Journal of Chromatography, 137 (1977) 41–52

CHROM. 9976

USE OF THIN-LAYER CHROMATOGRAPHIC SYSTEMS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS

PROCEDURE FOR SYSTEMATIZATION AND DESIGN OF THE SEPARATION PROCESS IN SYNTHETIC CHEMISTRY*

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(First received June 14th, 1976; revised manuscript received January 31st, 1977)

SUMMARY

A procedure is described for the simple scale-up of thin-layer chromatographic (TLC) systems to high-performance liquid chromatography (HPLC), which was developed in order to improve the separations involved in the work-up of synthetic reaction mixtures. The semi-quantitative compositional evaluation of the reaction products was performed by TLC, employing specially developed solvent systems. The retention of solutes was controlled by using TLC as a pilot technique, based on the correlation between TLC and HPLC mobilities. HPLC achieved complete removal of the reaction medium and reagents in addition to isolating the required products. According to the scheme described, single-step isolation and purification of synthetic products was accomplished without the need for any pre-treatment of the sample.

INTRODUCTION

Various techniques, such as thin-layer chromatography (TLC), gas chromatography, ultraviolet absorption spectroscopy and nuclear magnetic resonance spectroscopy, have been employed to trace the course of chemical reactions. TLC has been the most widely utilized because of its speed, its applicability to a wide range of compound types, from non-polar hydrocarbons to polar inorganic salts[1], and its ability to give detailed information on the component distribution in a crude reaction mixture.

The constituents of a crude synthetic reaction mixture, *i.e.*, substrates, reagents and products, are often routinely determined semi-quantitatively by TLC, and this procedure readily provides a means for the optimization of the reaction conditions. However, subsequent processes, including separation and isolation of the compounds of interest, generally involve time-consuming, tedious operations. The solvent is

* Presented at the *11th International Symposium on Advances in Chromatography, Houston, Texas, November 1–5, 1976*; the majority of the papers presented at this symposium has been published in *J. Chromatogr.*, Vol. 126 (1976).

generally removed by evaporation, subsequent solvent extraction transfers inorganic reagents into the aqueous layer and finally vacuum distillation, recrystallization, sublimation and/or other procedures are used to isolate a pure product.

Such classical treatment requires modernization if substantial improvements in ease of operation and resolution are to be achieved. The thin-layer chromatogram can be used to develop a high-performance liquid chromatographic (HPLC) separation that will enable one to obtain the desired reaction products while removing the reaction medium and reagents in a single operation. This paper presents a simple and efficient approach to the development of a laboratory-scale method for the separation and subsequent isolation of synthetic reaction products.

Diverse complex mixtures are encountered in modern synthetic research. In order to establish criteria for the classification of reaction products, a series of synthetic reactions can be considered as a model. The sequential reactions shown in Fig. 1 were utilized by Hara and co-workers[2–6] in the total synthesis of salamander alkaloids. All synthetic steps were monitored by TLC so as to provide a check on product composition and to help optimize the reaction conditions. Liquid chromatography was also needed in order to isolate and help in the characterization of intermediates, this information being required for the determination of subsequent synthetic pathways. Some of the thin-layer chromatograms are illustrated in Fig. 1.

It should be noted that, although the chromatographic patterns of the crude reaction mixtures are relatively simple, the actual procedures for product isolation are complicated, and that a single operation involving HPLC elution for the isolation of the compounds of interest would appear to be desirable.

Non-polar organic solvents are readily eluted from a silica column without any retention, and polar solvents are retained longer than the organic product fraction; however, they can be eluted from the column by employing a stepwise solvent gradient. Most inorganic reagents are strongly retained on a silica column and are therefore removed with a silica pre-column connected ahead of the main column. On the other hand, if a reversed-phase packing material, *e.g.*, ODS or C-18, is employed, polar solvents and inorganic reagents are eluted rapidly from the column and are easily removed.

Complete removal of an associating solvent such as dimethyl sulphoxide, dimethylformamide, higher alcohols, pyridine and acetic acid from a reaction mixture often presents a severe problem to the synthetic chemist. However, HPLC fractionation can readily solve this problem.

