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HIGH-PERFORMANCE DISPLACEMENT CHROMATOGRAPHY OF CORTICOSTEROIDS

SCOUTING FOR DISPLACER AND ANALYSIS OF THE EFFLUENT BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

High-performance displacement chromatography was used for preparative scale separation of corticosterone, deoxycorticosterone and Reichstein's substance S with high-performance liquid chromatographic (HPLC) columns and instrumentation. Thin-layer chromatography (TLC) in the displacement mode was used to find an adequate carrier as well as a suitable displacer and its appropriate concentration. Separation of a mixture containing 60 mg of each component was carried out with a 500 × 4.6 mm column packed with 5-μm silica gel. The carrier was chloroform and the displacer was 5% (v/v) diethylethanediamine in chloroform. Regeneration of the column was carried out with alcoholic acetic acid–chloroform mixtures and checked by an HPLC test. In order to evaluate the results, fractions of the column effluent were analyzed by TLC and the results were confirmed by HPLC. TLC was found to be a sufficiently accurate and highly convenient tool to determine product distribution upon separation by displacement chromatography.

INTRODUCTION

Recent works[1,2] evinced the advantages of preparative separations by displacement chromatography with column and instrumentation generally employed in analytical high-performance liquid chromatography (HPLC). The technique called high-performance displacement chromatography (HPDC) was found, by using HPLC for analyzing fractions of the column effluent, to yield excellent separations of aromatic substances and polymyxin antibiotics with silica-bound hydrocarbonaceous stationary phases.

In this report we shall illustrate the use of HPDC for preparative separation of corticosteroid hormones with columns packed with microparticulate silica gel. Furthermore we shall demonstrate that thin-layer chromatography (TLC) can be a powerful tool to scout for a suitable displacing agent prior to fractionation and to demarcate zones of the components upon analyzing the column effluent thereafter.

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A variety of chromatographic methods has been described for the separation of corticosteroids[3]. Traditionally, silical gel[4–6] but frequently also Sephadex LH-20[7,8] was employed as the stationary phase in conjunction with chloroform or other chlorinated hydrocarbons as eluent. In HPLC microparticulate silica columns were first used for the analysis of corticosteroids[9–14]. Recently however reversed-phase chromatography with non-polar bonded phases and polar eluents has found increasing application in this field[15–17]. TLC with silica gel has also widely been used for steroid separation in analytical work[16–21] or in the selection of optimum eluent composition for use in HPLC[16,17].

In preparative chromatography of such steroids on silica gel the upper limit of sample loading is usually estimated as 1‰ (w/w) of dry sorbent present in the column[22–24]. In order to separate sufficiently large quantities, therefore, preparative scale chromatography usually employs columns having relatively large diameter. On the other hand, for "micropreparative" separation of 0.87 mg of corticosteroid mixture the use of a 410 × 16 mm silica gel column, which is considered small by conventional standards, has been described.

The goal of the present work is to point out and demonstrate the benefits arising from the coadjutant use of TLC with HPDC for preparative scale separation of certain corticosteroids with a microparticulate silica column. We shall see that the role of TLC as an attendant technique in HPDC is more versatile than in preparative elution chromatography where it has been employed as a scouting method for suitable eluent composition[13].

The results suggest that, with this concomitant in particular, displacement chromatography, which received great attention in early chromatographic literature[25–30] and has a well developed theoretical foundation[31], offers a powerful and convenient method for the separation of biological substances on conventional stationary phases as well.

EXPERIMENTAL

Materials, columns and reagents

Type MK6F (25 × 75 mm, 200 μm layer) and K6DF (200 × 200 mm, 250 μm layer) silica gel precoated glass TLC plates were obtained from Whatman (Clifton, NJ, U.S.A.). Partisil PXS-525 columns (250 × 4.6 mm) packed with 5-μm silica gel were obtained from Whatman. A 500 × 4.6 mm column home-packed with 5-μm Partisil (Whatman) was also employed. According to the supplier, chromatographic properties of the silicas used for manufacturing the TLC plates and HPLC columns were sufficiently similar as far as retention behaviour is concerned. Corticosterone, deoxycorticosterone and 11-desoxy-17-hydroxycorticosterone were purchased from Sigma (St. Louis, MO, U.S.A.). Chloroform, methylene chloride, carbon tetrachloride, methanol, ethanol, propanol, butanol, hexanol, octanol, cyclohexanol, benzene, tetrahydrofuran, ethylene glycol (HPLC grade), triethylamine, triethanolamine, diethylethanediamine (DEEDA) and dimethylcyclohexylamine (certified) were, unless noted otherwise, reagent grade and purchased from Fisher (Pittsburgh, PA, U.S.A.).

Apparatus

The fractionator unit used for displacement chromatography has been described in a previous report[2]. The reservoirs of chloroform used as carrier, the displacer solution, and the regenerant are connected via a four-way valve to a Model No. 110 A solvent metering pump (Altex, Berkeley, CA, U.S.A.) equipped with a 1.0-ml feed loop. The column effluent was monitored by a Model LC 55 variable-wavelength detector (Perkin Elmer, Norwalk, CT, U.S.A.) and a Model SR-206 dual-pen strip-chart recorder (Heath, Benton Harbor, MI, U.S.A.). Fractions containing 0.5 ml of the effluent were collected with an Ultrarack II, No. 2070 fraction collector (LKB, Rockville, MD, U.S.A.). In some instances eluent fractions were also analyzed by an HPLC analyzer containing the same major components as the fractionator unit. Fractionation was carried out with two Partisil PXS-525 columns in series whereas only one column was used for analytical separations. All experiments were carried out at room temperature ranging from 21 to 25°C.

