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Review Article

Column Partition Chromatography in Pharmaceutical Analysis

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PERHAPS THE MOST widely applied separation process used in the analysis of pharmaceutical products is liquid-liquid extraction, wherein a solute is distributed between two immiscible phases. Its concentration in each of the two phases at equilibrium is governed by its distribution coefficient. Partition chromatography may be regarded as an extension of conventional liquid-liquid extraction, in which one of the two liquid phases is present in the form of a film sorbed by particles of an inert support. A very large surface area is required to reach equilibrium within a reasonable time interval, since interphase transfer of solute takes place at the liquid-liquid interface. The large surface area is furnished by the finely divided particles of the support.

The support is variously referred to as the solid phase, the supporting phase, the supporting medium, or the inert phase; the sorbed liquid is known as the immobile phase, the internal phase, the nonmobile phase, or the stationary phase; and the second liquid phase is termed the mobile phase, the developing solvent, or the eluant.

Column chromatography has been defined as "uniform percolation of a fluid through a column of more or less finely divided substance, which selectively retards certain components of the fluid" (1). Cassidy (2) defines partition chromatography as "a method of separating substances by distributing them between two liquid phases, one of which is mobile and the other essentially fixed by sorption to a support." He does not, however, restrict the process to liquid-liquid distribution; he states that "the support itself may or may not be active in the separation process." Catch, *et al.* (3), said that "doubtless the process (occurring on the column) is a combination of 'chemical' chromatography and 'partition' chromatography."

Meinhard (4) attributed to the supporting material a major role in the separation process. According to him, a layer of adsorbed liquid at a solid-liquid interface may extend to a depth of 100 molecules or more if the solid is "wetted" by the liquid. The structure of the adsorbed layer is modified to various degrees by the adsorbent. Therefore the liquid layer does not behave as an independent entity during the chromatographic process; the stationary phase is comprised of the layer of liquid as modified by the adsorbent. Hence the difference between ordinary chromatography and partition chromatography depends primarily on the media. Büchi and Soliva (5), on the other hand, exclude the effect of the support. They state that partition chromatography is not concerned with the equilibrium between adsorbed and dissolved material, but only with an equilibrium of dissolved material in two solvents which are immiscible or of limited miscibility.

The concept of partition chromatography was introduced in 1941 by Martin and Synge (6) during an investigation of the amino acid composition of wool. Initially they used a 40 stage countercurrent extraction apparatus to separate

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the acetylated amino acids (7). In seeking mechanical means to simplify this process, they sorbed the aqueous phase on finely divided silica gel and packed the mixture in a glass tube. By this means, one of the liquid phases was retained in a rigid position, while the second liquid phase simply percolated through it, making contact in passing.

Martin and Synge visualized the chromatographic column as a number of separate and consecutive regions, in each of which equilibrium of the solute is attained between the mobile and immobile phases. Thus each region or "theoretical plate" is equivalent to a separate vessel of a countercurrent extraction apparatus. In practice, they found that a column 20 cm. in length gave a separation equivalent to that of over 1000 theoretical plates.

In their characterization of the column as a series of "theoretical plates," Martin and Synge presented a mathematical analysis of the process which occurs on the column. In a column having a large number of theoretical plates, they let r = the serial number of the plate, Q_r = the quantity of solute in plate r, v = the volume of solvent used in the development of the chromatogram, α = the equilibrium partition coefficient of the solute = concentration in nonmobile phase/concentration in mobile phase, A = area of column, A_s = area of nonmobile phase, A_L = area of mobile phase, h = height of equivalent theoretical plate, $V = h(A_L + \alpha A_s)$, and R = (movement of band of maximum concentration)/(movement of mobile solvent).

Considering the case where a unit mass of a single solute is put onto the first plate and then followed by pure solvent, they constructed a table showing the quantity of solute in each plate after successive infinitesimal volumes of mobile phase have passed through. From this they showed that the quantity of solute in each plate is given by

$$Q_{(r+1)} = \frac{1}{\sqrt{2\pi r}} (v/rV)^r e^{r-v/V}$$

When $v/rV = 1$, $Q_{(r+1)}$ is a maximum and equal to $1/\sqrt{2\pi r}$ so that the position of maximum concentration has moved a distance hv/V directly proportional to the volume of liquid, v, which has passed through.

Now

$$R = \frac{hv/V}{v/A} = \frac{Ah}{V} = \frac{A}{A_L + \alpha A_s}$$

Then the partition coefficient, α, is $A/RA_s - A_L/A_s$. Martin and Synge found that partition coefficients calculated by this formula agreed closely with the values obtained by conventional procedures. They plotted a curve showing the ideal separations attainable by partition chromatography and discussed the factors contributing to deviations from their theoretical values. The principal source of the deviation is adsorption of solute by the supporting material. Among the other causes are diffusion of the mobile phase, which is affected by temperature, rate of flow, and changes in partition coefficient with change in concentration. Interaction of two solutes, with the more strongly adsorbed solute "eluting" the less strongly adsorbed, can result in separations better than theoretical.

Craig (8) did not accept the correlation between liquid-liquid partition ratios and rates of migration of bands on a column to be proof that partition chromatography is a liquid-liquid extraction process. He believed that "at best this evidence is only circumstantial, and probably reflects more the uniform shift in nearly any physical property in a homologous series than the particular property responsible for such an observed effect." He found the results of partition chromatography to be of very little use in selecting systems for countercurrent distribution. He stated that "certainly, the difference between countercurrent distribution and so-called partition chromatography is much greater than that between Tswett (adsorption) chromatography and partition chromatography. In fact there seems little to be gained by assuming that liquid-liquid partition plays any significant role in the latter process." Subsequent developments in partition column techniques support the interpretation of Martin and Synge. Separations based upon the degree of ionic dissociation, as discussed below, are patently liquid-liquid partition phenomena. The contribution of adsorption is subordinate and merely a complicating factor in these separations.

METHODOLOGY

Commercial silica, designated by Martin and Synge (6) as "pure precipitated" silica, was used as the supporting phase in the introductory application of column partition chromatography. Even in the initial publication on this technique, however, it was noted that separate lots of silica vary significantly with respect to their adsorptive power and their capacity for sorption of water. Several procedures have been reported for pre-

paring and standardizing silica gel suitable for use as a support (6, 9–12). Isherwood (13) and Resnik, *et al.* (14), described purification processes designed to decrease the adsorptive properties of the material.

A number of other materials have been used instead of silicic acid. Synge (15) and Elsden and Synge (16) used raw potato starch as the supporting medium for the aqueous phase. Various forms of cellulose have been used in column partition chromatography as well as in paper chromatography. In a note, Gordon, *et al.* (17), referred to the use of cellulose powder. Hough, *et al.* (18), prepared powdered cellulose by rubbing ashless filter tablets through an 80-mesh sieve. Baker, *et al.* (19), and Brindle, *et al.* (20), used Solka-Floc, a commercial powdered cellulose. Mitchell and Haskins (21) used a pile of 40 circles of filter paper as a column. Stoll and Kreis (22) used cotton linters, which are minute fibers of almost pure cellulose. In a discussion of the nature of supporting material, Martin (1) observed that the initial concept of cellulose as a completely inert support for aqueous phase has changed in favor of one considering the hydrated cellulose to be a strong polysaccharide solution or a complex gel. He concluded that the most ideal partition effect is achieved with siliceous earth (kieselguhr, diatomaceous earth, infusorial earth).

The first use of this material as supporting phase was reported by Catch, *et al.* (3), who used Hyflo Super-Cel[1] in the separation of the penicillins. The use of Celite[1] 545, which is now probably the most widely used supporting phase, was introduced by Peterson and Johnson (23). Other grades of Celite (503 and 535) have been used (24, 25). In a number of publications the material is simply termed "kieselguhr," with no further reference to its identity, source, or quality.

Hegedüs, *et al.* (26), washed Hyflo Super-Cel with a series of organic solvents to remove extractable impurities. Carol, *et al.* (27), removed alkaline buffering impurities of Celite 545 by an acid treatment. An acid-washed Celite 545 is now commercially available.