EXPERIMENTAL

TLC procedure

The adsorbent was standard TLC-grade silica (Wakogel B; Wako, Osaka, Japan) with an average pore size of 60 Å. TLC was carried out by the usual ascending procedures described previously[7].

HPLC procedure

The adsorbent was irregularly shaped, totally porous silica (Wakogel LC-10) with a mean particle size of 10 μm, of the same quality as the standard TLC adsorbent. Silica was deactivated by equilibration with ambient moisture.

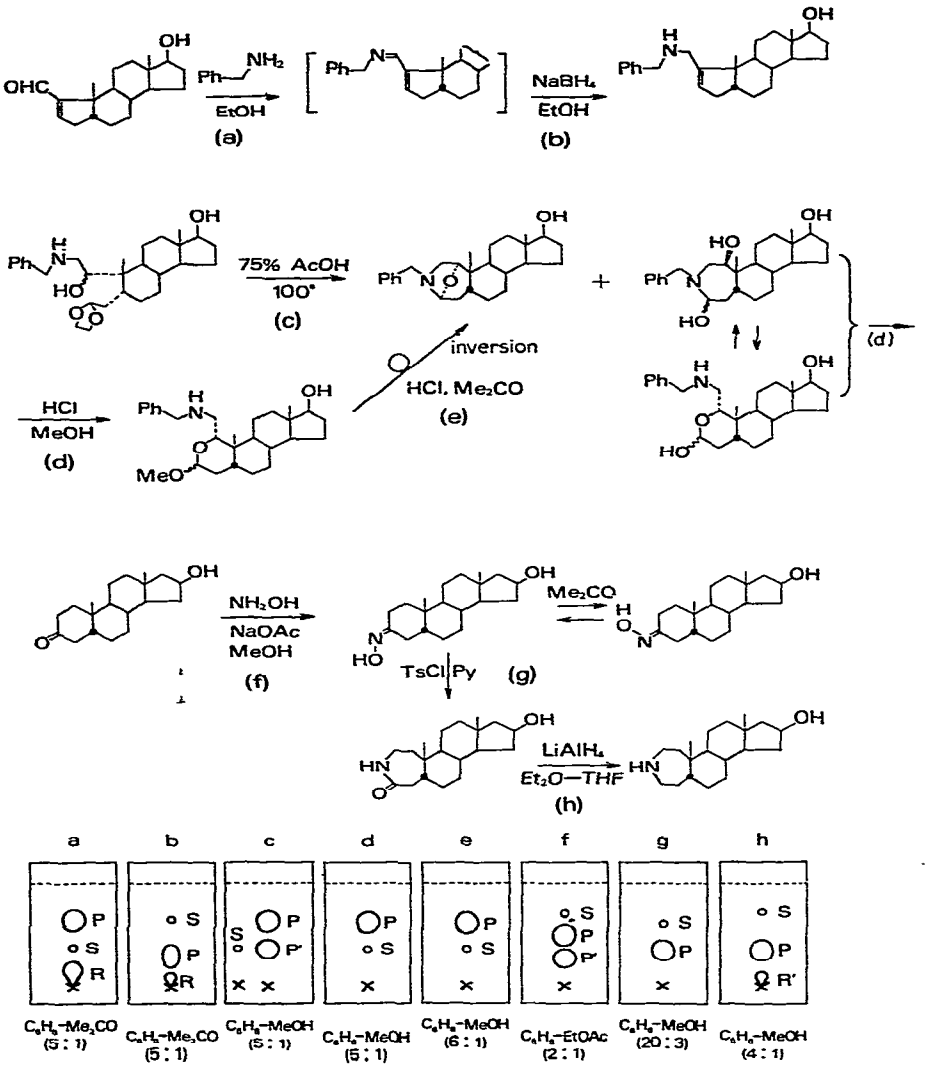

Fig. 1. Examples of thin-layer chromatograms in synthetic studies of salamander alkaloids. S = Substrate; P, P′ = products; R = reagent.