Methods

Frontal TLC. Silica-coated 25 × 75 mm TLC plates were used for frontal development in a 100-ml covered beaker that held the "displacer" solution for at least 2 h prior to the beginning of the experiments to saturate the vapor phase. Upon development the distance of the solvent front and the "displacer" front from the bulk liquid level were measured. The location of the displacer front was found by direct visual observation of the plate.

Displacement TLC. Corticosteroids (5 μg in 5 μl chloroform) were spotted at a distance of 2 cm from the bottom edge of the plates having the above dimensions. Thereafter the plates were dried and placed into the beaker containing the displacer solution for development. The development was terminated when the solvent front moved a distance of 5 cm from the liquid level on the plate. Thus, the distance between the solvent front and loci of sampling was 4 cm. After development both solvent front and displacer front were marked and the plates were dried. The spots were observed under UV light at 254 nm with a Model UVS-II lamp (Ultra-violet Products, San Gabriel, CA, U.S.A.) or after they were made visible upon spraying with 50% (v/v) sulfuric acid in water and subsequent heating at 120°C for 5 min. The shape and positions of the spots relative to both the solvent and displacer fronts were recorded.

Elution TLC in scouting for suitable displacer. Under the same conditions given in the preceding section 18 neat solvents were tested as potential eluents for the three corticosterones. After development and spot visualization the R_F values were recorded.

Analytical TLC of effluent fractions. Silica-coated 200 × 200 mm plates were used in a rectangular developing tank (Fisher) having dimensions of 300 × 100 × 250 mm. A 50-ml volume of a chloroform–acetone (9:1) mixture was placed into the covered tank 2 h before the experiment. The samples were spotted at a distance of 37 mm from the bottom of the plate, the distance between the individual spots was 8 mm so that 21 samples were chromatographed simultaneously and 20-mm margins were left at both sides of the plate. The solvent front moved 180 mm from the liquid level in the tank in 50 min. Thereafter the plate was dried and the spots were made visible in the way described above.

Fractionation by displacement chromatography. The 1.0-ml loop of the feed injector valve was filled up with the feed solution of the corticosterone mixture in chloroform. At the same time, the reservoir of DEEDA solution in chloroform used as displacer was connected to the pump by turning the four-way valve. Subsequently the displacer solution was pumped into the system at a flow-rate of 0.1 ml/min and the injector valve was moved into feed position. As the displacer solution moved through the column, the three corticosterones separated into juxtaposed concentration zones and emerged from the column consecutively. Fractions of the column effluent were collected in 5-min intervals and analyzed by TLC (see above), or by HPLC (see below). The collection and numbering of fractions began upon turning the feed valve.

Column regeneration. After the displacement front emerged from the column, the four-way valve was switched to the reservoir of regenerant A which contained 20% (v/v) methanol, 10% (v/v) acetic acid and 70% (v/v) chloroform. Regenerant A was pumped through the column for 30 min at a 2 ml/min flow-rate. Thereafter regenerant B containing 30% (v/v) propanol in chloroform was pumped through the system for 30 min. Finally, the four-way valve was turned back to the original position and chloroform, the carrier used in the next chromatographic run, was pumped through the system for one hour. No attempt was made to reduce regeneration time.

Test of column regeneration. After executing the steps described for column regeneration, 20 μl of a test mixture containing *p*-cresol, benzene and phenylethylalcohol was injected by using a 20-μl loop in place of the 1.0-ml feed loop in the injection valve of the displacer unit. The eluent was chloroform, the flow-rate was 1.0 ml/min and the column effluent was monitored at 254 nm. For measurement of t_0 it was assumed that *p*-cresol is eluted in the void volume of the column under the conditions used here. Column regeneration was considered complete when the retention factors were in the range of 1.2 ± 0.12 and 1.8 ± 0.18, for benzene and phenylethylalcohol, respectively.

Analysis of the fractions by HPLC. The analyzer unit was equipped with a Partisil PXS-525 column and the eluent was 6% (v/v) propanol in chloroform. The fractions were diluted with two-fold volume of propanol containing the third component of the feed mixture which was not present in the fractions analyzed, as the internal standard and 20-μl aliquots were injected. The flow-rate was 1 ml/min and the column effluent was monitored at 254 nm.

Data evaluation

Measurement of retention in TLC. R_F values in elution chromatography were evaluated in the usual way. In both frontal and displacement TLC the front of the displacer with respect to the solvent front and the liquid level in the chamber, *viz.*, the actual starting line, was characterized by an equivalent dimensionless parameter, R_D.

Frontal chromatography of displacer with the HPLC unit. The breakthrough volume of the displacer front was measured under various conditions as far as the composition of the displacer solution is concerned. In these experiments the procedure used for the introduction of the displacer solutions was essentially the same as described for fractionation except no sample loop was employed and no sample was introduced. The fractionator unit was equipped with two Partisil PXS-525 columns and operated at a flow-rate of 1 ml/min. The carrier was chloroform and diethyleth-

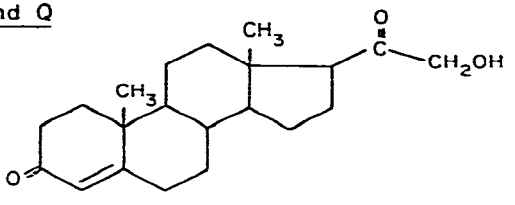
anediamine solutions of different concentrations in chloroform were used as displacer. The column effluent was monitored at 300 nm and the retention volume of the displacer was measured at the inflection point of the breakthrough curve.

