(19) K. K. Kaistha and J. H. Jaffe, paper presented at the 33rd Meeting of the Committee on Problems of Drug Dependence, National Academy of Sciences, National Research Council, Toronto, Ontario, Canada, Feb. 16–17, 1971, **1**, 576–600(1971).
(20) K. K. Kaistha and J. H. Jaffe, *J. Chromatogr.*, **60**, 83(1971).
(21) U. M. Senanayake and R. O. B. Wijesekara, *ibid.*, **32**, 75 (1968).
(22) K. K. Kaistha and J. H. Jaffe, to be published.
(23) K. K. Kaistha and J. H. Jaffe, *J. Pharm. Sci.*, **61**, 305(1972).

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Versatile System for Partition Chromatography of Corticosteroids and Prediction of Their Elution Curves

D. J. WEBER▲, T. R. ENNALS, and H. MITCHNER

Abstract □ The versatile solvent system of *n*-hexane–chloroform–dioxane–water in the ratio 90:10:40:5 was used for the partition chromatographic isolation of corticosteroids. The relative amounts of the components may be adjusted if necessary to give a convenient partition coefficient on a diatomaceous earth column. Data are presented for 17 steroids, showing the close agreement between partition coefficients predicted from chromatographic elution curve data and those calculated from solvent extraction data. A procedure for the experimental determination of column parameters is given, and the necessary equations are outlined. The prediction of peak elution volumes and solute bandwidths as a function of partition coefficients from the derived equations is demonstrated, and the quantitative effect of varying the stationary phase volume and column length is calculated. The effect of steroid side-chain structure and fluoride substitution on the partition coefficient was studied. The effect of the substituents can be estimated from their expected polarity.

Keyphrases □ Corticosteroids—separation by partition chromatography, versatile solvent system, prediction of elution curves □ Partition chromatography, 17 corticosteroids—versatile solvent system, prediction of elution curves from partition coefficients, substituent effect □ Chromatography, partition—versatile solvent system for corticosteroids, prediction of elution curves

A common problem in the partition chromatographic separation of steroids is the choice of an efficient procedure from among the variety of solvent systems and column designs quoted in the literature (1, 2). It would be convenient if a versatile solvent system, coupled with a column of predictable characteristics, was available. Other chromatographic systems for the analysis of corticosteroids are available such as TLC (3, 4), GLC (5, 6), and paper chromatography (7). The problems of high temperature operation, long analysis times, and uncertain recoveries of these methods make the column partition chromatographic method a useful alternative. An efficient three-component partition system was described for a selection of four corticosteroids (8). Attempts to evaluate the usefulness of the system for a wider variety of steroids resulted in inconveniently large elution volumes for many steroids. Much more convenient elution times and a more versatile system were obtained after addition of chloroform to produce a four-component system.

The mathematical basis of partition chromatography was first demonstrated by Martin and Synge (9). Glueckauf (10) later extended the theory to nonequilibrium conditions such as exist in columns at normal flow rates. A truly versatile partition system must have properties that can be predicted by theory if its full usefulness is to be realized. Therefore, an outline of the pertinent equations and the demonstration of their applicability to the present system is presented.

EQUATIONS

Column Parameters—A dry-packed column was found to be more convenient and rapid to prepare in this laboratory than a slurry-packed column. This presents a problem in the determination of the effective volumes of stationary and mobile phases on the column, since not all of the open space between particles is taken up by the mobile phase on a dry-packed column. If a column of standardized inside diameter, weight of diatomaceous earth, and length is prepared using a slurry- and a dry-packing technique, then the total volumes occupied by the two columns are given by:

$$V_{ST} = V_{SS} + V_{SM} + V_{Si} \qquad \text{(Eq. 1)}$$

and:

$$V_{DT} = V_{DS} + V_{DM} + V_{Di} + V_{DO} \qquad \text{(Eq. 2)}$$

for the slurry- and dry-packed columns, respectively, where V_{DT}, V_{DS}, V_{DM}, V_{Di}, and V_{DO} are the total, stationary, mobile, inert, and void volumes for the dry-packed column and V_{ST}, V_{SS}, V_{SM}, and V_{Si} are the total, stationary, mobile, and inert volumes for the slurry-packed column. The sum of V_{SS} and V_{SM} is determined by measuring the volume needed to slurry pack a column with mobile phase only. The total volume of the slurry-packed column, V_{ST}, is equal to V_{DT} and is calculated from the length and cross-sectional area of the packed portion of the column. The inert volume of the