Stoll and Kreis (22) prepared "diatom stone" (diatomitstein) for use as support by calcination of a mixture of kieselguhr and clay. Chilton and Partridge (28) used 60-mesh Pyrex glass powder as support in the separation of the pomegranate alkaloids.

Although siliceous earth has a much lower adsorptive capacity than silicic acid, losses of alkaloid due to adsorption on this support have been reported (28, 29). Modification of the immobile phase (30) or of the mobile phase (31) has been very effective in eliminating this loss by adsorption. Graf (32) used activated alumina as supporting phase, but he recognized that the process occurring on the column was not simple partition chromatography.

Celite 545 is the most uniform in particle size of the siliceous earths commercially available in this country. The particle size of Celite 545 is 20–40 μ; of Celite 535, 10–40 μ; of Celite 503, 6–40 μ; of Hyflo Super-Cel, 2–20 μ (33).

Howard and Martin (34) modified kieselguhr to permit "reverse phase" partition chromatography. They treated Hyflo Super-Cel with dimethyldichlorosilane, thereby attaching hydrophobic alkyl groups to the surface of the particles. Using this modified material, a nonpolar organic solvent which is normally used as mobile phase may be used as immobile phase, while an aqueous solution of a polar solvent becomes the mobile phase. Boldingh (35) used a column of powdered vulcanized rubber, partly swollen by treatment with benzene. The rubber functions as both supporting phase and immobile phase.

Water was used as immobile phase in the initial applications of partition chromatography (6). Catch, *et al.* (3), used a solution of alkali carbonate. Following this, various aqueous solutions were substituted for water as immobile phase. Isherwood (13) used 0.5N sulfuric acid to suppress the ionization of weak organic acids. Peterson and Johnson (23) used 27 N to 35 N sulfuric acid, and Graham (36) used concentrated hydrochloric acid. Ferric chloride solution was used (37) to achieve complexation with phenolic compounds.

Nonaqueous fluids may also be used as immobile phase in partition chromatography, in conjunction with immiscible organic solvents. Among those which have been used are nitromethane (38), propylene glycol (19), formamide (39), absolute ethanol (40), and a mixture of concentrated and fuming sulfuric acid (41).

A solution of the sample itself may be used as immobile phase in the partition column, eliminating a preliminary extraction step. Graf (32) and Schill and Ågren (42) added sufficient supporting phase material directly to an aqueous extract of alkaloid-bearing plant material to sorb the liquid. Baker, *et al.* (19), mixed Solka-Floc with an oil solution of adrenocorticosteroids. Banes (43) extended this mode of sample preparation to drugs in various dosage forms.

The apparatus used in partition chromatog-

[1] Marketed by Johns-Manville Corp.

raphy may be quite simple. It requires only a glass tube to which an outlet tube is attached. Flow of the mobile phase may be regulated by a stopcock or by a screw clamp on rubber tubing attached to the outlet tube. A support is generally placed at the lower end of the column to retain the column packing. This may be a perforated or a sintered disk, either sealed in or fitted, or it may be a simple wad of cotton or glass wool. Although catalogs of most laboratory equipment suppliers list chromatographic columns with stopcocks, disks, and ground glass connections, the simple column without such fittings is not commercially available at this time.

Several procedures have been described for mixing the immobile phase with the support and for packing the column with this mixture. The immobile phase may be added directly to the solid (6) or to a stirred suspension of the supporting phase in immobile solvent (44). In preparing columns using silica gel, Fischbach, *et al.* (11), determined the ratio of immobile phase to support by adding the fluid to the solid in small increments, mixing after each addition until the mixture became sticky and began to "ball." They then used 5 to 10% less immobile phase than this quantity to prepare the column. Haenni, *et al.* (45), found that a ratio of slightly less than 1 ml. of aqueous immobile phase per gram of Celite 545 is about the correct proportion to prepare a pack with suitable physical characteristics. However, satisfactory separations have been reported (37) with a ratio of 7 ml. of aqueous phase to 5 Gm. of Celite 545. Chilton and Partridge (28) found that 7 Gm. of powdered glass was required to support 1 ml. of aqueous phase.

A number of variaitons in the preparation of the columns have been described. Martin and Synge (6) transferred a slurry of the prepared mixture in chloroform to the column in small portions. As the chloroform ran through, the gel, which floated in the chloroform, packed down and did not refloat during addition of further amounts of slurry. Ramsey and Patterson (12) applied compressed air at a definite pressure to pack the mixture, while Bergström and Sjövall (46) applied reduced pressure at the outlet of the column. Many authors stipulate that a head of fluid be maintained above the packing. Graf (32) simply transferred the prepared mixture to the empty column and compressed it by tamping with a glass rod.

For the resolution of compounds which have relatively small differences in partition coefficients, it is essential that the column be carefully and uniformly packed. This high degree of uniformity in the column packing is not necessary for the resolution of mixtures which are separated because of relatively wide differences in their solubility characteristics, their ionic species or their ionic strengths. Nevertheless, many published analytical procedures which are based upon these properties have specified the preparation of the very meticulously packed column, although the same results can be obtained with a very simple, rapidly prepared column. (Compare, *e.g.*, References 47 and 48.)

The rate of elution or development may be controlled in several ways. The mobile phase may flow over the partition column by gravity, with the aid of air pressure above the column, or by connecting the outlet of the column to a suction flask and applying reduced pressure. The rate of flow may be controlled by the amount of pressure or vacuum applied, with a stopcock or a screw-clamp on tubing attached to the outlet of the column, by the height of the head of fluid above the column, or by the density of the column packing. Rates of flow of mobile solvent have been reported over a range of from $1^1/_4$ ml. per hour (26) to 10 ml. per minute (49). Haenni, *et al.* (45), discuss critically the factors involved in determining optimum flow rate. The same authors describe an apparatus for obtaining a highly uniform flow rate throughout the development of the chromatogram (44).

TECHNIQUES

Partition chromatography was first conceived as a form of countercurrent distribution. Most of the early applications of the technique were designed to achieve separations based on relatively small differences in distribution coefficients. It was soon found that processes other than simple countercurrent extraction can be conducted very conveniently and efficiently on the partition chromatographic column. These range in complexity from the isolation of a single substance from its dosage form to the conducting of a chemical reaction on the column in the immobile phase. Frequently the modifications of the partition process were patterned on techniques used in adsorption chromatography, but many were designed specifically for the partition column. In adsorption chromatography, a mixture of materials may be selectively desorbed by changing solvents after the elution of the first component. In partition chromatography, the same process is applied to substances having significantly different partition coefficients. This

procedure was introduced by Evans and Partridge (50), who separated hyoscine and hyoscyamine on a *kieselguhr:pH 6.2 buffer* column,[2] eluting the hyoscine with ether and the hyoscyamine with chloroform. Marvel and Rands (51) used mixed solvents as eluant, increasing the proportion of polar solvent stepwise; Donaldson, *et al.* (52), devised an apparatus for achieving this increase continuously.

The influence of buffers upon the separation of substances which form dissociable salts has been discussed by several investigators. Jensen and Svendsen (53) showed that while strychnine and brucine cannot be separated by elution from a column in which water is immobile phase, they are readily separated if buffers at pH 7 or lower are used.

The relationship between degree of dissociation and rate of elution is derived from the equation (54): $\log \alpha' = \log \alpha + \text{pH} - \text{pK}$, where α' is the partition coefficient of the dissociated compound, α is the partition coefficient of the undissociated compound, and K is the dissociation constant.

Büchi and Soliva (5) point out that the partition coefficient of a weak acid or base in a buffer solution is a simple function of pH. In an extension of this equation to encompass mixtures of bases, they showed that, at a constant pH, the degree of separation which is achieved conforms to

$$\beta = \frac{\alpha' B_1}{\alpha' B_2} = \frac{\alpha B_1}{\alpha B_2} \cdot \frac{K B_2}{K B_1}$$

where β is termed the "separability factor" and B_1 and B_2 are weak bases. Bettschart and Flück (55) treated the subject in a similar manner. Bottomley and Mortimer (56) stated that alkaloids are eluted from a buffered column in order of their $\text{pH}_{1/2}$ values (where the term $\text{pH}_{1/2}$ designates the pH at which $\log \alpha' = 0$; *i.e.*, the compound is distributed equally in the two phases).

