nuclear.

highly efficient separations. Unlike many liquid–solid chromatographic methods, no water deactivation of the column nor careful control of the solvent water content was required for this method to give consistently good results.

A high speed liquid chromatographic method has been developed for the separation and quantitative determination of isomeric mixtures of the cyano-, carboxamido-, and carboxy-substituted pyridines. A 1-m. × 2.1-mm. i.d. column filled with a strong cation-exchange resin (1% sulfonated fluorocarbon) coated on Zipax was employed. Elution was effected with an aqueous solution of 0.1 N sodium nitrate and 0.1 N phosphoric acid at a flow rate of 1.70 ml./min. at an inlet pressure of 1500 p.s.i. Detection of the eluting substances was accomplished with a fixed-wavelength (254 nm.) UV detector.

The analysis of trisulfapyrimidines by cation-exchange liquid chromatography was accomplished using a phosphate buffer solution for the mobile phase; an application for the quantitative determination of sulfadiazine, sulfamerazine, and sulfamethazine was presented (180).

Miscellaneous—A liquid–solid adsorption chromatographic technique, using microbore glass columns packed with dry silica gel, was developed by Gordon and Peters (181) to separate 4,4'-diaminodiphenyl-sulfone from its monosubstituted and disubstituted acetylated derivatives. 4,4'-Diaminodiphenylsulfone has had a great amount of pharmacological interest since it has been shown to be a highly effective antileprotic agent as well as an antimalarial drug.

The procedure was adapted from a TLC separation, with anhydrous ethyl acetate as the eluting solvent. A satisfactory separation of all three compounds was obtained in less than 30 min. by pumping the mobile phase through a 30-cm. column at a pressure of 100 p.s.i. and a flow rate of 0.25 ml./min. Detection of the compounds at the microgram level was accomplished by measuring the UV absorption at 280 nm. A linear relationship existed between the peak areas obtained and the amount of substance, thereby providing a rapid quantitative estimation of each compound.

N,N-Dimethyl-p-aminobenzeneazobenzoyl chloride reacts with a wide range of organic compounds to form colored products with differing polarities. This reaction was used in conjunction with HPLC, using small bore glass columns, and the effects of the various parameters were discussed (182). Either high speed cation-exchange chromatography using a strongly acidic sulfonated styrene-divinylbenzene resin or high speed liquid-solid adsorption chromatography on silica columns permitted satisfactory separations. A microvolume flow-through photometric detector continuously monitored the column effluent stream. The effect of the hydrochloric acid concentration in aqueous methanolic or aqueous ethanolic solutions and also the effect of the alcohol concentration in the eluting solutions were determined.

The acidic and neutral catabolites from catecholamines were quantitatively and rapidly separated on a cation-exchange resin, PA-28 (183). A scheme for the elution of the catabolites formed in $vivo$ from dopamine,

noradrenaline, and adrenaline was outlined: the column was successively eluted with sodium citrate buffers, pH 3.28 for 15 min., followed by a sodium citrate–boric acid buffer, pH 4.53, for 100–235 min., and, finally, with 0.2 N sodium hydroxide for 60 min. The flow rate was maintained at 50 ml./hr. with a back-pressure of 140–200 p.s.i. in the 60-cm. × 9 mm. column. All analyses were performed at a constant temperature of 55°. The eluting peaks were detected with a spectrophotometer[21] at 280 nm.

In a similar manner, Lange and Hempel (184) demonstrated that a wide range of aromatic acids, aldehydes, and alcohols could be separated. The eluting substances were determined by: (a) measuring the absorbance of the eluate at 280 nm. or (b) determining the fluorescence at 315 nm. of the effluent fractions by means of a spectrofluorometer[22]. The elution times for 50 aromatic compounds were tabulated, with the order of elution generally being dependent upon the polarities of the substances.

Efficient separations of aromatic monosulfonates and disulfonates and of isomeric and homologous aromatic sulfonates were achieved (185) by use of high speed anion-exchange chromatography, using narrow bore columns packed with a cross-linked polyalkylene amine resin. Elution of the sorbed substances was accomplished with an aqueous organic solution of water-acetonitrile-methanol (a 1:1:1 mixture provided the best performance) and a linear salt gradient of lithium chloride. A monochromator was utilized for the absorbance measurements over the UV range from 220 to 400 nm., which permitted the maximizing of general or selective sensitivities for the sulfonates being determined.