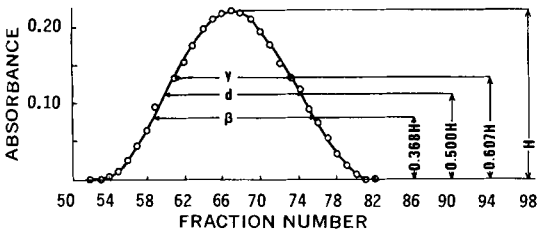

Figure 1—*Typical elution curve of prednisone in a hexane-chloroform-dioxane-water system (90:10:40:5), demonstrating relation of peak height to functions of standard deviation. Eluant fractions are 5.00 ml. Column length, internal diameter, grams of diatomaceous earth, and milliliters of stationary phase are 22.0 cm., 19 mm., 18 g., and 12.0 ml., respectively.*

slurry-packed column, V_{Si}, is given by:

$$V_{Si} = V_{ST} - (V_{SS} + V_{SM}) \qquad \text{(Eq. 3)}$$

which is also equal to V_{Di} for columns of equal amounts of diatomaceous earth. The value of V_{DM} is determined directly on a dry-packed column containing the desired ratio of milliliters of stationary phase to grams of diatomaceous earth by measuring the volume taken up by the column when it is eluted with the mobile phase. The value of V_{SM} is determined directly on a slurry-packed column containing the same ratio of milliliters of stationary phase to grams of diatomaceous earth used to determine V_{DM}. The stationary phase volume of a dry-packed column, V_{DS}, is given by:

$$V_{DS} = V_{SS} = V_{ST} - (V_{SM} + V_{Si}) \qquad \text{(Eq. 4)}$$

The value of the void space in a dry-packed column, V_{DO}, is given by:

$$V_{DO} = V_{SM} - V_{DM} \qquad \text{(Eq. 5)}$$

Dividing V_{DS} and V_{DM} by the column length, L, gives A_{DS} and A_{DM}, the stationary and mobile phase cross-sectional areas of the column. A tabulation of the values of A_{DS} and A_{DM} for dry-packed columns for various column loadings of stationary phase is given in Table I.

Volume of Theoretical Plate from Column Parameters—The fraction of solute extracted from a single theoretical plate is given by:

$$f = \frac{C_M V_M}{C_M V_M + C_S V_S} \qquad \text{(Eq. 6)}$$

where C_M and C_S are the concentrations of solute in the mobile and stationary phases, respectively, and V_M and V_S are the effective volumes of the respective phases. Dividing the right side of Eq. 6 by C_M and defining C_S/C_M as the partition coefficient, P_1, give:

$$f = \frac{V_M}{V_M + P_1 V_S} \qquad \text{(Eq. 7)}$$

The denominator of Eq. 7 is the effective volume of a theoretical plate, V_h. In column chromatographic terms:

$$V_h = h(A_M + P_1 A_S) \qquad \text{(Eq. 8)}$$

where h is the height and $A_M + P_1 A_S$ is the effective area of a theoretical plate.

Volume of Theoretical Plate from Elution Curves—The equation for a chromatographic elution curve was derived by Glueckauf (10) and is based on a continuous flow model. The model assumes the equilibration of solute between the stationary and mobile phases in each theoretical plate. However, Glueckauf (10) showed that moderate nonequilibrium conditions can be accommodated by the model as well. Each plate is defined as a unit of column length within which the solute concentrations in both mobile and stationary phases are uniform and at equilibrium. Large increases in flow rate significantly increase the nonequilibrium condition and result in larger *theoretical* plate heights and a less efficient column.

The chromatographic elution curve equation is given by Eq. 9:

$$C = C_{\max.} e^{[-N'(V_r' - V)^2]/2VV_r'} \qquad \text{(Eq. 9)}$$

Table I—Effect of Stationary Phase Loading on Effective Mobile and Stationary Phase Areas[a] and Volumes

Diatomaceous Earth (g.)–Stationary Phase (ml.)	V_M, ml.	A_{DM}, cm.^{2}	V_S, ml.	A_{DS}, cm.^{2}
18:18	32.3	1.468	15.8	0.719
18:12	38.3	1.740	9.8	0.445
18:9	41.0	1.863	7.1	0.322
18:6	43.6	1.981	4.8	0.218

[a] Column packing was 22.0 cm. in length and 19.0 mm. in diameter and contained 18 g. of diatomaceous earth support.

The value of $C_{\max.}$ is given by Eq. 10 and is equal to C when the exponential term of Eq. 9 is unity:

$$C_{\max.} = \frac{m\sqrt{N'}}{V_r'\sqrt{2\pi}} \qquad \text{(Eq. 10)}$$

The symbols of Eqs. 9 and 10 have the following significance: C, the concentration of solute per unit volume of mobile phase; m, the quantity of solute being chromatographed; N', the effective[1] number of theoretical plates on the column; V, the total volume of mobile phase eluted; and V_r', the peak elution volume of the solute band. When $C = C_{\max.}/e$, the exponent of Eq. 9 is equal to unity and V_e is the value of V necessary to elute $1/e$ of the total elution peak height. Setting the exponent of Eq. 9 equal to unity, solving for N', and setting $2(V_r' - V_e) = \beta$ (see *Appendix*) give the equation to be used for calculating the effective number of theoretical plates for all types of elution chromatography:

$$N' = \frac{8V_eV_r'}{\beta^2} \qquad \text{(Eq. 11)}$$

Since $\beta = 2\sigma\sqrt{2}$ (see *Appendix*), Eq. 11 can be written[2] as $N' = V_r'V_e/\sigma^2$. Since N' can also be expressed as the column length, L, in centimeters divided by the height of a theoretical plate, h, it is possible to write:

$$\frac{V_r'V_e}{\sigma^2} = \frac{L}{h} \qquad \text{(Eq. 12)}$$

Substituting hN' for L and rewriting V_r' as $N'V_h$ give:

$$V_h = \frac{\sigma^2}{V_e} \qquad \text{(Eq. 13)}$$

where V_h is the effective volume of a single theoretical plate. Equation 13 is used to determine the volume of a theoretical plate from elution curves.