Schill and Ågren (42) performed on the partition column the counterdistribution of a base between an organic solvent and aqueous acid or alkali, which constitutes the basis of the classical alkaloidal assays. They used 0.5 *M* H_3PO_4 as immobile phase to trap the alkaloids from ether solution while neutral or acidic components passed through the column. They then transformed the immobile phase to an alkaline pH *in situ* with ammonia-saturated chloroform, which eluted the alkaloid as the liberated free base. Solutions of diethylamine in ether (57) and of triethylamine in chloroform (58) have been used for the same purpose. Bitman and Sykes (25) bubbled gaseous carbon dioxide onto a column containing sodium hydroxide as immobile phase to convert it to sodium bicarbonate.

Carless (57) changed the pH of the buffer used as immobile phase gradually and continuously, progressing from pH 3.0 to pH 4.8 by adding 0.1% pyridine to the developing solvent. He considered this process analogous to gradient elution methods, since a pH gradient existed down the column. To achieve the inverse process, acidification of an alkaline immobile phase *in situ*, a chloroform solution of glacial acetic acid has been used (59).

Multistage columns to separate mixtures of drugs (59) have been reported. Separate stages having acid and alkali as immobile phase were used either as layers in the same column or in separate columns arranged in series (58). These layers "trap" alkaloidal or acidic drugs as their water-soluble salts from solution in the nonaqueous mobile phase. The separated drugs were recovered after reversal of the pH of the immobile phase as described above.

PROCEDURES

Steroids.—Column partition chromatography has been widely used in the separation of the steroid hormones, particularly the estrogenic steroids. Nyc, *et al.* (60), separated estriol, estradiol, and estrone by reverse-phase partition on a rubber column. The stationary phase was powdered rubber swollen in 10% methanol saturated with benzene. A mixture of the estrogens in 20% methanol was passed onto the column; estriol was eluted with this same solvent. Estradiol was removed with 40% methanol, and estrone with 60% methanol. Swyer and Braunsberg (61), using a column of *Celite 535:2.3 N sodium hydroxide*, with benzene as eluant, separated estrone and estradiol in order; estriol remained on the column. Stern and Swyer (62), using the same system, successfully eluted the estriol with chloroform-butanol (3 + 1). Haenni, *et al.* (45), described in detail the optimum conditions for quantitative separation of estrogenic diols. They used Celite 545 as support and 0.4 *N* sodium hydroxide as stationary phase. This concentration of sodium hydroxide was selected as requiring the minimum volume of developing solvent to achieve a desired degree of separation of β-estradiol from α-dihydroequilin. They weighed the importance of temperature, flow rate of eluant, ratio of immobile to mobile phase, and degree of pack-

[2] This terminology will be used to designate the supporting phase: immobile phase combination.

ing of the column as factors in the separation. The effect of temperature is twofold. The partition coefficients of the diols change at different rates with temperature, and the band volume of the individual compound varies inversely with temperature. Two columns were described. The estradiols and dihydroequilins as a group were separated from the dihydroequilenins on one and the estradiols were separated from α-dihydroequilin on the second. They defined the volume of mobile solvent required for the elution of each component, giving the correction of these volumes for variations in temperature. The same authors (44) described the analysis of commercial estrogenic preparations, including oil solutions, tablets, and suspensions. The mixture of α-dihydroequilin and α- and β-estradiol was separated from nonestrogenic impurities, traces of estrone and equilin, and the dihydroequilenins, and the individual components of the mixture were determined by a differential colorimetric procedure. A collaborative study (63) of the combined chromatographic separation and colorimetric procedure was conducted for the determination of the physiologically active estradiol-17β. (At the time of publication the configurations of the estradiols had not been established. It was later shown that the compound then referred to as α-estradiol is actually estradiol-17β.)

Bitman and Sykes (25) separated estrone, estradiol, and estriol in a two-step operation. They eluted estrone and estradiol successively with benzene from a column of sodium hydroxide on Celite, in a procedure analogous to those described above. The more strongly acidic estriol was not eluted under these conditions. After the pH of the immobile phase was lowered by bubbling carbon dioxide over the column, benzene eluted the liberated estriol.

Carol, *et al.* (64), described the preparation of the previously unreported diol, β-dihydroequilin. They reduced the ketosteroid equilin with aluminum isopropoxide to a mixture of α- and β-dihydroequilin, and separated the two isomers on a *Celite:0.4 N sodium hydroxide* column similar to that described above. The β-isomer was eluted with benzene and the α-isomer with ether. Using the same partition system, Banes, *et al.* (65), resolved the "isoequilin A" of Hirschman and Wintersteiner (66) into two major constituents, 8-dehydro-14-isoestrone and 9-dehydro-14-isoestrone. They also isolated the latter compound from equine pregnancy urine extracts.

Beall and Grant (67) showed that the water-soluble conjugated estrogens in concentrates prepared from equine pregnancy urine can be extracted with chloroform from aqueous solution as their cyclohexylamine complexes. However, troublesome emulsions frequently formed during the extraction with conventional liquid-liquid extraction techniques. Carol, *et al.* (68), employed partition chromatography for the extraction. They mixed the sample with Celite 545, added water to dissolve the conjugated estrogens in the preparation and simultaneously constitite an immobile phase, and transferred the mixture to a column. Chloroform eluted the nonconjugated estrogenic materials, together with extractable impurities from the urine concentrate and the soluble tablet excipients. This elution was followed by a chloroform solution of dicyclohexylamine acetate which eluted the chloroform-soluble complex. Graham (69) later found that chloroform eluted significant proportions of conjugated estrogens from tablets containing surface-active agents in their formulations, and substituted ether for chloroform as pre-wash. A mixture of ether and methylene chloride was used to dissolve the dicyclohexylamine acetate and elute the conjugated estrogen complex. In an extension of this procedure, Graham (36) passed the elute containing the conjugated estrogen-dicyclohexylamine complex directly onto a second column, in which concentrated hydrochloric acid is the immobile phase. In a single operation the complex is dissociated, the conjugated estrogen cleaved, and the liberated estrogen eluted.

Snair and Schwinghamer (70) employed partition on *Celite 545 : polyethylene glycol 600* for the assay of estradiol propionate in oil solution. Without the chromatographic purification, sterols present in the oil base interfered with the colorimetric determination of the liberated estradiol. They reported analyses of about 115% for samples in sesame oil and of about 90% for solutions in corn, cottonseed, or peanut oil. After the purification, recoveries in the range of 99.6% to 102.4% were obtained for all samples.

Wolff (71) used a *Celite 545:nitromethane* column, with *n*-heptane as mobile phase, in the quantitative determination of progesterone in oil. Chromatography yielded pure progesterone uncontaminated by oil constituents.

Butt, *et al.* (72), separated progesterone, desoxycorticosterone, and testosterone on a Hyflo Super-Cel column, using 70% methanol as stationary phase and *n*-hexane as mobile phase.

Carol (73) used Celite 545, with 80% ethanol as stationary phase and iso-octane as mobile phase, for the quantitative separation of progesterone and testosterone in commercial aqueous suspensions.

Katzenellenbogen, *et al.* (40), separated androsterone and 5β-androsterone on a silica gel column, using absolute ethanol as immobile phase and petroleum ether–methylene chloride (1:1) as eluant. Bergström and Sjövall (74) employed reverse-phase partition to separate the cholic acids. They used siliconized kieselguhr, prepared as described by Howard and Martin (34), as supporting phase, chloroform-heptane (9 + 1) as immobile phase, and 58% methanol as mobile phase. Cholic acid, deoxycholic acid, and lithocholic acid were eluted in order, with recoveries of 70 to 90%. Hauton (75) applied gradient elution to the same separation. Siliconized kieselguhr was used as support and chloroform–*sec*-octanol (1 + 1) as immobile phase. The mobile phase entered the column from an apparatus consisting of two reservoirs, one containing methanol and the other water, delivering a mixture with a continuously increasing concentration of water. Sharp separations of cholic, deoxycholic, and lithocholic acids were obtained, with recoveries of 97 ± 2%.