RESULTS AND DISCUSSION

The chemical structure of the three adrenal corticosteroids investigated here is given in Fig. 1. On the preparative scale they are most conveniently chromatographed on silica gel with chloroform containing some polar solvent such as methanol, acetone or propanol[32]. Therefore, silica gel was selected here as the stationary phase also for their separation by displacement chromatography with HPLC systems. In searching for a suitable carrier solvent and displacer we have found that TLC with silica-coated plates offers a rapid and convenient means to that end.

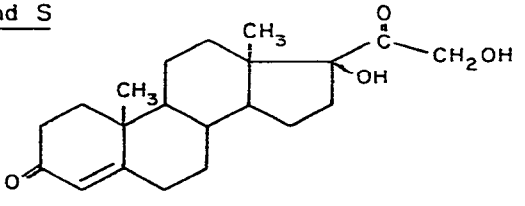
The criteria for an appropriate carrier with the stationary phase under investigation is that the components are strongly but selectively retarded when the carrier is

Compound Q



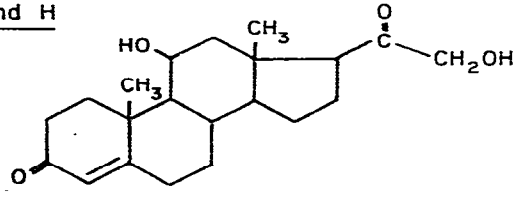
Deoxycorticosterone,
Reichstein's "Substance Q"

Compound S



11-Deoxy-17-hydroxycorticosterone,
Reichstein's "Substance S"

Compound H



Corticosterone,
Reichstein's "Substance H"

Fig. 1. Corticosteroids investigated in present study.

used as the eluent. As an arbitrary rule we consider a solvent an acceptable carrier when it yields, if used as an eluent in TLC with the same stationary phase as that present in the column, unequal R_F values smaller than 0.1 for the sample components.

In a series of screening experiments the three corticosteroids were subjected to elution development in TLC with silica gel coated plates by using a variety of neat solvents. The R_F values obtained with 18 solvents are listed in Table I. As expected the corticosteroids under investigation do not migrate upon development with nonpolar solvents. Perusing the data in Table I and applying the above criteria we concluded that chloroform may be the best carrier.

TABLE I

R_F VALUES OF CORTICOSTEROIDS IN SOLVENTS EXAMINED AS POTENTIAL CARRIERS FOR DISPLACEMENT CHROMATOGRAPHY BY USING ELUTION TLC DEVELOPMENT WITH SILICA GEL COATED PLATES

Solvent	R_F *Values*		
	Corticosterone	*11-Deoxy-17-hydroxycorticosterone*	*Deoxycorticosterone*
Hexane	–*	–	–
Cyclohexane	–	–	–
Xylene	–	–	–
Carbon tetrachloride	–	–	–
Chloroform	–	0.03	0.09
Methylenechloride	0.02	0.08	0.25
Acetonitrile	0.86	0.83	0.95
Dioxane	0.90	0.90	0.98
Acetone	0.90	0.90	0.90
Tetrahydrofuran	0.95	0.95	0.99
Acetic acid	0.95	0.98	0.98
Octanol	0.65	0.70	0.85
Hexanol	0.75	0.78	0.85
Butanol	0.85	0.85	0.90
Propanol	0.85	0.85	0.90
Ethanol	0.85	0.88	0.93
Methanol	0.85	0.88	0.95

* – = Very small value: $R_F < 0.01$.

After the carrier solvent had been selected, the appropriate displacer was found in an ensuing series of experiments by using TLC in the displacement mode. Positions of both the solvent front and the displacer front were recorded upon development and the location of the displacer front was measured by its R_D value evaluated in a fashion equivalent to the measurement of R_F values. The three corticosteroids were individually spotted on silica-coated plates and the solution of a potential displacer in chloroform was used for the development of the chromatogram. The criterion for a suitable displacer was simply that all three components moved with the displacer front on the TLC plate, *i.e.*, the R_F value of each component was about the same or slightly greater than the R_D value. We have found that no displacement occurred and

the R_F value of each component was significantly smaller than R_D, when 10% (v/v) chloroform solutions of the following substances were used as developing agent: ethanol, *n*-propanol, *n*-butanol, *n*-hexanol, *n*-octanol, cyclohexanol, tetrahydrofuran, acetone, dimethylcyclohexylamine and triethylamine. On the other hand, with 10% (v/v) solutions of methanol, acetic acid, DEEDA and triethanolamine in chloroform displacement chromatography took place on the TLC plates.

Subsequently, the last four solutions described above were also used to carry out displacement chromatography with silica gel columns. We found that with 10% (v/v) methanol as the displacer corticosterone and 11-deoxy-17-hydroxycorticosterone did not separate whereas corticosterone was not displaced by 10% (v/v) acetic acid in chloroform and emerged from the column after the displacer front. However, DEEDA and triethanolamine in chloroform solution both were suitable displacers in these experiments and the results strongly advanced the notion that for our purposes chloroform solutions of these amines are the most promising displacers.