Partition Coefficients from Elution Curves—The peak elution volume, V_r', can be written as $V_r' = N'V_h$. Recalling that $N' = L/h$ and substituting, one gets:

$$h = LV_h/V_r' \qquad \text{(Eq. 14)}$$

Solving for P_1 in Eq. 8 and substituting for h give:

$$P_1 = \frac{V_r'}{LA_S} - \frac{A_M}{A_S} \qquad \text{(Eq. 15)}$$

which is linear with a slope of $1/LA_S$ and an intercept of $-A_M/A_S$ when P_1 is plotted *versus* V_r'.

EXPERIMENTAL

Apparatus—The chromatographic columns were glass and were equipped with Teflon stopcocks; the dimensions were 500 mm. length and 19 mm. i.d. An automatic fraction collector[3] was used to

[1] The effective number of theoretical plates, N', is the number of theoretical plates from the center of the original band to the bottom of the column and is given by $N' = N - (No/2)$ (11).

[2] A common approximation (11) is to set $V_r' = Ve$ so that $N' = V_r'^2/\sigma^2$ and $V_h = \sigma/\sqrt{N'}$.

[3] Gilson Medical Electronics, Inc., model LBI with LVM1 volumetric unit.

collect fractions of eluant, and a spectrophotometer[4] was used to determine their UV absorbance. A recording UV spectrophotometer[5] was used in the determination of partition coefficients by solvent extraction.

Reagents—Analytical grade hexane, chloroform, and methanol and fresh spectrograde *p*-dioxane were suitable. The chloroform contained 0.75% ethanol as a preservative. The solvent system is prepared by shaking the solvents mixed in the appropriate proportions (*n*-hexane, 90; $CHCl_3$, 10; dioxane, 40; water, 5) and allowing the phases to separate. The lower phase is the aqueous (stationary) phase and the upper phase is the organic (mobile) phase.

The diatomaceous earth[6] was acid washed by slurrying about 300 g. diatomaceous earth with 1 l. of concentrated hydrochloric acid and allowing the mixture to stand for about 24 hr., mixing at intervals. The acid is decanted off and the diatomaceous earth is filtered and washed with distilled water until acid free and it is then washed with 500 ml. methanol and dried overnight at 100°.

All steroid samples[7] were used as received.

Determination of Elution Curves—Fifteen grams of acid-washed diatomaceous earth is thoroughly mixed with the appropriate volume of aqueous phase and packed in six or seven portions into a chromatographic column containing a glass wool plug, tamping down firmly with a glass tamping rod after each addition to give a column of diatomaceous earth 18.5 ± 0.25 cm. long. The column is washed with 75 ml. organic phase, leaving about 1–2 cm. liquid above the level of the diatomaceous earth. Five milliliters of an approximately 100-mcg./ml. solution of steroid in methanol is taken to dryness in a 50-ml. beaker, dissolved in the appropriate volume of aqueous phase (3, 2, 1.5, and 1 ml., respectively, for the diatomaceous earth aqueous phase ratios of 1:1, 3:2, 2:1, and 3:1) and mixed with 3 g. diatomaceous earth. This is added to the column in one portion and tamped down to give a total column length of 22.0 ± 0.25 cm., and a glass wool plug is inserted. Columns packed as described gave flow rates of 2.5 ± 0.3 ml./min. The column is eluted with the organic phase, collecting 5-ml. fractions of eluant. The absorbance of each fraction is determined at the absorbance maximum for the steroid, and an elution curve of absorbance *versus* fraction number is plotted. Each column was used once and discarded.

Reduced flow rates (1.5 and 0.5 ml./min.) were achieved by partially closing the stopcock. The flow rates were measured by measuring the effluent over a recorded time interval.

The slurry-packed columns were prepared using exactly the same amounts of materials and packing the column to the same dimensions as for the dry-packed column. The only difference was the presence of the mobile phase in the column to give a slurry during the packing.

Determination of Partition Coefficients by Solvent Extraction—Partition coefficients, using equal volumes of the two phases, are obtained by taking 5 ml. of a 100-mcg./ml. standard solution in methanol to dryness in a glass-stoppered tube and shaking the residue with a mixture of 10 ml. of each phase. After dissolution of the solid and phase separation, a 5-ml. aliquot of the organic phase is evaporated and the residue is dissolved in 5 ml. methanol. The absorbance of this solution, further diluted if necessary, is compared with that of a fivefold dilution of the standard, and the total amount of steroid in the organic phase is calculated. The partition coefficient is then calculated by the following formula:

$$P_1 = \frac{\text{steroid added (mcg.)} - \text{steroid in organic phase (mcg.)}}{\text{steroid in organic phase (mcg.)}} \times \frac{\text{organic phase volume}}{\text{aqueous phase volume}} \quad \text{(Eq. 16)}$$

This procedure gives accurate values of partition coefficients up to 4.0. For coefficients out of this range, the relative volumes of organic and aqueous phases are adjusted and suitable aliquots of organic phase are taken to give an absorbance of the sample solution close to that of the diluted standard. The procedure is otherwise identical.