Baker, *et al.* (19), isolated steroids from a hog adrenal extract, using Solka-Floc as support, propylene glycol as immobile phase, and toluene as mobile phase. They mixed the extract with Solka-Floc and placed it above the propylene glycol segment of the column. The oily components were eluted initially, followed by cortisone and hydrocortisone. Haines (76) used a *silica gel*:*ethylene glycol* column, with cyclohexane–methylene chloride as mobile phase, to separate (in order) 11-desoxycorticosterone, corticosterone, cortisone, and hydrocortisone. Heftmann and Johnson (77) separated 11-desoxycorticosterone, 11-dehydrocorticosterone, cortisone, hydrocortisone, and Δ-4-pregnene-17α, 21-diol-3,20-dione, on a *silicic acid*:*water* column. They used as developing phase petroleum ether mixed with increasing proportions of methylene chloride, added as described by Donaldson, *et al.* (52). Morris and Williams (78) separated corticosteroids from lipids by reverse-phase chromatography, using petroleum ether as immobile phase on siliconized Celite with 50% methanol as mobile phase. The initial eluate contained a mixture of 11-dehydrocorticosterone, corticosterone and cortisone, and the next fraction contained a mixture of cortisone and hydrocortisone. These were further resolved on a *Celite*:*ethylene glycol* column, with petroleum ether–toluene (2 + 8) as mobile phase.

Banes (79) described a procedure for the complete chemical assay of adrenal cortex extracts in their commercial dosage forms. Two columns were employed for the complete separation of the steroids. In the first column, Celite 545 was the support, formamide-water (1 + 1) the immobile phase, and benzene the mobile phase. The first fraction of eluate contained a mixture of 11-dehydrocorticosterone and corticosterone, the second fraction cortisone, and the final fraction hydrocortisone. The mixture of 11-dehydrocorticosterone and corticosterone was completely resolved upon the second column in which formamide-water (4 + 1) was immobile phase on Celite and benzene-isooctane (3 + 2) the mobile phase. In evaluating the procedure he obtained recoveries of 95 to 102% from a mixture of 1 to 2 mg. of each of the four compounds.

Jones and Stitch (80) separated dehydroepiandrosterone from epiandrosterone on a *silicic acid*:*nitromethane* column, using 3% chloroform in petroleum ether as mobile phase.

Stoll and Kreis (22) applied partition chromatography to the separation of the squill glycosides. They used cotton linters as supporting phase, water as immobile phase, and ethyl acetate as mobile phase. Hegedüs, *et al.* (26), chromatographed the strophanthus glycosides on *Hyflo Super-Cel*:*water*, with mobile solvents of increasing polarity, progressing from petroleum ether-benzene to chloroform. They isolated several new glycosides.

Banes and Carol (81) described a procedure for the quantitative determination of digitoxin, gitoxin, and digitoxigenin in digitoxin tablets. They used a mixture of formamide and water (2 + 1) as immobile phase on Celite 545. An extract of the sample was added to the column and the chromatogram was developed with chloroform-benzene (1 + 9). The forerun contained digitoxigenin, which was followed by digitoxin. To recover gitoxin, they extruded the column contents into a separator and extracted the gitoxin with chloroform. Recoveries of 95.6 to 102.3% of standard preparations were obtained. In a modification of this procedure (43), a solution or suspension of the sample itself in formamide-water was used as immobile phase. Iso-octane was used to elute digitoxigenin, benzene-chloroform (3 + 1) the digitoxin, and chloroform the gitoxin. The gitoxin content of digoxin preparations was determined (82) by converting it to dianhydrogitoxigenin and

isolating it by elution with iso-octane from a *Celite:50% ethanol* column.

Antibiotics.—Among the earliest applications of partition chromatography was the separation of the penicillins. Catch, *et al.* (3), used a solution of an alkali carbonate with either silicic acid or Hyflo Super Cel as support. Ether or amyl acetate was the mobile phase. Fischbach, *et al.* (83), separated penicillin K quantitatively from mixtures with the other penicillins, on a column of *silicic acid:20% phosphate buffer at pH 6.4*, using ether as mobile solvent. The same authors (11) separated penicillins K, dihydro F, F, and G using a similar system. Recoveries of known mixtures were 99% for penicillin K, 82% for penicillin dihydro F, 99% for penicillin F, and 98% for penicillin G. Low recoveries of penicillin dihydro F (80–85% in all cases) was attributed to destruction during the treatment. Higuchi and Peterson (84) identified the individual penicillins in mixtures by hydrolyzing them to obtain their characteristic acids and separating the acids on Celite columns. Either 30 *N* sulfuric acid or a mixture of sulfuric and phosphoric acids (8 + 11) was used as immobile phase and benzene as mobile phase.

Higuchi, *et al.* (85), separated intact chloramphenicol from its degradation products, eluting the latter from a *silica gel:water* column with chloroform and the intact antibiotic with chloroform–ethyl acetate (2–10%).

Analgesics.—Higuchi and Patel (86) applied partition chromatography to the analysis of mixtures of acetylsalicylic acid, acetophenetidin, and caffeine. After removing the acetylsalicylic acid in a separator, they separated the other two components on a column of 20 Gm. of silicic acid and 20 ml. of water. Chloroform–isopropyl ether (1 + 3) was used to elute the acetophenetidin, and chloroform to elute the caffeine. Smith (87) used Celite 545 as support and chloroform-ether (1 + 9) as eluant for the same separation. Banes (43) used a column segment of 5 ml. of 2 *N* hydrochloric acid on 5 Gm. of Celite 545 for the separation. Above this was placed a segment prepared by mixing 6 Gm. of Celite with 6 ml. of a solution of the caffeine and acetophenetidin in formamide-water (2 + 1). Ether was used for elution of the acetophenetidin and chloroform for the caffeine. Levine (59) used a two-stage column to separate the three components without prior extraction of acetylsalicylic acid. The first stage, 2 ml. of 4 *N* sulfuric acid on 2 Gm. of Celite, trapped caffeine from an ether solution, and the second stage, 2 ml. of 1 *N* sodium bicarbonate on 2 Gm. of Celite, trapped acetylsalicylic acid as its salt. Ether eluted the neutral acetophenetidin and chloroform the caffeine. To recover the acetylsalicylic acid, the bicarbonate stage was acidified *in situ* with a solution of acetic acid in chloroform, and the liberated acetylsalicylic acid was eluted with chloroform. The caffeine and acetylsalicylic acid eluates were received directly in volumetric flasks and their concentrations measured spectrophotometrically, without further treatment. Indemans and Mulder (88) substituted 4 *N* sodium hydroxide for the 1 *N* sodium bicarbonate to increase the capacity of the column, in order to chromatograph samples large enough to permit gravimetric measurement. Stroes (89) reported that the 50 ml. of chloroform specified by Levine is insufficient to elute the caffeine quantitatively, and that 100 ml. is necessary. In a collaborative study of the procedure conducted by Smith (90), however, quantitative recoveries with the specified volumes of eluants were obtained.

Heuermann and Levine (58) separated combinations of acetylsalicylic acid, acetophenetidin, and caffeine with codeine, antihistamines, and/or barbiturates, using multiple columns. For the combinations with codeine or the antihistamines, the sodium bicarbonate and sulfuric acid stages were placed in separate columns arranged in such a manner that effluent from one ran directly onto the second. After elution of the acetylsalicylic acid, acetophenetidin, and caffeine exactly as in the procedure for this combination alone, the codeine or antihistamine was eluted after conversion from its salt to the chloroform-soluble free base with a solution of triethylamine in chloroform. For combinations with barbiturates, a third column, with tripotassium phosphate solution as immobile phase, was placed in series between the sodium bicarbonate and the sulfuric acid columns. The very weakly acidic barbiturate passed through the sodium bicarbonate column, but was held on the more strongly alkaline tripotassium phosphate column, while the other components passed through. The barbiturate was eluted after acidification of the column with acetic acid in chloroform.

To separate acetophenetidin from isopropylantipyrine, Indemans and Mulder (88) suspended the mixture in 4 *N* sulfuric acid, precipitated the isopropylantipyrine with potassium ferrocyanide, added Celite, and transferred the mixture to a column. Ether eluted the acetophenetidin alone.