Columns and apparatus

A new column system, shown in Fig. 2, was developed for the isolation of pure products from a crude reaction mixture. A conical-inlet glass (CIG) column, having a fan-shaped inlet* that was adopted for the effective introduction of the sample into a large-diameter column, was used. Various features of this system can be summarized as follows:

(i) A large number of theoretical plates was obtained, possibly owing to the

* A silica pre-packed disposable glass column and a stainless-steel column, having similarly shaped inlets, have been developed by E. Merck and Varian Aerograph, respectively.

smooth inside wall of the CIG column and its inlet shape. The number of theoretical plates (N) for 4-, 5- and 8-mm I.D. silica dry-packed columns was 1700 per 30 cm using 5α-cholestan-3-one as the solute and n-hexane–ethyl acetate (20:1, v/v) as the solvent.

(ii) A PTFE plug was designed to fit CIG columns of various sizes. The procedure for fitting and removing the column plug with a metal clip is extremely simple.

(iii) The upper pressure limit of the CIG column is sufficiently high for our preparative programme. Columns of length (excluding conical inlet parts) 30 cm and I.D. 4, 5, 8, 15 and 30 mm were prepared. For example, 4- and 8-mm I.D. columns were used with pressures up to 50 and 30 kg/cm^2, respectively.

(iv) A pre-column can be connected directly to the main column. The preferred length of the pre-column was 5 cm.

More detailed data for the CIG column system will be reported elsewhere.

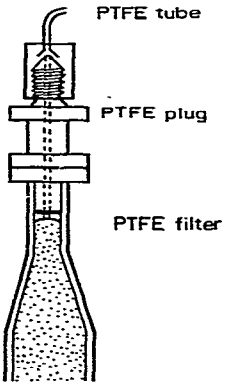

Fig. 2. CIG column.

HPLC was performed using a reciprocating piston pump (SF-0396, Milton Roy, Philadelphia, Pa., U.S.A.), an RI detector (RI-401, Waters Assoc., Milford, Mass., U.S.A.), a recorder (DR-1S, Ohkura Electric Co., Tokyo, Japan) and 10-, 100- and 500-μl microsyringes (Kusano Scientific Co., Tokyo, Japan). The experiments were carried out at room temperature (15 $\pm$ 5°).

Examples of applications

(a) 5α-Cholestan-3β-ol (366 mg) and p-toluenesulphonyl chloride (1.3 g) were dissolved in 3 ml of pyridine and the mixture was allowed to stand overnight at room temperature. The crude reaction mixture was injected into the silica column. Evaporation of the solvent from the tosylate fraction gave 500 mg of crystalline product, which was identified with an authentic sample by measuring the infrared absorption.

(b) α-Phenylpropionic acid (288 mg) and dicyclohexylcarbodiimide (DCC) (198 mg) were dissolved in 3.0 ml of tetrahydrofuran (THF), then cholesterol (389 mg) and triethylamine (97 mg in 2.0 ml of THF) were added. The crude reaction mixture was allowed to stand overnight and then injected directly into the silica column. A crystalline product (370 mg) which was identified with an authentic sample was obtained.

(c) Dihydrocholesteryl acetate, which was obtained from 550 mg of cholesteryl acetate by catalytic hydrogenation, was hydrolyzed with sodium hydroxide in methanol–water[8]. The reaction mixture was pumped through the reversed-phase column in methanol–water as the mobile phase. The residual dihydrocholesterol was injected into the silica column, and 5β- and 5α-cholestanol (55 and 430 mg, respectively) were isolated.

RESULTS AND DISCUSSION

Design of a solvent system

The mobile phases employed in liquid chromatography are often selected by trial and error in a non-systematic manner. Current schemes for the mechanism of liquid–solid chromatography (LSC) stress the contribution of hydrogen bonding provided by the solvent as an acceptor or a donor[9–11]. Based on this consideration, a new classification of a solvent system for LSC has been attempted.

Solvents with non-bonded electron-pair donors, including hetero atoms such as nitrogen and oxygen, and solvents with proton donors have been classified as "class B" and "class AB", respectively[12]. Solvents that do not take part in hydrogen bond formation are now designated as "class O", which is further subdivided into three categories, *viz.*, O (aliphatic hydrocarbons), P (aromatic hydrocarbons) and N (haloalkanes). For preparative work, the solvents used are usually limited to commonly available volatile substances, which can be listed as follows:

"class O": hydrocarbons, halides —*n*-hexane (O-type), benzene (P-type), methylene chloride, chloroform (N-type);

"class B": *n*-donors (base) —diethyl ether, ethyl acetate, acetone;

"class AB": H-donors (acid) —methanol, ethanol, 2-propanol, acetic acid.