Determination of Column Parameters—To determine the mobile phase volume, V_{DM}, 18 g. of diatomaceous earth is mixed with the appropriate volume of stationary phase, weighed, and packed into a column as already described to give a 22-cm. column. A known volume of mobile phase, usually 75 ml., is passed through the column and collected in a measuring cylinder until the liquid is level with the top of the column. The difference between the amount added and collected is one measure of V_{DM}. The difference between the weight of the column saturated with mobile phase and the weight of diatomaceous earth plus stationary phase is converted to V_{DM} by use of the density of the liquid as measured by a conventional method.

The mobile phase volume V_{SM} for a slurry-packed column made to the same specifications as the dry-packed column is determined by the weighing method previously described.

The inert phase volume, V_i, is determined by preparing a slurry-packed column 22 cm. long containing no stationary phase. The mobile phase volume is determined by the weight method. The value

[4] Beckman Instruments, Inc., model DU.
[5] Cary Instruments, model 15.
[6] Johns-Manville, Celite 545.
[7] Obtained commercially or from Syntex Research.

Table II—Peak Elution Volumes and Partition Coefficients from Elution Curves

Steroid	Peak Elution, V_r' (ml.)[a]	Partition Coefficient, P_1[b] Elution Curve[c]	Partition Coefficient, P_1[b] Solvent Extraction[d]
Prednisolone	562.5	53.48	45.30
Flumethasone	386.0	35.41	33.12
Betamethasone	356.5	32.45	29.63
Prednisone	335.0	30.21	28.57
Hydrocortisone	304.0	27.04	26.35
Paramethasone	293.0	26.00	23.55
Cortisone	248.0	21.45	17.89
Fluocinolone acetonide	156.0	11.94	11.47
Triamcinolone acetonide	136.0	9.90	9.50
Prednisolone acetate	95.5	5.85	5.96
Hydrocortisone acetate	70.0	3.24	3.36
Flumethasone acetate	68.0	2.96	2.90
Paramethasone acetate	67.5	2.98	2.90
Fluocinolone acetonide-21-aldehyde	55.0	1.63	1.68
Fluocinonide	49.0	1.09	1.08
Betamethasone valerate	45.7	0.752	0.746
Progesterone	—	—	0.31

[a] Ratio of grams diatomaceous earth to milliliters stationary phase is 3:2. [b] Defined as [stationary phase]/[mobile phase]. [c] Calculated from $P_1 = V_T'/LA_S - A_M/A_S$. [d] Determined from equilibrium distribution of solute between phases in a test tube.

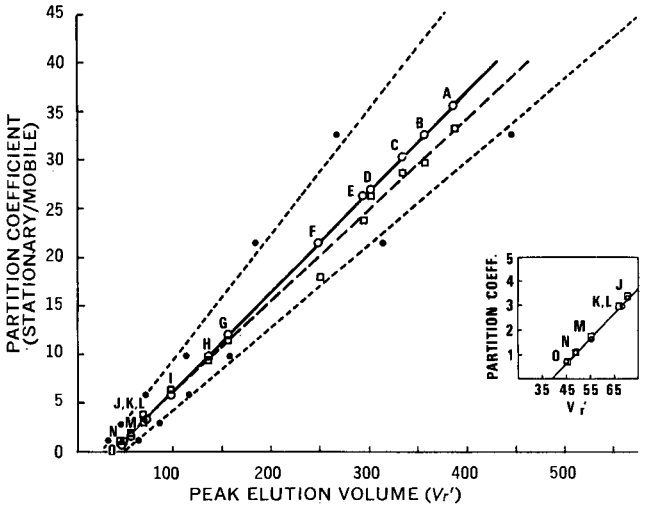

Figure 2—*Comparison of partition coefficients from elution curves* (○) *and extraction studies* (□). *Key: ---, calculated 99.9% bandwidths; and* ●, *experimental bandwidth values. Steroids are: A, flumethasone; B, betamethasone; C, prednisone; D, hydrocortisone; E, paramethasone; F, cortisone; G, fluocinolone acetonide; H, triamcinolone acetonide; I, prednisolone acetate; J, hydrocortisone acetate; K, flumethasone acetate; L, paramethasone acetate; M, fluocinolone acetonide-21-aldehyde; N, fluocinonide; and O, betamethasone valerate. The insert is an expansion of the data for steroids J through O. Eluant fractions are 5.00 ml.*

Table III—Elution Curve Parameters of Steroids as a Function of Stationary Phase Loading