In order to investigate the effect of displacer concentration in chloroform on the migration rate of the displacer front, TLC of the three corticosteroids was performed again in the displacement mode by using amine solutions of different concentrations. Beside DEEDA and triethanolamine, dimethylcyclohexylamine and triethylamine were included in the experimental series in order to verify that indeed the first two amino compounds, and only they, are appropriate displacers in the chloroform

TABLE II

DISPLACEMENT TLC OF THE THREE CORTICOSTEROIDS ON SILICA GEL WITH VARIOUS AMINES IN CHLOROFORM SOLUTION

Positions of displacer fronts are characterized by R_D values listed here for the various displacer solutions differing in the nature and concentration of the amine. The locus of all three spots after development was at the displacer front unless otherwise indicated.

Amine in chloroform (% v/v)	R_D *values*			
	Diethylethane-diamine	*Triethanol-amine*	*Triethyl-amine*	*Dimethylcyclo-hexylamine*
1	0.09	0.02	0.51*	<0.01
2	0.17	0.08	0.51*	0.19*
3	0.26	0.12	0.51*	0.31*
4	0.36	0.16	0.51*	0.38*
5	0.40	0.19	0.51*	0.44*
6	0.43	0.22	0.53**	0.53**
7	0.46	0.28	0.60**	0.58**
8	0.50	0.36	0.63**	0.61**
9	0.53	0.41	0.65**	0.63**
10	0.57	0.43	0.67**	0.63**
20	0.81	0.56		
30	0.91	0.86		
40	0.98	0.98		
50	1.00	1.00		

* All sample components were eluted behind the displacer front.

** Corticosterone was not displaced but eluted behind the displacer front.

carrier. The results of these experiments are displayed in Table II. In chloroform solution both DEEDA and triethanolamine were good displacers for all these corticosteroids over a concentration range from 1 to 50% (v/v), whereas the other amines were not. The R_D values, equivalent to the R_F values of the displacer front and listed in Table II, however, do not give information about the optimum concentration of the displacer solution. Evidently, too rapid migration observed at relatively high concentration of the displacer is not desirable as the displacement train may not be fully developed in a given column. Alternatively at low displacer concentrations, isotachic conditions may be reached too slowly in a given column[1,31]. According to our experience the useful range of R_D values is between 0.20 and 0.50. However, this is not a sufficient criterion for selecting the displacer concentration and therefore it was necessary to examine not only retention behavior but also the shape of the spots in actual displacement TLC development. When a suitable displacer is employed in such experiments the spots of sample component should be closely spaced at the front of the displacer and have narrow oblong shapes as shown in Fig. 2. In fact, the most effective way to use TLC in scouting for carrier solvent and displacer is to carry out TLC of the mixture to be separated in both frontal (without displacer in the prospective carrier solvent) and displacement mode and examine both the position and shape of the spots as well as the R_D value obtained for the front of the potential displacer substance.

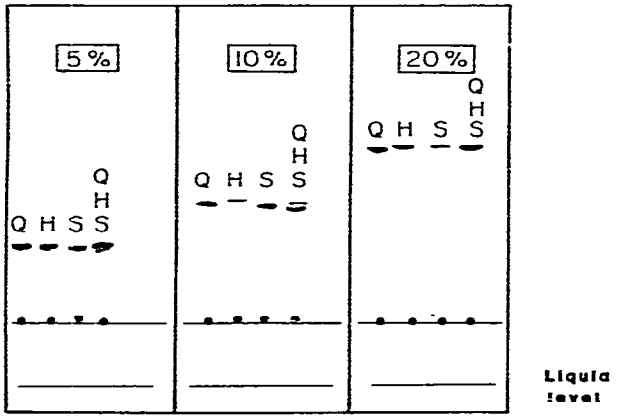


Fig. 2. Displacement TLC of the three corticosteroids by using 5, 10 and 20% (v/v) DEEDA in chloroform for development. Sample: 1 μg of each components in 10 μl of chloroform. Development time 8 min. The spot was made visible upon spraying with 50% (w/v) sulfuric acid in water and subsequent heating at 120°C for 5 min.

In our case both DEEDA and triethanolamine were found to be equally suitable displacers in chloroform solution in the concentration range from 2 to 10% (v/v). The decision to use DEEDA as the displacer in the subsequent experiments was based on its relatively low boiling point, 145°C, in comparison to 207°C the boiling point of triethanolamine. The rationale for this stems from the belief that a lower boiling displacer is easier to remove from the product if contamination occurs.

After having chosen DEEDA as the displacer we reexamined and confirmed the results of the carrier selection process. Displacement was carried out with the

three corticosteroids by using DEEDA dissolved not only in chloroform but also in carbon tetrachloride and methylene chloride, the two closest prospective carrier solvents according to Table I. The result of these experiments are presented in Fig. 3 and corroborate that chloroform is the most suitable carrier. It is seen that when carbon tetrachloride, which for all practical purposes is not an eluent for the corticosteroids, is used as the solvent DEEDA does not displace the sample components and they migrate slower than the DEEDA front. As seen in Fig. 3 they are eluted by the DEEDA solution in carbon tetrachloride and their spots are located behind the DEEDA front. On the other hand, Fig. 3 also shows that the three corticosteroids are eluted ahead of the DEEDA front by methylene chloride used as the carrier since it is a weak eluent as suggested by the R_F values of the carriers listed in Table I. Similar experiments with solutions of triethanolamine, triethylamine and dimethylcyclohexylamine in the above three solvents over a wide concentration range yielded essentially the same results. We may conclude, therefore, that the expediency of choosing chloroform and DEEDA as the carrier solvent and displacer, respectively, has been confirmed by these results.