For moderately polar compounds, binary solvent systems and silica as the adsorbent[13] have usually been preferred, as in the examples of the synthetic reactions shown in Fig. 1. Depending on the character of the solute, a binary solvent system can be readily formulated by choosing a pair of solvents, *i.e.*, O + O, O + B, O + AB, B + B or B + AB.

Solvent combinations. To find a suitable solvent, consideration of the solvent selectivity is necessary[9,10,14] and for this purpose the relationship between mobility and solvent system was examined by using several steroid compounds. R_m [$= \log(1/R_F - 1)$] values for pairs of compounds that differ only by the presence of a characteristic functional group were obtained by employing TLC R_F values[15]. ΔR_m values for particular functional groups using various binary solvents were calculated according to Martin's additive rule. The results are illustrated in Fig. 3.

Even though the non-polar component was changed, similar ΔR_m values were obtained if the polar component remained the same. The selectivity of the solvent system is controlled by the more polar solvent.

Ethyl acetate (class B) systems afforded comparatively small ΔR_m values for carbonyl and hydroxyl groups, especially for the acidic phenolic hydroxyl group. Acetone (class B) systems gave smaller ΔR_m values than others, particularly for the keto group. Methanol (class AB) systems afforded large positive ΔR_m values for the phenolic hydroxyl group, and affected the alcoholic hydroxyl ΔR_m values in a non-systematic manner.