Steroid	Phase[a] Loading	$\bar{\sigma}$	N'	V_r'	V_h[b]	h[b]	P_1[b]	V_h[c]	h[c]	P_1[c]
Prednisone	1:1	49.08	88.28	493.0	5.22	0.23	29.22	5.65	0.25	29.19
Fluocinolone acetonide	1:1	24.19	78.30	229.0	2.73	0.26	12.48	2.96	0.28	12.41
Triamcinolone acetonide	1:1	17.93	89.53	180.5	1.89	0.23	9.37	2.02	0.25	9.35
Fluocinolone acetonide-21-aldehyde	1:1	6.60	68.99	60.0	0.794	0.29	1.75	0.880	0.32	1.75
Prednisone	3:2	30.03	109.15	335.0	2.87	0.19	30.30	3.07	0.20	30.20
Fluocinolone acetonide	3:2	15.07	93.73	156.0	1.56	0.22	11.99	1.66	0.23	12.07
Triamcinolone acetonide	3:2	12.11	111.75	136.0	1.15	0.18	10.04	1.23	0.20	9.95
Fluocinolone acetonide-21-aldehyde	3:2	6.34	62.55	55.0	0.802	0.32	1.71	0.894	0.36	1.71
Prednisone	2:1	22.9	126.26	272.0	2.04	0.16	32.66	2.18	0.18	32.47
Fluocinolone acetonide	2:1	9.91	146.78	126.0	0.818	0.14	12.00	0.870	0.15	11.98
Triamcinolone acetonide	2:1	8.45	156.55	111.0	0.676	0.13	9.91	0.718	0.14	9.85
Fluocinolone acetonide-21-aldehyde	2:1	4.49	117.10	52.5	0.415	0.17	1.62	0.457	0.19	1.61
Prednisone	3:1	14.84	155.13	194.5	1.19	0.135	31.46	1.27	0.144	31.34
Fluocinolone acetonide	3:1	7.08	189.40	102.0	0.515	0.111	12.19	0.545	0.118	12.00
Triamcinolone acetonide	3:1	5.73	226.19	90.0	0.381	0.093	9.70	0.403	0.098	9.72
Fluocinolone acetonide-21-aldehyde	3:1	3.99	140.31	50.0	0.337	0.148	1.36	0.361	0.159	1.33

[a] The stationary phase loading is defined as diatomaceous earth (grams)/stationary phase (milliliters). [b] Calculated using the approximation $V_h = \sigma/\sqrt{N'}$. [c] Calculated using the relation $V_h = \sigma^2/V_e$.

of V_i is the difference between this value and the calculated total volume of the column.

The remaining parameters are calculated from the given equations.

RESULTS

Column Parameters—The total volume of the packed portion of the column, V_{DT} or V_{ST}, calculated from the length and cross-sectional area was 62.4 cm.^{3}. The inert volume, V_i, was found to be 9.10 cm.^{3}. The values of A_{DM} and A_{DS} for several ratios of grams of diatomaceous earth to milliliters of stationary phase are summarized in Table I.

Elution Curves—An example of a typical elution curve is shown in Fig. 1 and demonstrates the determination of parameters from which σ can be obtained (see *Appendix*). The number of theoretical plates was calculated using Eq. 11, while V_h and h were calculated from Eqs. 13 and 14, respectively. A summary of the peak elution volumes and partition coefficients for the 3:2 loading ratio (grams diatomaceous earth–milliliters stationary phase) is given in Table II. Plots of the data in Table II according to Eq. 15 are given in Fig. 2. Excellent linearity of the elution data and satisfactory agreement between partition coefficients obtained from elution and extraction data are obtained, especially for values of P_1 up to 15. The agreement of the data with theory, even when each column is used only once, indicates the ease of reproducible packing of a dry column.

Equation 15 states that its slope is a linear function of the effective stationary phase area of a theoretical plate. Accordingly, columns containing varying stationary phase areas were prepared and used to determine the partition coefficients of selected steroids. These partition coefficients plus the corresponding standard deviations, number of theoretical plates, effective volumes of a theoretical plate, and heights equivalent to a theoretical plate are given in Table III. The effect on the partition coefficient of calculating V_h and hence h, assuming $V_e = V_r'$, is essentially nil, as can be seen by comparison of the calculated partition coefficients.

Effect of Flow Rate—Flumethasone was eluted using flow rates of 2.5, 1.5, and 0.5 ml./min. No change in the shape of the eluted peak was seen and the peak elution volume was constant within 0.5 ml. The partition coefficients calculated for the three flow rates were: 2.5 ml./min., 35.41; 1.5 ml./min., 35.66; and 0.5 ml./min., 35.46.

Comparison of Slurry- and Dry-Packed Columns—Flumethasone and fluocinolone acetonide were also chromatographed using a slurry-packed column at a flow rate of 2.5 ml./min. and a column loading of 3:2 (grams of diatomaceous earth to milliliters of stationary phase). The partition coefficients calculated from the elution curves using slurry-packed columns were: flumethasone, 35.83; and fluocinolone acetonide, 12.77. The elution peaks from the slurry-packed columns were slightly tailed and the peak elution volumes were about 10.0 ml. larger for the slurry-packed than the dry-packed column data. The cross-sectional area of the slurry-packed column taken up by the mobile phase was 1.98 cm.^{2}.

DISCUSSION

Partition Coefficients—The agreement between the partition coefficients determined from elution curves with those calculated from extraction data is excellent below a value of about 15 (Fig. 2 and Table II). However, at higher values of P_1 the elution curve value is slightly higher than the extraction value. Since the difference occurs in the case where the solubility in one phase is many times different from that in the other phase, it is possible that activity coefficient effects are becoming important.