The separation of caffeine from acetophenetidin by the procedure of Higuchi and Patel (86) is based upon partition of caffeine between water, the immobile phase, and the mobile phase. It is for this reason that the relatively long column, containing 20 ml. of immobile phase, is required. In the procedure of Levine (59), on the other hand, the separation is based upon salt formation. Caffeine, while feebly basic, will form salts with strong acids; therefore only 2 ml. of immobile phase is sufficient to trap the caffeine as its salt from ether solutions. If a less concentrated acid (*e.g.*, 2 *N* sulfuric acid) is used, the caffeine is not quantitatively retained; if a more concentrated acid (*e.g.*, 6 *N* sulfuric acid) is used, the caffeine is not eluted quantitatively by the specified volume of chloroform.

The procedure for the analysis of acetylsalicylic acid combinations was adapted to apply to the analysis of acetylsalicylic acid in its various dosage forms, including flavored, colored, buffered, and enteric-coated tablets (37). The same publication describes the determination of the free salicylic acid content of acetylsalicylic acid. Ferric chloride solution is used as immobile phase to retain salicylic acid as its complex while acetylsalicylic acid, which has no phenolic hydroxyl group, passes through. In this separation, only dilute solutions of ferric chloride will remove salicylic acid from chloroform, and acetylsalicylic acid removes ferric chloride from the column as the chloroform-soluble salt. A balance must therefore be maintained to provide a sufficiently low concentration of ferric chloride to trap the salicylic acid, and at the same time provide a sufficiently large quantity to insure an excess over that removed by the acetylsalicylic acid. For a 200-mg. sample, an effective balance was achieved by using 5 Gm. of Celite and 7 ml. of 2% ferric chloride solution. The salicylic acid remains on the column as its purple ferric ion complex. The salicylic acid is eluted after the complex is dissociated with a solution of acetic acid in chloroform.

Barbiturates.—In a procedure for the determination of phenobarbital in mixtures with salicylates (91), a chloroform solution of the mixture is passed over a *Celite 545:2 M dipotassium phosphate* column. The salicylates remain on the column while phenobarbital passes through. Byers (92) applied this procedure to phenobarbital–acetylsalicylic acid combinations. Lach, *et al.* (93), used a column of pH 9.2 buffer on Celite 535 to separate diphenylhydantoin from phenobarbital. Chloroform eluted the diphenylhydantoin and butanol-chloroform (1 + 9) the phenobarbital. Recoveries of 99 to 100% were obtained. Sabatino (94) separated phenobarbital from other barbiturates (secobarbital, pentobarbital, amobarbital, or butabarbital) on a Celite column, using formamide-water (2 + 1) as immobile phase. Iso-octane–chloroform (1 + 1) eluted the barbiturate other than phenobarbital, and chloroform eluted the latter. Stroes (89) separated phenobarbital from aprobarbital on a Celite column with pH 9 buffer as immobile phase. Chloroform eluted the aprobarbital and ether the phenobarbital. The separation of barbiturates from combinations with acetylsalicylic acid, acetophenetidin, and caffeine (58) has been described above.

Alkaloids.—Graf (32) applied a combination of adsorption and partition chromatography to the assay of alkaloid-bearing crude drugs. Although adsorption has a much greater role in this procedure than in the others discussed in this review, sufficient features of partition chromatography are retained to warrant its inclusion. In this procedure, an alkaline aqueous suspension of the powdered crude drug, constituting the immobile phase, is stirred with sufficient activated alumina to prepare a dry mixture. The alkaloid is eluted with an immiscible solvent. Strong or weakly polar solvents may be used, depending upon the activity of the alumina. This procedure was used in the assay of lobelia, ephedra, nux vomica, hydrastis, cinchona, yohimbe, ipecacuanha, solanacea, and other drugs. In applying this procedure to opium, Graf found alumina to be too strongly adsorbent and substituted kieselguhr as supporting phase. Ethylene chloride was used to elute the alkaloids other than morphine, along with the coloring matter. The morphine was eluted with chloroform-isopropanol (3 + 1). When alumina was used as support, elution of the by-alkaloids and coloring matter with ethylene chloride was incomplete; these contaminated the morphine in the ensuing elution.

Belladonna Alkaloids.—Schill and Ågren (42) extracted belladonna alkaloids in a similar manner. They made the crude extract alkaline with sodium carbonate, sorbed the solution on kieselguhr, and packed the mixture in a column. They passed the chloroform eluate from this column, containing the alkaloids together with neutral extractives, over a second kieselguhr column in which 0.5 *M* phosphoric acid was immobile phase. This column retained the alkaloids, while the neutral components passed through. They eluted the alkaloids with

ammonia-saturated chloroform, which made the immobile phase alkaline *in situ*.

Atropine, in solutions containing phenol as preservative, has been separated from its hydrolytic product, tropic acid (95). Two columns were used in series. The upper contained the sample solution, made alkaline with sodium bicarbonate, as immobile phase on Celite 545; the lower column contained *Celite:0.2 N sulfuric acid*. Chloroform extracted the atropine and phenol from the upper column; the alkaloid was retained on the lower column, while phenol passed through. Tropic acid was eluted from the upper column with a solution of acetic acid in ether. To recover the atropine from the lower column, a segment of 1 Gm. of Celite and 1 ml. of concentrated ammonium hydroxide was added to the column, and chloroform was passed over it. The added segment was effective in saturating the chloroform with ammonia.

The combined effect of partition coefficient and dissociation constant upon the separation of alkaloids has been discussed above. Evans and Partridge (50) applied this effect to the separation of hyoscine and hyoscyamine. They used kieselguhr as support and, as immobile phase, a series of phosphate buffers ranging in pH from 6.2 to 7.4. The 6.2 buffer was most efficient in separating the two alkaloids. At this pH, the hyoscine is completely eluted with ether long before any hyoscyamine appears in the eluate. The latter is readily eluted with chloroform. The same authors (96) used pH 7.3 buffer to separate the alkaloids of Australian *Datura Ferox* and Indian henbane. Using ether as mobile phase, they eluted (in order) hyoscine, hyoscyamine, and metaloidine; chloroform then eluted tropine from extracts of the latter species. Evans and co-workers (97–100) isolated a number of alkaloids from the individual plant parts of several *Datura* species on *kieselguhr:phosphate buffer* columns at pH 5.4 to 6.6, using petroleum ether, ether, and chloroform as developing solvent. Santoro and Matz (101) separated the belladonna alkaloids from dosage forms containing chlorpheniramine and phenylpropanolamine, using a Hyflo Super-Cel column with pH 7.0 buffer as immobile phase. Chlorpheniramine was eluted with carbon tetrachloride–cyclohexane (1 + 1) and the belladonna alkaloids were eluted with chloroform. Phenylpropanolamine remained on the column.

Veratraea Alkaloids.—In a series of publications, Parks and associates described the application of partition chromatography to the separation of the sabadilla alkaloids. Hennig, Higuchi, and Parks (102) using *silicic acid:pH 8.5 phosphate buffer*, with chloroform containing 1–3% ethanol as mobile phase, separated the water-soluble sabadilla alkaloid group into five fractions, including the new alkaloid sabatine. Svoboda and Parks (103) prepared pure veratridine and cevadine by chromatography of a crude alkaloid mixture on *silicic acid:pH 4.0 or 4.25 buffer*. Stuart and Parks (104) chromatographed fractions obtained by countercurrent distribution on a *silicic acid:pH 5.0 buffer* column, with chloroform as eluant. They isolated the pure alkaloid vanilloylcevine. Mitchner and Parks (29) used two systems, silicic acid as support and pH 5.90 buffer with benzene as mobile phase, or pH 3.95 buffer with chloroform as mobile phase, in an investigation of the separation of veratridine and cevadine. They compared the chromatographic separations with those obtained by countercurrent distribution, and found that effects other than partition occurred on the column. They attributed this to the tailing effect due to adsorption.