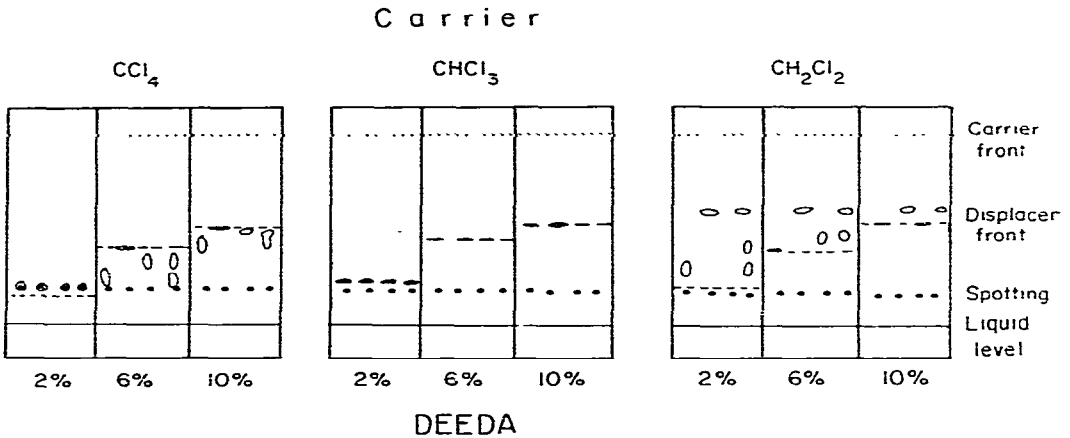


Fig. 3. Experiments with displacement TLC of corticosteroids to select the suitable carrier solvent. DEEDA was used as the displacer in carbon tetrachloride, chloroform and methylenechloride. The displacer concentration in % (v/v) is indicated at the bottom. The order of spots of sample components from left to right on each plate is the same and given by Q, S, H, Q + S + H; see Fig. 1 for symbols.

The effect of DEEDA concentration in chloroform on the migration rate of the displacer front was also investigated by using the HPDC unit in the frontal chromatographic mode. The breakthrough time of the displacer front at the inflection point was measured and the velocity of the displacer front u_D, was calculated. According to the literature[31] this velocity is given by

$$u_D = u_C/(1 + k_D^*) \quad (1)$$

where u_C is the velocity of the carrier solvent through the column and k_D^* is the "retention factor" of the displacer in the column that is given by the chord of the adsorption isotherm[1]. Fig. 4 shows the dependence of the parameter k_D^* on the con-

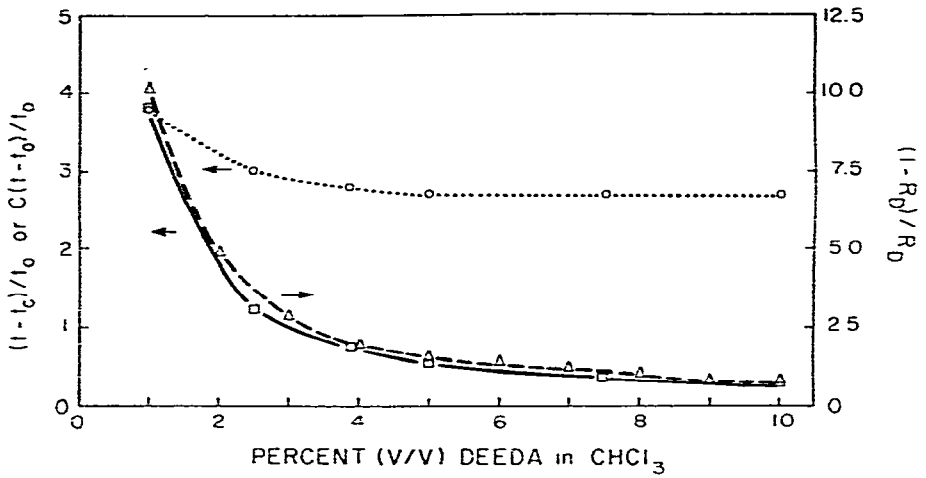


Fig. 4. Graph illustrating the dependence of frontal retention factor of DEEDA on the concentration of its solution in chloroform. Retention factors were measured with silica gel column in HPLC as $(t_D - t_0)/t_0$ where t_D is the breakthrough time of the displacer front and t_0 is the carrier hold-up time in the column, both measured at the same flow-rate. In TLC the retention factors were evaluated as $(1 - R_D)/R_D$. The dependence of $C_D(t_D - t_0)/t_0$, where C_D is the displacer concentration in the carrier, on C_D is also shown by the dotted line.