The possibility that the observed difference in partition coefficients might be due to solute adsorption onto the diatomaceous earth support was also considered. The equilibration of the adsorbed and desorbed solute must be very rapid since the data are linear, indicating that the equilibration rate between mobile and stationary phases is fast compared to the flow rate. Moreover, no change in the calculated partition coefficient was found over a fivefold change in flow rate (0.5–2.5 ml./min.), which again indicates a rapid equilibration between phases. Therefore, if adsorption is occurring, it is highly reversible. Reversible adsorption would function as an apparent increase in A_S and would result in a lower slope and a less negative intercept in Eq. 15. Thus, reversible adsorption could explain the difference in slopes in Fig. 2 and the slight difference between cal-

Table IV—Comparison of Calculated[a] and Theoretical[b] Slopes and Intercepts

Loading	Calculated		Theoretical	
	Slope	Intercept	Slope	Intercept
1:1	0.0621	−2.00	0.0632	−2.04
3:2	0.0967	−3.75	0.102	−3.91
2:1	0.129	−5.50	0.141	−5.77
3:1	0.194	−8.40	0.208	−9.08

[a] Calculated from plots of partition coefficient *versus* peak elution volume, assuming a column length of 22.0 cm. [b] Calculated according to $P = V_r'/LA_S - A_M/A_S$ from values of A_{DM} and A_{DS} independently determined and a column length of 22.0 cm.

culated and theoretical slopes and intercepts of Table IV. The estimated errors in A_{DM} and A_{DS} are insufficient to account for the observed difference.

The partition coefficients of flumethasone and fluocinolone acetonide determined using slurry-packed columns were slightly larger than the dry-packed column data and further away from the extraction data. Therefore, the discrepancy between the elution and extraction data of Fig. 2 is not caused by some artifact of a dry-packed column.

The effect of flow rate in dry-packed columns on the partition coefficients of flumethasone and fluocinolone acetonide was essentially nil over a range of 0.5–2.5 ml./min. This means that the peak elution volume, V_r', was constant and that the shape of the elution peak was gaussian.

Prediction of Column Loading—If the column is operating under reproducible conditions, the partition coefficients for each steroid should be a constant, independent of the stationary phase loading and equal to the partition coefficient determined by solvent extraction. Thus, according to Eq. 15, a plot of partition coefficient (independently determined by solvent extraction) *versus* the desired peak elution volume, V_r', allows the calculation of A_S and A_M if a choice of column length, L, is made. Alternatively, if the column parameters and partition coefficient are known, then the peak elution volume can be predicted. A plot using the peak elution volumes of Table III and assuming a column length of 22.0 cm. is shown in Fig. 3. Good linearity is obtained. Comparison of the slopes and intercepts of Fig. 3 with those calculated using the values of A_{DM} and A_{DS} of Table I is good, as shown in Table IV. The significance of the data in Table IV is that the procedure for determining the values of A_{DM} and A_{DS} is valid and, in this system, allows the calculation of the necessary stationary phase loading to achieve a desired peak elution volume. The prediction is quite accurate for values of P_1 up to about 15. Beyond this value, deviations occur (Table II) so that the prediction is only approximate but still useful. The analytical usefulness of such plots is obvious, but they can only be obtained from true partition systems such as the one described.

The usual procedure for calculating the stationary phase area (9) is to divide the volume, in milliliters, of stationary phase by the column length. This procedure assumes that all of the stationary phase is equally available to the mobile phase. This is not true, as indicated by the data in Table I. The effective stationary phase volume is significantly less than the volume added. The data are plotted in Fig. 4 and show a slightly nonlinear relationship. It is easier to predict the peak elution volume of a solute in a given column than to try and construct a column having a desired elution volume for a given solute. However, if data such as Fig. 4 are available, it is a simple matter.

Calculation of Bandwidths and Degree of Separation—It is of major interest from an analysis viewpoint to know the volume over which a solute is eluted as well as the peak elution volume. Mathematically, elution curves can be represented by the normal curve of error and, therefore, have associated with them a standard deviation (see *Appendix*). From a table of areas of the standard normal distribution (12) the number of standard deviations covering 99.9% of the normal curve is 6.582 σ. This distance in milliliters, centered on the peak elution volume, includes essentially 100% of the solute band. The data in Fig. 2 demonstrate the agreement between calculated and experimental bandwidths. In an actual analysis, it would be wise to include a small increase in the collected fractions to allow for slight variations in the column parameters.

The data in Table III show that less stationary phase loading gives a more efficient column, since the values of V_h and h are the least.