Linear gradient elution chromatography has provided an efficient means of separation of aromatic hydrocarbons having widely different boiling points (186). Analyses were carried out on small bore glass columns packed with alumina, using a pentane-ether gradient at a flow rate of 45 ml./hr. The eluting peaks were detected with the aid of a fixed-wavelength photometer at 260 nm., and identification was made either by comparison of retention times or by addition of pure standards to the mixture being analyzed.

Beyer (187) developed a quantitative HPLC analytical method for sulfonylurea antidiabetic agents. A column of 1% hydrocarbon polymer on Zipax eluted with citrate or borate buffers separated glyburide, chlorpropamide, tolazamide, tolbutamide, and acetohexamide.

HPLC was used for the analysis of N,N-dimethyl-2,2-diphenylacetamide and its metabolites in soybean plants (188). The analysis was obtained on a 1-m. hydrocarbon polymer column eluted with a water-methanol (4:1) mixture.

APPENDIX: LIST OF SYMBOLS

A = eddy diffusion term in the Van Deemter equation

B = longitudinal molecular diffusion in the Van Deemter equation

[21] Beckman DB.
[22] Aminco-Bowman.

C = mass transfer term in the Van Deemter equation

D_m = diffusion coefficient of solute in the mobile phase

D_{is} = diffusion coefficient of solute in the stationary phase

d_c = internal diameter of the column

d_p = particle diameter of the packing

f = total porosity in the column

h = reduced plate height (H/d_p)

H = height equivalent to a theoretical plate

K = distribution coefficient

K^0 = specific permeability of a column

k' = capacity factor = $K(V_s/V_m)$

L = column length

N = number of theoretical plates

N_{eff} = number of effective plates

N_{eff}/t = effective plates per second

ΔP = column pressure drop

R_s = resolution

T_m = interparticle tortuosity factor

T_s = intraparticle tortuosity factor

t = time of analysis

t_r = retention time of peak measured from start

t_0 = retention time of a nonsorbed species

V = mobile phase velocity

V_m = column mobile phase volume (dead volume)

V_s = column stationary phase volume

W = peak width

ϵ_α = fraction of column volume occupied by phase α

ϵ_m = fraction of column volume occupied by the moving phase

ϵ_s = fraction of column volume occupied by the static phase

ϵ = fraction of the column volume occupied by the two phases

$1 - \epsilon$ = fraction of column volume occupied by the support material

η = mobile phase viscosity

$\lambda, \lambda 2$ = dimensionless constants

σ_l = length-based standard deviation of the Gaussian band

μ = micron (micrometer)

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RESEARCH ARTICLES

Absorption and Distribution of Naloxone in Rats after Oral and Intravenous Administration

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Abstract ☐ The effect of route of administration on the absorption and distribution of naloxone, a narcotic antagonist, was investigated in rats. Plasma levels were determined by GLC. Five minutes after intravenous administration of 1 mg./kg., the plasma concentration was 258 ng./ml. Plasma levels after low oral doses were undetectable; but after 100 mg./kg. orally, the peak level of unchanged drug was almost 5000 ng./ml. In terms of percent of administered dose, the maximum amount of naloxone in the calculated plasma volume is 1.04% of the intravenous dose *versus* 0.19% of the oral dose. Pharmacokinetic parameters were generated with a computer program; the models constructed are of a rapidly absorbed and rapidly excreted and/or metabolized drug. These results, together with results from absorption studies with ligated intestinal loops, indicate that poor absorption of naloxone is not the cause of its relatively low oral potency. *In vitro* metabolic studies with rat liver slices confirmed rapid naloxone metabolism, suggesting that the lower potency of oral naloxone compared to parenteral naloxone is due to rapid first-pass liver metabolism.

Keyphrases ☐ Naloxone hydrochloride—absorption and distribution after oral and intravenous administration, rats ☐ Absorption and distribution, naloxone hydrochloride—after oral and intravenous administration, plasma levels, liver metabolism, rats

Naloxone [(−)-N-allyl-14-hydroxynordihydromorphinone] is a potent narcotic antagonist upon parenteral administration to laboratory animals (1, 2) or man (3), but it is approximately one-fiftieth as po-