centration of DEEDA in chloroform as evaluated from frontal chromatographic experiments with both HPLC column and TLC plate. From experiments with columns k_D^* is evaluated from the carrier hold-up time, t_0, and the inflection point of the displacer front, t_D, as

$$k_D^* = (t_D - t_0)/t_0 \tag{2}$$

In planar chromatography under ideal circumstances, when uniform conditions prevail along the TLC plate, the following relationship holds

$$k_D^* = (1 - R_D)/R_D \tag{3}$$

Results obtained with both silica columns and TLC plates and shown in Fig. 4 indicate a similar dependence of k_D^* on concentration. The adsorption behavior of DEEDA on silica in both chromatographic systems, therefore, can be considered comparable. This finding supports the use of data obtained from TLC experiments in the design of displacement chromatographic systems for column chromatography. In Fig. 4, the product of the retention factor of the displacer, k_D^* and its concentration in chloroform, C_D, is also plotted against C_D. If the adsorption isotherm of DEEDA on silica has a plateau at sufficiently high value of C_D, as Langmuir isotherms do at saturation, the product $k_D^* C_D$ should reach a constant value with increasing C_D. It is seen in Fig. 4 that the value of $k_D^* C_D$ first decreases with increasing C_D and remains constant at DEEDA concentrations higher than 5% (v/v). This behavior suggests that the adsorption isotherm of DEEDA from chloroform on silica gel reaches a plateau.

Despite the conformity observed between results of experiments with columns and TLC plates, conclusions reached on the basis of TLC experiments still require confirmation by column chromatography before their applicability in the latter can be accepted. A caveat has already been implied by our experience with the selection of displacer as described above. Non-uniformity of flow in space and time as well as non-uniformity of equilibrium conditions along the migration path may give rise to deviations of TLC results from those obtained in HPLC with a homogeneous column under precisely controlled conditions.

As expected from theory[31] and shown in Fig. 4, the velocity of displacer front increases with the concentration of DEEDA. In view of these data the concentration of DEEDA in chloroform should be higher than 2% (v/v) in order to avoid lengthy development. The separation of the three corticosteroids by displacement chromato-

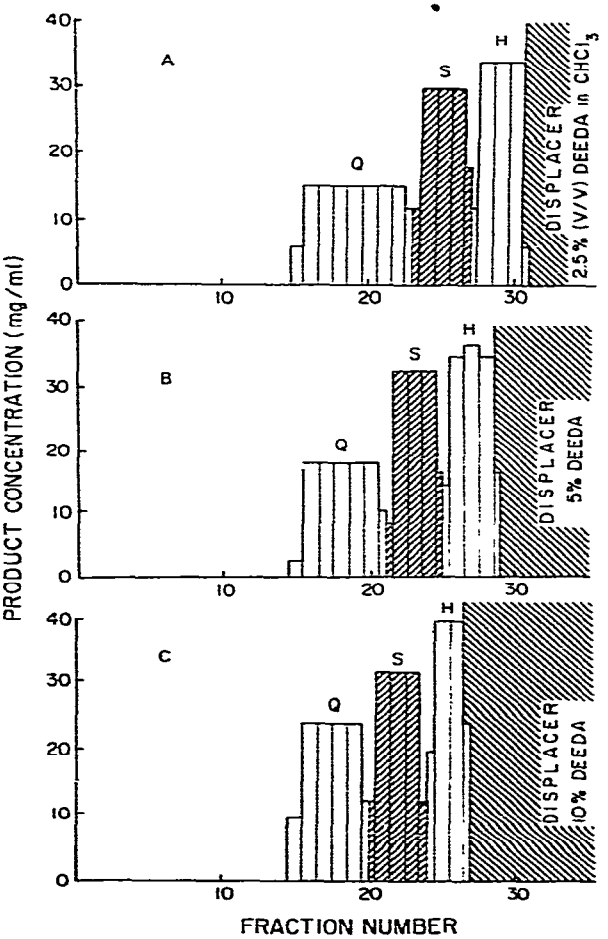


Fig. 5. Displacement diagrams of corticosterones as obtained by HPLC analysis of the effluent fractions. A, 2.5; B, 5.0 and C, 10% (v/v) DEEDA in chloroform was used as displacer. Symbols of sample components are given in Fig. 1. Column (2 × 250) × 4.6 mm, Partisil PXS-525 packed with 5 μm silica gel; feed, 60 mg of each component in 1 ml chloroform; fraction volume, 0.5 ml; flow-rate, 0.1 ml/min; temperature, 22°C.

TABLE III

RESULTS OF TLC ANALYSIS OF THE FRACTIONS OBTAINED IN DISPLACEMENT CHROMATOGRAPHY OF CORTICOSTEROIDS BY USING DIFFERENT CONCENTRATIONS OF THE DISPLACER UNDER CONDITIONS GIVEN IN FIG. 5

The symbols Q, S and H represent the three sample components, see Fig. 1, and D stands for DEEDA, the displacer. No feed component was found in fractions before No. 16.

DEEDA (%, v/v)	*Fraction number*																
	16	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*
2.5	Q	Q	Q	Q	Q	Q Q	Q	Q S	S	S S	S	S	S H	H H	H	H	H D
5.0	Q	Q	Q	Q	Q Q	S	S	S	S S	H	H	H H	H	D			
10.0	Q	Q	Q	Q	S	S	S	S	H	H	H	D					

graphy at three different displacer concentrations, 2.5, 5 and 10% DEEDA is shown in Fig. 5. The displacement diagrams were constructed from solute concentrations found by HPLC analysis of the effluent fractions. Demarcation of the component zones does not require the use of HPLC, however. As seen in Table III analysis of the fractions by TLC offers a very simple way to identify the product and estimate its purity in each fraction. Such TLC results are eminently suitable to find zone boundaries that contain more than one component and therefore require rechromatography for their separation.