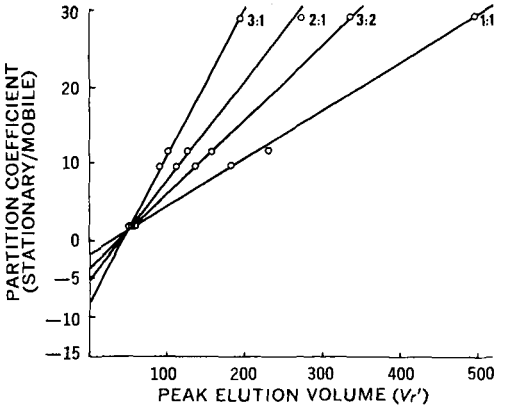

Figure 3—*Effect of stationary phase loading on peak elution values* (V_r'). *The loading is defined as grams diatomaceous earth–milliliters stationary phase.*

However, the peak elution volumes are inconveniently small in some cases. Therefore, more efficient separations can be achieved by using a lower stationary phase loading and lengthening the column. Equation 15 can be used to calculate the length necessary for a desired convenient elution volume for a particular partition coefficient. The limiting factors in reducing the stationary phase volume are the capacity of the stationary phase volume and a convenient column length.

Channeling—Glueckauf (10) cited three cases when near equilibrium conditions can be expected to exist in a chromatographic column. One case is when the plate height is determined by channeling effects in the column. In a well-prepared dry-packed diatomaceous earth column, the stationary phase can be expected to accumulate on each particle and at their points of contact. An

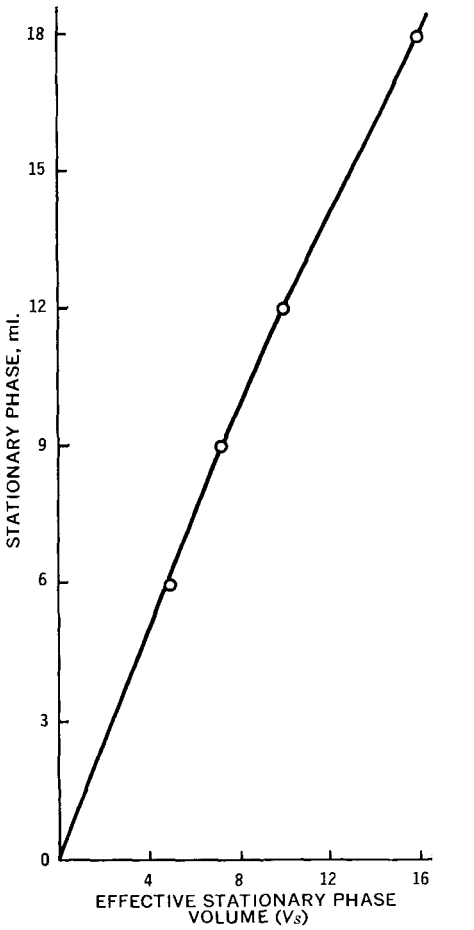

Figure 4—*Relation of milliliters of stationary phase used to effective stationary phase volume* (V_s).

appreciable void space is formed which is only partially filled by the mobile phase (A_o = 0.236 cm.^{2} in a 22-cm. column with a 40% stationary phase loading and 1.90 cm. i.d.). The mobile phase is channeled in a flowing layer on the surface of the particles. The mobile phase in a slurry-packed column fills all of the interstices of the column and results in a longer average diffusional path length for the solute. Snyder (13) showed that the van Deemter equation parameters give significantly lower plate heights for dry-packed (tamped) columns then for slurry-packed (wet) columns and that the principal difference is in the term corresponding to the rate of mass transfer between mobile and stationary phases. Thus, the increase in diffusional path length requires more time for equilibration to occur and slower flow rates.

Effect of Number of Components in a Partition System—Some liquid–liquid partition systems in the literature (1) contain just two components, often water and an immiscible organic solvent. The solute capacity of such systems is nearly always low and it is usually not possible to adjust to achieve a convenient elution volume. Moreover, two-component systems are prone to interfacial solute adsorption (14).

Most partition systems (1, 2) have three components, but disadvantages still exist in many cases. The third component is often chosen to modify the solute solubility in only one of the other two solvents (H_2O plus CH_3OH, $CHCl_3$ plus benzene, *etc.*). Since the partition coefficient is approximated by the ratio of solubilities (15), the possibility of changing the solute solubility in only one of the phases limits the range of partition coefficients (and therefore solutes) that can be usefully covered. A four-component system is expected to be much more versatile and to accommodate a wider range of partition coefficients, since the solubility in each phase can be varied more easily.

In the present system, it would be possible to obtain a range of partition coefficients with the basic three-component system hexane–dioxane–water, but the substitution of chloroform for some of the hexane allows wide changes in partition coefficients without drastically altering the basic system. This has also been of value in gradient elution applications. For analytical applications, if the partition coefficient is too large, the proportion of chloroform should be increased by substitution for hexane; if too small, the proportion of chloroform should be reduced and, if necessary, some of the water should be replaced by methanol.

Effect of Steroid Structure—The substitution of acetate or valerate at the 21-position significantly decreased partition coefficients. This is in agreement with the usual behavior of such substitution seen in GC. Comparison of the partition coefficients of prednisolone and hydrocortisone, whose structures differ by a Δ^1 bond, show that prednisolone is significantly more polar and elutes much later. Thus, in otherwise equal structures, the solvent system can be sensitive to changes in the A ring. Comparison of prednisolone with prednisone and hydrocortisone with cortisone shows that an 11-keto function is less effective than the OH in increasing the elution volume.