Brown and Kupchan (24) incorporated bromthymol blue in the immobile phase of a Celite column to monitor the separation of the alkaloids, the location of the bands being shown by the indicator. The polar layer of an equilibrated mixture of ethylene chloride–Skellysolve B, methanol, and water (3 + 12 + 2 + 0.24) was used as immobile phase and the nonpolar layer as developing phase. They separated commercial "Veratrine" into two completely homogeneous bands, cevadine and veratridine. Using the same system, they obtained three bands from *Zygadenus paniculatus:* the first contained neogermitrine, the second a mixture of neogermidine and germidine, and the third pure zygacine.

Levine and Fischbach (30) separated "protoveratrine" into its components, protoveratrines A and B, on a Celite column, using as immobile phase pH 3.5 buffer–ethylene glycol (2 + 1). Benzene eluted the protoveratrine A and ethylene chloride the protoveratrine B. When aqueous buffer was used without the glycol, losses occurred due to adsorption. The same authors (105) modified the procedure for application to commercial protoveratrine preparations which contained associated alkaloids. They substituted pH 3.0 buffer–ethylene glycol (2 + 1) as immobile phase. Carbon tetrachloride–ethylene chloride (3 + 2) eluted protoveratrine A; ethylene chloride eluted alkaloids intermediate between protoveratrines A and B in hydrophilic strength; chloroform–ethylene chloride (2 + 1) eluted protoveratrine B; and finally chloroform

eluted the more strongly hydrophilic alkaloids. Paper chromatography was used to monitor the separations.

Hegi and Flück (106) used *kieselguhr:pH 4.2 buffer* columns for the separation of pairs of veratrum alkaloids. They used chloroform-ether (40 + 160), chloroform-ether (90 + 110), and chloroform to separate the alkaloid pairs protoveratrine A–protoveratrine B, germitetrine, and jervine-veratrobasine. They used this same chromatographic procedure in isolating new alkaloids from the above-ground portions of *Veratrum album* (107). Kupchan and Gruenfeld (108) resolved the components of a commercial *Veratrum viride* alkaloid preparation on a *Celite:pH 4.25 buffer* column, eluting successively with benzene, benzene-chloroform, chloroform, and chloroform-methanol. They rechromatographed certain of the fractions on alumina, and isolated and identified a total of 13 alkaloids.

Ergot Alkaloids.—Brindle, *et al.* (109), separated the water-insoluble ergot alkaloids ergocristine and ergocristinine. They used Solka-Floc as support, pH 3.4 buffer as immobile phase, and ether as mobile phase. Carless (57) used a column of pH 3.0 buffer on ashless cellulose powder in the chromatography of the ergot alkaloids. With ether as eluant, the alkaloids moved down the column in the order ergocristinine, ergocristine, ergosine, and ergotamine. The water-soluble alkaloids, ergonovinine and ergonovine, remained at the top of the column. Ergosine and ergotamine moved very slowly, requiring inordinate volumes of eluate. Increasing the pH of the immobile phase accelerated their movement, but resulted in incomplete separation of the faster-moving ergocristinine and ergocristine. Pyridine (0.1%) was added to the developing solvent, thereby establishing a pH gradient on the column; the pH was raised from 3.0 to 4.8 after passage of 100 ml. of solvent. With this procedure, the slower moving alkaloids were eluted more rapidly and the zones compacted. To elute the water-soluble alkaloids, the column was made alkaline with a solution of diethylamine in ether. Recovery studies using the individual pure alkaloids yielded 90–95% except for ergotamine, for which recoveries were about 80%.

Graf and Neuhoff (110) described the quantitative determination of the water-insoluble and water-soluble groups of ergot alkaloids. They triturated the sample of ground ergot with ammonium hydroxide and methanol, added sufficient silica gel to prepare a dry mixture, and transferred it to a column above a segment of *silica gel:pH 7 buffer*. They eluted the alkaloids with chloroform-trichloroethylene (1 + 1), monitoring the separation by fluorescence under ultraviolet illumination. In control experiments with mixtures of ergotamine and ergonovine they obtained recoveries of 96 to 100%. Their assays of crude ergot gave somewhat higher values than those obtained by non-chromatographic procedures, particularly for the water-soluble group.

Alexander and Banes (111) separated the water-soluble and water-insoluble groups on a *Celite:0.1 M citric acid* column. They passed a chloroform solution of ergot extract over the column and eluted the water-insoluble group with chloroform. They then extruded the column, added sodium bicarbonate to render the immobile phase alkaline, and reconstituted the column. The water-soluble group of alkaloids was eluted with chloroform. Recoveries of 95 to 98% of ergonovine were obtained. To determine the extent to which the water-insoluble alkaloids appear in the water-soluble alkaloid fraction, they chromatographed the pure water-insoluble alkaloids ergotamine and ergocristine by the same procedure. The quantity of the alkaloids found in what would be the water-soluble fraction was equivalent to no more than 3% of the ergonovine present in a normal sample. These authors, like Graf and Neuhoff (110), found that the chromatographic procedure gave higher values, particularly for the water-soluble group, than the conventional shake-out procedure of the National Formulary XI (112).

Van de Langerijt (113) separated ergotamine and ergotaminine on a *Celite:formamide* column, using benzene-petroleum ether (9 + 1) as developing solvent. Ergotaminine was eluted first, followed by ergotamine. The cutting of fractions was monitored by fluorescence of bands under ultraviolet light. Lysergic acid and lumiergotamine present in the sample remained on the top of the column.

Alexander (114) described a procedure for the determination of ergotamine and ergotaminine in its dosage forms, including combinations with acetophenetidin, caffeine, barbiturates, etc. The sample was first chromatographed on a column of *Celite:50% citric acid solution* with ether. The ergot alkaloids remained on the column while the neutral or acidic components passed through. The column was extruded, the aqueous phase was made alkaline, and the combined ergotamine and ergotaminine

were extracted with chloroform. The extract was passed over a second Celite column, in which 25% (w/v) citric acid was the immobile phase. Ergotaminine was eluted with chloroform, with recoveries of 98 to 100%. The column was extruded and ergotamine extracted as before; recoveries of 97 to 98% were obtained. Pin Liang and Tonh-Hui Chou (115) separated ergonovine from the water-insoluble ergot alkaloids on a *Celite:pH 3.4 buffer* column. Chloroform eluted the latter group; ergonovine remained on the top of the column, and was eluted with ammonia-saturated chloroform. Recoveries of over 95% were obtained. Voight and Kaehler (116) adapted a paper chromatographic separation of ergot alkaloids to a column, using cellulose powder as support, 0.2% tartaric acid as immobile phase, and ether-acetone-water (2:4:2) as developing solvent. The peptide alkaloids were eluted first, followed by the clavine fraction. Ammonium hydroxide (1 drop per 100 ml.) was then added to the eluant. Ergonovine, ergonovinine, and lysergic and isolysergic amides were eluted serially.

Rauwolfia Alkaloids.—Pure reserpine was isolated from commercial reserpine preparations by chromatography on a Celite column, using a mixture of 0.05 *M* citric acid and alcohol (5 + 2) as immobile phase and chloroform–alcohol–iso-octane (100 + 40 + 200) equilibrated with water as eluant (117). After a forerun was discarded, spectrophotometrically pure reserpine was eluted.

The weakly basic alkaloids, mainly reserpine and rescinnamine, were separated from *Rauwolfia serpentina* preparations, with a slightly modified immobile phase (118). Chloroform was used as eluant, and 0.5 *N* sulfuric acid and 10% dipotassium phosphate were used as immobile phase on separate segments of the column, to separate reserpine from its acidic degradation products and the more polar alkaloids (119).

Hayden, *et al.* (120), separated the three weakly basic rauwolfia alkaloids, reserpine, deserpidine, and rescinnamine. They equilibrated *n*-heptane, chloroform, morpholine, and formamide (715 + 110 + 1 + 25), and used the lower layer as immobile phase and the upper layer as eluant. With a column of 25 Gm. of Celite and 20 ml. of immobile phase, and quantities of about 1.5 mg. of alkaloid, they found that after a 125 ml. forerun, deserpidine was eluted in the next 75 ml.; after the next 25 ml., reserpine was eluted in 110 ml.; and after the following 60 ml., rescinnamine was eluted in the next 200 ml. Recoveries of deserpidine, reserpine, and rescinnamine averaged 99%, 97%, and 95%, respectively.