Results presented here corroborate earlier finding that the loading capacity of a column for a sample containing a few components is at least one to two order of magnitude higher when the mode of chromatography is displacement rather than elution.

Another displacement diagram illustrating the separation of the three corticosteroids is depicted in Fig. 6. The feed contained 60 mg of each component and the displacer solution was 5% (v/v) DEEDA in chloroform. However, the column was different than that used to obtain the results shown previously. In the latter experiment the volume of the fractions was 0.2 ml compared to 0.5 ml fraction volume in the previous experiments. Solute concentrations shown in the displacement diagram were determined by HPLC. For zone demarcation TLC was also used and

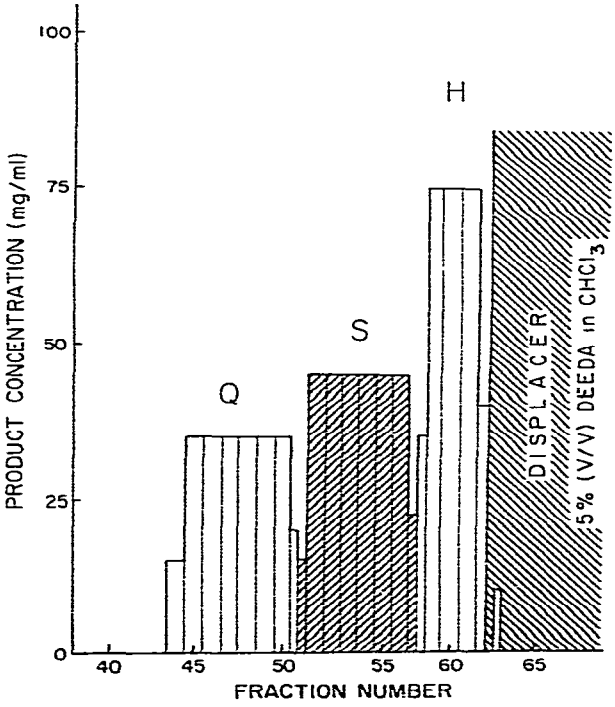


Fig. 6. Displacement diagram of three corticosterones obtained with a 500 × 4.6 mm column home packed with 5-μm Partisil silica gel by using chloroform and 5% (v/v) of DEEDA in chloroform as the carrier solvent and displacer solution, respectively. The flow-rate was 0.1 ml/min and the feed contained 30 mg of each of the three corticosterones, see Fig. 1 for symbols, in 1 ml of chloroform. Each fraction contained 0.2 ml of column effluent and was analyzed by HPLC.

typical results are shown in Fig. 7. Under conditions employed here 0.1 μg of any of the corticosteroids gives a well discernible plot. Comparison of Figs. 6 and 7 shows good agreement between the results obtained by TLC and HPLC as far as the qualitative analysis of the individual fractions is concerned. On the basis of our experience the most convenient approach to the evaluation of the results in displacement chromatography is first TLC analysis of the fraction to identify fractions containing the separated individual components and those which contain more than one component. Subsequently, the concentration of each product in the individual or combined fractions is determined by using HPLC.

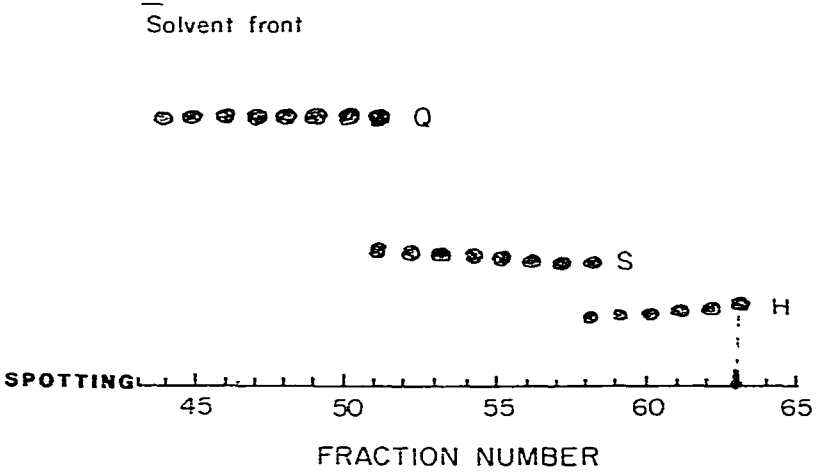


Fig. 7. TLC analysis of the fractions obtained in the displacement chromatographic separation depicted in Fig. 6.

The use of relatively high concentration of amine in the displacer solution did not impede regeneration of the column. We found that washing the column first with a mixture of acetic acid, alcohol and chloroform, then a chloroform–propanol mixture and finally with chloroform results in complete regeneration in less than 90 min. In the course of our study the "status" of the column was tested before every displacement run by using linear elution chromatography of a standard mixture as described in the experimental section. Although no particular detrimental effect of DEEDA on silica was noted, the displacer was immediately washed out from the column after each chromatographic run and after regeneration the column was filled with chloroform for storage.