Fluoride substitution causes an increase in partition coefficient. Fluocinolone acetonide (6,9-F) and triamcinolone acetonide (9-F) differ by two partition coefficient units. Comparison of flumethasone, betamethasone, and paramethasone shows that the 6-F is much less effective in raising the partition coefficient than the 9-F derivative.

Analytical Applications—The partition columns described here have been used in conjunction with suitable extraction techniques in routine analytical control procedures for steroidal pharmaceutical products such as creams, ointments, tablets, and solutions. With a minimum of experience, the equilibration of the phases, packing of the column, initial washing, and addition of sample packing require no more than 45 min. With a flow rate of 2.5 ml./min., elution of the sample is complete in 80 min., provided the solvent system and/or column loading has been adjusted to give a convenient peak elution volume of about 150 ml. Since the columns have been shown to be very reproducible, it is possible to specify accurately the eluate volume fraction containing the solute peak of interest without monitoring small fractions of the eluate. No special temperature control beyond a thermostated room temperature is needed. Temperature variations of 1 or 2° have no observable effect on column operation. A column can be used at least six times if the sample portion of the column is carefully removed each time. The principal disadvantage of these partition columns is the analytical time required. The time is usually comparable to TLC but is much longer than GC procedures.

APPENDIX

The equation for the normal curve of error (12) is used to derive an expression for β, used in Eq. 11, in terms of the standard deviation, σ, of an elution curve. Expressed in terms of concentration, C, the normal curve of error is written as:

$$C = \frac{1}{\sigma\sqrt{2\pi}} e^{[-(V-V_r')^2]/(2\sigma^2)} \qquad \text{(Eq. A1)}$$

where V_r' is the peak elution volume and V is the elution volume to any point on the curve. When $V = V_r'$, then the exponential term is unity and $C = C_{max.} = 1/\sigma\sqrt{2\pi}$. Therefore, Eq. A1 becomes:

$$C = C_{max.} e^{[-(V-V_r')^2]/2\sigma^2} \qquad \text{(Eq. A2)}$$

Setting $V = V_e$, the point on the elution curve where the height is $1/e$ of the peak height, Eq. A2 becomes:

$$\frac{C_{max.}}{e} = C_{max.} e^{[-(V_e-V_r')^2]/(2\sigma^2)} \qquad \text{(Eq. A3)}$$

Therefore:

$$1 = (V_e - V_r')^2/2\sigma^2 \qquad \text{(Eq. A4)}$$

Setting $V_e - V_r' = \beta/2$ gives:

$$\beta = 2\sigma\sqrt{2} \qquad \text{(Eq. A5)}$$

Similarly, setting $V = V_{0.5}$, the point on the elution curve that is half the peak height, gives:

$$d = 2\sigma\sqrt{2 \ln 2} \qquad \text{(Eq. A6)}$$

where $d = 2(V_{0.5} - V_r')$, or setting $V = V_{0.607}$ gives:

$$y = 2\sigma \qquad \text{(Eq. A7)}$$

REFERENCES

(1) J. Levine, *J. Pharm. Sci.*, **52**, 1015(1963).

(2) R. Neher, "Steroid Chromatography," 2nd ed., Elsevier, New York, N. Y., 1964, pp. 58–86.

(3) D. Waldi, in "Thin Layer Chromatography," 1st ed., E. Stahl, Ed., Academic, New York, N. Y., 1965, pp. 249–278.

(4) R. Neher, in "Thin Layer Chromatography," 2nd ed., E. Stahl, Ed., Springer-Verlag, New York, N. Y., 1969, pp. 311–362.

(5) E. C. Horning and W. J. A. Vandenheuvel, in "Advances in Chromatography," vol. 1, J. C. Giddings and R. A. Keller, Eds., Marcel Dekker, New York, N. Y., 1965, pp. 153–198.

(6) H. H. Wotiz and S. J. Clark, "Gas Chromatography in the Analysis of Steroid Hormones," 1st ed., Plenum, New York, N. Y., 1966.

(7) I. E. Bush, "The Chromatography of Steroids," Pergamon, New York, N. Y., 1961.

(8) F. Bailey, A. Holbrook, and R. J. Miller, *J. Pharm. Pharmacol., Suppl.*, **18**, 12S(1966).

(9) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35**, 1358 (1941).

(10) E. Glueckauf, *Trans. Faraday Soc.*, **51**, 34(1955).

(11) H. Purnell, "Gas Chromatography," 1st ed., Wiley, New York, N. Y., 1962, pp. 94–109.

(12) D. V. Huntsberger, "Elements of Statistical Inference," 1st ed., Allyn and Bacon, Boston, Mass., 1961, p. 103.

(13) L. R. Snyder, *Anal. Chem.*, **39**, 698(1967).

(14) D. C. Locke, in "Advances in Chromatography," 1st ed., J. C. Giddings and R. A. Keller, Eds., Marcel Dekker, New York, N. Y., 1969, p. 69.

(15) H. Purnell, "Gas Chromatography," 1st ed., Wiley, New York, N. Y., 1962, p. 10.

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