Opium Alkaloids.—Lindblad and Ågren (121) used a *Hyflo Super-Cel:pH 2.0 phosphate buffer* column to separate several opium alkaloids. Ether eluted narcotine, chloroform eluted papaverine, and ammonia-saturated chloroform eluted codeine. Büchi and Huber (122), using a *kieselguhr:pH 4.6 buffer* column and benzene-ether (1 + 3) as developing solvent, eluted first a mixture of narcotine and papaverine and then thebaine. Codeine was eluted with ammonia-saturated chloroform. Lach, *et al.* (123), separated morphine from pseudomorphine on a *Celite 535:pH 6.5 buffer* column. Chloroform containing 15% butanol eluted pure morphine, leaving the pseudomorphine on the column. Yeh and Lach (124), using a similar column, eluted degradation products of pseudomorphine with chloroform containing 5% butanol; pure morphine was then recovered as before.

Other Alkaloids.—Welsh (125) determined epinephrine and norepinephrine in mixtures by chromatographing their triacetyl derivatives on a *Celite:water* column. Benzene eluted the triacetyl epinephrine and chloroform the triacetyl norepinephrine. Buffer (pH 4) was used as immobile phase and chloroform as eluant to separate triacetylepinephrine from its degradation products (126).

Jensen and Svendsen (53) separated strychnine and brucine on a kieselguhr column with pH 7 buffer as immobile phase. The strychnine was eluted with ether and the brucine with chloroform.

Chilton and Partridge (28) separated the pomegranate alkaloids of the commercial mixture pelletierine tannate, using powdered glass as support and pH 6.8 buffer as immobile phase. They found that with kieselguhr excessive loss was incurred due to adsorption. The aconite alkaloids were separated on a *kieselguhr:pH 7 buffer* column, with first ether and then chloroform as mobile phase (127).

To avoid gelation which occurs in the separator on extraction of pilocarpine from solutions containing methylcellulose, the sample solution has been used to constitute the immobile phase on Celite (128). Chloroform eluted the alkaloid quantitatively.

Salts of Bases.—The classical analytical procedures for alkaloids are based upon the general rule that the nondissociated form (free base) will enter the organic phase and the dissociated form (the salt) will enter the aqueous

acidic phase during distribution between the immiscible solvents. With selection of the proper acid, however, it is possible to extract the specific salt of most alkaloids and organic bases with chloroform directly from the acidic aqueous solution. Although the distribution coefficient may be relatively unfavorable, quantitative recoveries are obtained because of the efficient extraction which is obtained by column partition chromatography. Application of this means of distribution to the analysis of a large variety of alkaloids and related bases in their dosage forms is thus feasible. Schill and Ågren (129) applied this property to the separation of hyoscyamine hydrochloride from scopolamine hydrochloride. They used a *kieselguhr:1 N hydrochloric acid* column for the separation; chloroform eluted the hyoscyamine hydrochloride quantitatively while the scopolamine salt remained on the column. The authors attributed the separation simply to the solubility of hyoscyamine hydrochloride and the insolubility of scopolamine hydrochloride in chloroform. It has been shown (31) that solubility of the salt in chloroform is only one factor governing the extractability of the salt from aqueous solution. Salts having comparable solubility in chloroform may vary widely in their partition characteristics. In the distribution of diphenhydramine hydrochloride and of cyclizine hydrochloride (both of which are readily soluble in chloroform) between chloroform and aqueous hydrochloric acid solution, the proportion of diphenhydramine in the chloroform phase at equilibrium increases with increasing concentration of acid, while that of cyclizine decreases.

The procedure of Haddock and Evers (130) for the separation of strychnine from quinine was adapted to partition chromatography (43). The separation of the two alkaloids was more complete, the analyses more accurate, and the manipulations much more simple than in the original procedure, in which the extractions were performed in separators. A *Celite 545:2 N hydrochloric acid* column was used for the separation; chloroform eluted strychnine while quinine was retained on the column. Dihydrocodeinone was separated from pheniramine on a similar column; the latter is retained on the column while dihydrocodeinone passes through (131).

It has been shown that acids other than hydrochloric may be used to effect the differential extraction of salts of alkaloids and organic bases, and that by proper selection of acid, significant degrees of specificity can be achieved. Nitric acid (1 *N*) was used as immobile phase (132) for the separation of codeine from pyrilamine, methapyrilene, and pheniramine; chloroform eluted codeine from this column while the antihistamines were retained. The chloroform eluate was passed onto a *Celite:0.5 N sulfuric acid* column, which retained the codeine while the neutral and acidic extractives of the sample (*e.g.*, a cough syrup) passed through. This partition system was not applicable to the separation of codeine from phenindamine, since this antihistamine is not retained on the nitric acid column. To separate this combination, a *Celite:0.5 N sulfamic acid* column was used, with chloroform as mobile phase. The phenindamine passed through while codeine was retained.

Miller (133) reported that while the chromatographic procedure of Banes (43) for the determination of strychnine in elixir of iron, quinine, and strychnine was applicable to freshly prepared elixir, high values were obtained for aged samples. He showed (134) that this was caused by the partial degradation of quinine to one or more products not separated from strychnine in the chromatographic step. Pure strychnine was isolated from the aged elixir on a three-stage column (135). In the first stage, a solution of *p*-toluenesulfonic acid in the sample constituted the immobile phase. The second stage was *Celite:2 N hydrochloric acid*, and the third stage *Celite:1 N tartaric acid*. Ether, passed over the first stage alone, eluted one or more of the quinine degradation products which accompanied strychnine in the previous procedure. Chloroform then eluted the quinine, strychnine, and the remaining quinine degradation products. The chloroform eluate was passed through the second stage, which retained the quinine, and over the third stage, which retained the strychnine, while the remaining quinine degradation products passed through. Strychnine was recovered from this stage with chloroform-triethylamine as described above. If 1 *N* sulfuric acid was used rather than tartaric acid in the third stage, the final separation was not obtained; the quinine degradation product was retained together with the strychnine.

Levine and Ottes (136) showed that the analysis of alkaloidal preparations by extraction of the salt with chloroform from aqueous solution of *p*-toluenesulfonic acid is applicable to a large variety of alkaloids. A solution of the dosage form in 10% *p*-toluenesulfonic acid is used as immobile phase on Celite. Ether elutes neutral and acidic constituents of the sample, and chloro-

form elutes the alkaloid-*p*-toluenesulfonic acid complex. This eluate is passed over a *Celite:1 N sodium hydroxide* column, which retains the acid moiety while the alkaloid passes through. Centigram quantities of alkaloid can be isolated by this procedure. Quantitative recoveries were obtained with strychnine, quinine, atropine, codeine, emetine, physostigmine, dihydrocodeinone, quinidine, and homatropine. Low recoveries resulted with cocaine, apomorphine, and arecoline, while morphine, pilocarpine, procaine, and phenylephrine were retained completely. This procedure was adapted (31) to the quantitative analysis of those antihistamine preparations for which the procedure *Salts of Organic Nitrogenous Bases* is official in U.S.P. XVI (137). *p*-Toluenesulfonic acid could not be used for all of the compounds, since the ether used in the purification step eluted varying quantities of several bases. It has been found that this phenomenon is the cause of the low recoveries of cocaine, arecoline, and apomorphine mentioned above. For these compounds the *p*-toluenesulfonic acid was replaced by 2 *N* hydrochloric acid or, in the case of those which were eluted by ether from this acid, 5% sulfamic acid solution. In the final procedure as adopted, 10% *p*-toluenesulfonic acid is used for chlorpheniramine, doxylamine, pyrilamine, and tripelennamine; 2 *N* hydrochloric acid for chlorcyclizine, cyclizine, and diphenhydramine; and 5% sulfamic acid for phenindamine and promethazine. Tailing occurred during the elution of several of the compounds with chloroform, but was completely eliminated by adding 1% glacial acetic acid to the chloroform eluant; quantitative recoveries of all of the compounds were obtained.