CONCLUSIONS

The use of TLC for exploring optimum conditions and analyzing product fractions in displacement chromatography with HPLC columns and instrumentation greatly facilitates the exploitation of the potential of this technique for preparative scale separations. When thin-layer plates coated with the same stationary phase as that used in the column are available, scouting for appropriate carrier and displacer as well as establishing optimum displacer concentration are conveniently performed by displacement TLC.

Due to the possibility of analyzing simultaneously a large number of samples containing similar analytes, TLC is an eminently suitable technique to establish which effluent fractions contain pure product and which contain unresolved component pairs. Since the number of components to be separated by TLC is expected to be not greater than three in well-executed experiments in HPDC, TLC analysis can be performed rapidly. Of course, electrophoresis or isoelectric focussing also offer similar advantages in product analysis.

The employment of reversed-phase TLC in conventional fashion for scouting for suitable carrier and/or displacer is limited to chromatographic systems with organic rich mobile phase. On the other hand, in overpressured, *mutato nomine* "forced flow", TLC eluent flow is maintained by an external pressure gradient[33,34] that can cause even a plain aqueous eluent to migrate on a hydrophobic alkyl-silica plate. Due to the controlled conditions and relatively high speed "forced flow" TLC appears to be a particularly appropriate adjunct to displacement chromatography provided suitable instrumentation is available.

Whereas the employment of TLC greatly facilitated the selection of appropriate carrier and displacer solution, column length, sample loading, flow-rate and temperature were not optimized in the present study. We feel that such an endeavor will be more successful by taking advantage of the results of an investigation concerning the fundamentals, including the theoretical aspects, of displacement chromatography that is currently being carried out in our laboratory.

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REFERENCES

1 Cs. Horváth, A. Nahum and J. F. Frenz, *J. Chromatogr.*, 218 (1981) 365.
2 H. Kalász and Cs. Horváth, *J. Chromatogr.*, 215 (1981) 295.
3 E. Heftmann, *Chromatography of Steroids* (*Journal of Chromatography Library*, Vol. 8), Elsevier, Amsterdam, 1976.
4 N. S. Lamontagne and D. F. Johnson, *J. Chromatogr.*, 53 (1970) 225.
5 N. S. Lamontagne and D. F. Johnson, *Steroids*, 17 (1971) 365.
6 R. Noiret and R. Davis, *Anal. Biol. Clin. (Paris)*, 21 (1963) 373.
7 A. Castro, D. Bartos, B. Jelen and M. Kutas, *Steroids*, 22 (1973) 851.
8 T. Seki and K. Matsumoto, *J. Chromatogr.*, 27 (1967) 423.
9 B. H. Shapiro and F. G. Péron, *J. Chromatogr.*, 65 (1972) 568.
10 V. Cejka and E. M. Venneman, *Clin. Chim. Acta*, 11 (1965) 188.
11 J. Q. Rose and W. J. Jusko, *J. Chromatogr.*, 162 (1979) 273.
12 N. A. Parrish, *J. Chromatogr. Sci.*, 12 (1974) 753.
13 C. Hesse, K. Pietrizik and D. Hotzel, *Z. Klin. Chem. Klin. Biochem.*, 12 (1974) 193.
14 F. K. Trefz, D. J. Byrd and W. Kochen, *J. Chromatogr.*, 107 (1975) 181.
15 M. Schöneshöffer and H. J. Dulce, *J. Chromatogr.*, 164 (1979) 17.
16 N. R. Scott and P. F. Dixon, *J. Chromatogr.*, 164 (1979) 29.
17 M. Schöneshöffer, R. Skobolo and H. J. Dulce, *J. Chromatogr.*, 222 (1981) 478.
18 S. Hara and M. Nakahata, *J. Liquid Chromatogr.*, 1 (1978) 43.
19 S. Hara, *J. Chromatogr.*, 137 (1977) 41.
20 S. Görög and G. Hajós, *J. Chromatogr.*, 43 (1969) 541.

21 B. Matkovics and G. Göndös, *Mikrochim. J.*, 13 (1968) 171.
22 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
23 P. Pei, S. Ramachandran and R. S. Henry, *Amer. Lab.*, 7 (1975) 37.
24 E. Godbille and P. Devaux, *J. Chromatogr.*, 122 (1976) 317.
25 A. Tiselius, *Ark.-Kemi., Minerol. Geol.*, 16A (1943) 1.
26 A. Tiselius and L. Hagdahl, *Acta Chem. Scand.*, 3 (1950) 394.
27 J. Porath, *Acta Chem. Scand.*, 6 (1952) 1237.
28 J. Porath and C. H. Li, *Biochim. Biophys. Acta*, 13 (1954) 268.
29 C. H. Li, A. Tiselius and K. O. Pedersen, *J. Biol. Chem.*, 190 (1951) 317.
30 S. Claeson, *Ark.-Kemi, Mineral. Geol.*, 23A (1947) 1.
31 F. Helfferich and G. Klein, *Multicomponent Chromatography*, Marcel Dekker, New York, 1970, pp. 225–254.
32 E. Godbille and P. Devaux, *J. Chromatogr. Sci.*, 12 (1974) 564.
33 E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.*, 174 (1979) 75.
34 E. Mincsovics, E. Tyihák and H. Kalász, *J. Chromatogr.*, 191 (1980) 293.