Miscellaneous Applications.—Theivagt and Campbell (138) separated vitamin D from vitamin A on a Celite 545 column, using polyethylene glycol 600 as immobile phase and isooctane as eluant. The *R* values of the two vitamins, calculated by the formula developed by Martin and Synge (6), predicted that vitamin D should come off the column in $^1/_3$ the volume required to elute vitamin A. The experimental values were very close to the theoretical. The procedure was applied to pharmaceutical products, including multivitamin tablets and liquids and fish liver oils. Quantitation of the isolated vitamin D agreed closely with results obtained by the U.S.P. bioassay. A similar chromatographic system, substituting *n*-hexane for iso-octane as eluant, was used (139) to separate vitamin A from β-carotene, vitamin E, and oxidized vitamin A. The progress of the vitamin A band in the column was monitored by occasional examination in low intensity ultraviolet light. Recoveries of 98 to 101% were obtained for vitamin A added to multivitamin preparations; 98 to 103% from mixtures with oxidized vitamin A; 95% for mixtures with vitamin E; and 92 to 102% for fortified margarine.

Higuchi, *et al.* (140), separated the parabens (*p*-hydroxybenzoic esters) on a silicic acid column, using methanol–water–sulfuric acid (7.5 ml. + 3 ml. + 1 drop) as immobile phase and carbon tetrachloride–Skellysolve B (1 + 1) as eluant. The parabens were eluted in the order butyl, propyl, ethyl, methyl.

In a procedure for the determination of piperazine by acetylation and extraction of the diamide, Perlmutter (141) reported the usual separator extraction to be quite laborious. Because of the unfavorable distribution coefficient, a minimum of ten extractions is necessary for quantitative recovery. To improve the procedure, he mixed an alkaline solution of piperazine with Celite 545 and stirred the mixture with acetic anhydride to form the diamide. The material was transferred to a column (142) and the diamide was eluted with 200 ml. of chloroform.

In the determination of glyceryl trinitrate in its dosage forms, Levine and Hohmann (143) separated the ester from its degradation products chromatographically. They eluted the ester with iso-octane from a column in which a solution of the sample in 50% acetic acid was the immobile phase and Celite 545 the support.

De Ropp (144) chromatographed the phenolic fraction of *Cannabis sativa* "red oil" on a *Celite:dimethylformamide* column with cyclohexane. He separated tetrahydrocannabinol, cannabinol, and cannabidiol in that order.

Schwartz, *et al.* (145), used two chromatographic systems for the determination of naphazoline and its hydrolytic products, 1-naphthylacetic acid and its ethylenediamine monoamide. Chloroform eluted 1-naphthylacetic acid from a *silicic acid:water* column while the amide and intact naphazoline remained on the column. The latter two compounds were separated on a *Celite:pH 8.5 buffer* column. The intact material was eluted with *n*-heptane–chloroform (65 + 35) and the amide with chloroform. The naphthylacetic acid was retained on this column. The chromatographic behavior of the other hydrolytic product, ethylenediamine, was not determined.

Taraszka and Marcus (146) separated adiphenine from its hydrolytic product, β-diethylaminoethanol, on a *silicic acid:pH 4.8 buffer* column, using chloroform as eluant. The adiphenine was eluted and determined by nonaqueous titration. Recoveries of 99.1 to 100.7% were obtained. It was not established whether the second hydrolytic product, diphenylacetic acid, remained on the column, but this compound did not interfere in the titration.

Several procedures have been described in which operations other than simple extraction take place in the partition system. The hydrolysis of the conjugated estrogens on a chromatographic column, in which the hydrolytic reagent, concentrated hydrochloric acid, is the immobile phase, has been described above (22). Levine and Fischbach (30) adapted to the partition column the familiar acid-dye procedure for the determination of alkaloids. They used a solution of chlorophenol red in a mixture of ethylene glycol and pH 3.5 buffer as immobile phase. Portions of chloroform containing various quantities of protoveratrine were passed over the column successively. The eluate contained quantities of dye proportional to the alkaloid content of each aliquot of chloroform. Clark (147) determined phenylephrine in combinations with other bases in formulations such as cough syrups. The sample, made alkaline with dipotassium phosphate, constituted the immobile phase. Chloroform eluted the accompanying bases, such as codeine and antihistamines, together with neutral extractives. A chloroform solution of acetic anhydride was then passed onto the column, acetylating the phenylephrine *in situ*. The product, diacetylphenylephrine, was then eluted quantitatively with chloroform.

APPLICATIONS

Partition chromatography constitutes an integral step in the analysis of four products which are included in the official compendia of the United States: digitoxin, digitoxin injection, and digitoxin tablets (148), based upon the procedure of Banes, *et al.* (81); ouabain and ouabain injection (149), based upon the procedure of Banes and Houk (150); ergotamine tartrate injection (151), based upon the procedure of Alexander (114); acetylsalicylic acid, acetophenetidin, and caffeine capsules and tablets (152), based upon the procedure of Levine (59).

Analytical procedures employing partition chromatography have been adopted as official methods of analysis of the Association of Official Agricultural Chemists, following validation by collaborative studies. These include: ergotamine in tablets, applicable in presence of caffeine, acetophenetidin, phenobarbital, and belladonna alkaloids (153), based upon the procedure of Alexander (154); elixir of iron, quinine, and strychnine (155, 156), based upon the procedures of Banes (43), Miller (134), and Levine (135); phenobarbital in mixtures with salicylates (157), based upon the procedure of Banes (91); phenobarbital in mixtures with acetylsalicylic acid (47), based upon the procedure of Byers (92); acetophenetidin, acetylsalicylic acid, and caffeine (48), based upon the procedure of Levine (59) and collaborative study by Smith (90); β-estradiol (158), based upon the procedure of Haenni, *et al.* (45); norepinephrine in preparations of epinephrine (159), based upon the procedure of Welsh (160); phenylpropanolamine (161), based upon the procedure of Smith (162); digitoxin (163), based upon the procedure of Banes (164); determination of codeine in presence of antihistamines (165), based upon the procedure of Levine (132); determination of conjugated estrogens (166), based upon the procedure of Carol, *et al.* (68), and collaborative study of Banes (167); determination of piperazine (168), based upon the procedure of Perlmutter (142); determination of dihydrocodeinone in presence of antihistamines (169), based upon the procedure of Levine (131).

SUMMARY

The importance of liquid-liquid extraction in pharmaceutical analysis makes column partition chromatography particularly useful in this field. This review of the literature shows how several of the unsatisfactory features of early applications of the technique have been overcome or circumvented. These include availability of material suitable for use as supporting phase, means for limiting or preventing loss due to adsorption, further means for monitoring separations, and simplification of experimental technique. The recent introduction of such variations as multistage columns and the separation of combinations of organic bases by differential solvent extraction from solution in a specific acid facilitates the analysis of complex mixtures which are resistant to analysis by conventional procedures. Communications from colleagues indicate that these developments have given new impetus to the use of column partition chromatography in pharmaceutical analysis.

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Research Articles

Powdered Particle Interactions: Suspension Flocculation and Caking II

By BERNARD ECANOW and ROBERT G. WILSON

The flocculation of a bismuth subnitrate suspension has been explained as principally due to forces other than van der Waals acting between the suspended particles. Two basic types of bonds are described. The conclusions are supported by microscopic data and by data on relative suspension heights obtained from a series of controlled flocculation experiments. These findings disagree with a proposed concept, which views the flocculation of a bismuth subnitrate suspension as the end result of the action of van der Waals forces, following the neutralization of the zeta potential of the suspended particles.

IN THE INITIAL paper of this series (1), a 2% sulfamerazine suspension was chosen for a study of the phenomena of flocculation and of caking. Particles of sulfamerazine were suspended in dilute dioctyl sodium sulfosuccinate solution, and either aluminum or ferric ions were introduced as the flocculating agent. It was shown that flocculation was influenced both by wetting agent concentration and by the chemical reaction that took place between the above trivalent cations and the anions of the wetting agent. Where the chemical reaction was a dominant factor in flocculation, the mechanics were visualized as the adsorption of wetting agent anions on the surface of the sulfamerazine particles, followed by a chemical coprecipitation between the flocculating agent cations and the adsorbed anions. An analogous reaction is obtained when common soaps react with the heavy metal ions in "hard" water.

The sulfamerazine suspension system was previously discussed in the literature (2, 3).

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