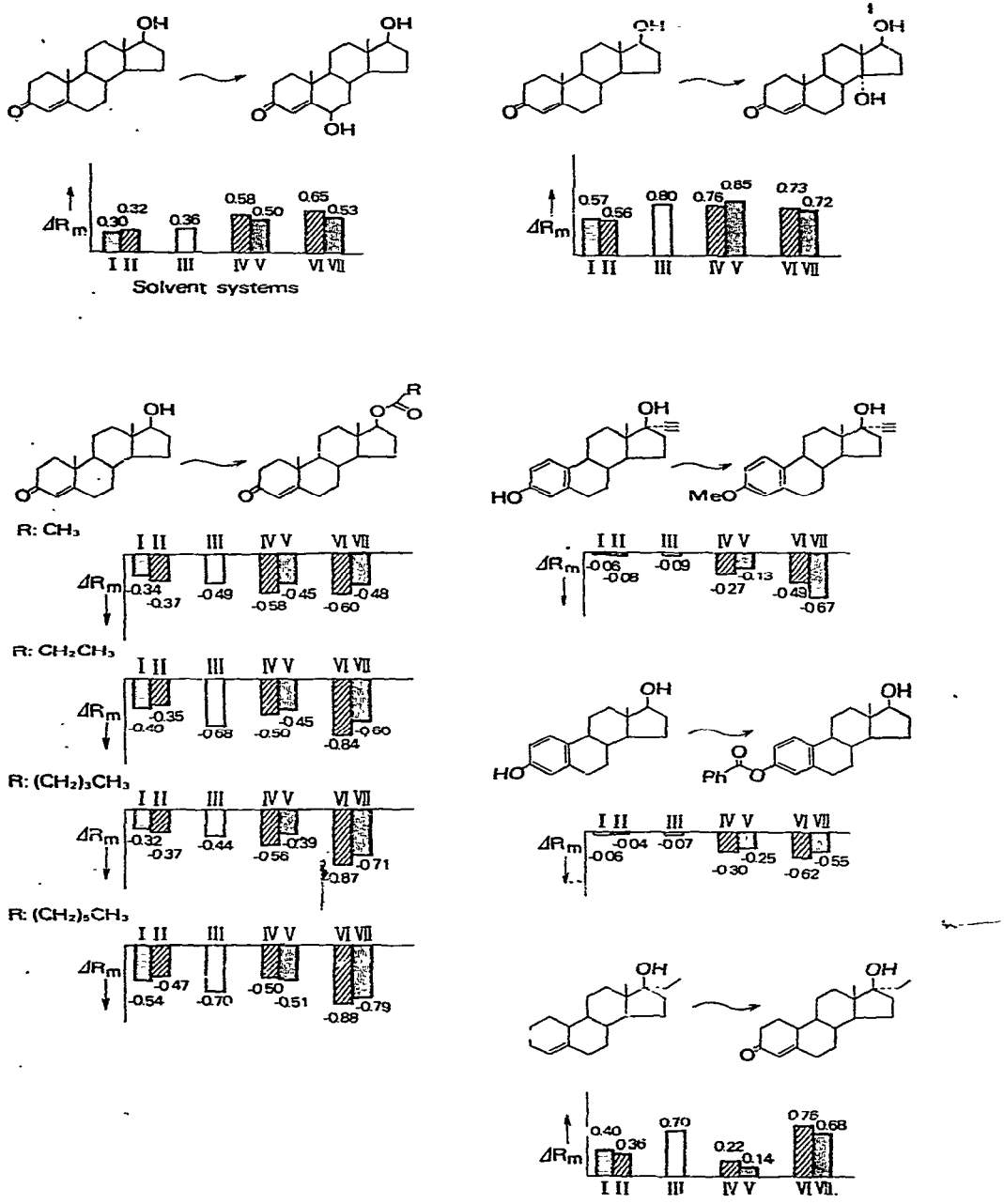

Fig. 3. ΔR_m values for functional groups of steroids in several solvent systems. The values represent the arithmetic means of results from 10 runs[15]. Solvent systems: I = *n*-hexane–ethyl acetate (2:8, v/v); II = benzene–ethyl acetate (3:7, v/v); III = diethyl ether; IV = benzene–acetone (4:1, v/v); V = chloroform–acetone (3:1, v/v); VI = benzene–methanol (9:1, v/v); VII = chloroform–methanol (97:3, v/v).

These observations indicate that a "strong" solvent specifically affects the ΔR_m values of a solute with a functional group which is similar to that contained in the "strong" solvent. Of course, this conclusion is based upon the limited material presented here; additional data will be reported in a later paper.

Solvent composition. With binary solvent systems, the solvent strength for a given solute can be optimized by adjusting the strong solvent composition. Soczewiński[11] described an effect of strong solvent composition on retention in LSC, which can be expressed by

$$R_m = \text{constant} - n \log N_B \quad (1)$$

where N_B denotes the molar fraction of polar component B and $n > 1$. Eqn. 1 was supported by TLC data for some phenols and amines[16–18]. To confirm this relationship, the effect of solvent composition on the retention of some steroids was examined by employing binary solvent HPLC*, because accurate measurements of the mobility require HPLC rather than TLC[10].

Data obtained with mono- and difunctional steroids and two types of solvent systems, O + B and O + AB, are illustrated in Fig. 4. Here the log k' values in HPLC are analogous to the R_m values in TLC. The actual correlations between mobilities in TLC and HPLC will be discussed later.

In the graphs of log k' *versus* N_B, the slope (n) increased from acyloxy to keto groups and then to hydroxyl groups in steroids from mono- to difunctional compounds. For a particular solute, the slope decreased from the O + B type of solvent system to the O + AB type.

The intercept on the abscissa, corresponding to the constant in eqn. 1, increased from acyloxyl to keto and then hydroxyl groups and from mono- to difunctional steroids, and decreased from the O + B type of solvent system to the O + AB type.

There is an additive tendency in the slopes and intercepts for difunctional compounds relative to the values for monofunctional compounds. Therefore, an appropriate binary solvent composition for analogous compounds with similar functional groups can be predicted.

Correlation between TLC and HPLC mobilities

The use of TLC data to provide an insight into HPLC has often been suggested[21–25], but no simple and reliable relationship has been reported. Some characteristic phenomena that have been observed in TLC are the volume of mobile phase, which varies with respect to the height of the thin-layer plate, pre-adsorption of solvent vapour and solvent demixing[26]. Considering these effects, extensive investigations finally led to rather simple conclusions[15]. A simple relationship has also been observed by Soczewiński and Gołkiewicz[27].

At first, the TLC R_F values and HPLC mobilities (R) of some steroids were directly compared by applying the same binary solvents as mobile phases. Irregularly shaped, totally porous silica, with properties similar to those of TLC-grade silica,

* A similar relationship has been observed for some binary solvent systems in HPLC by Scott and Kucera[19] and Gołkiewicz[20].

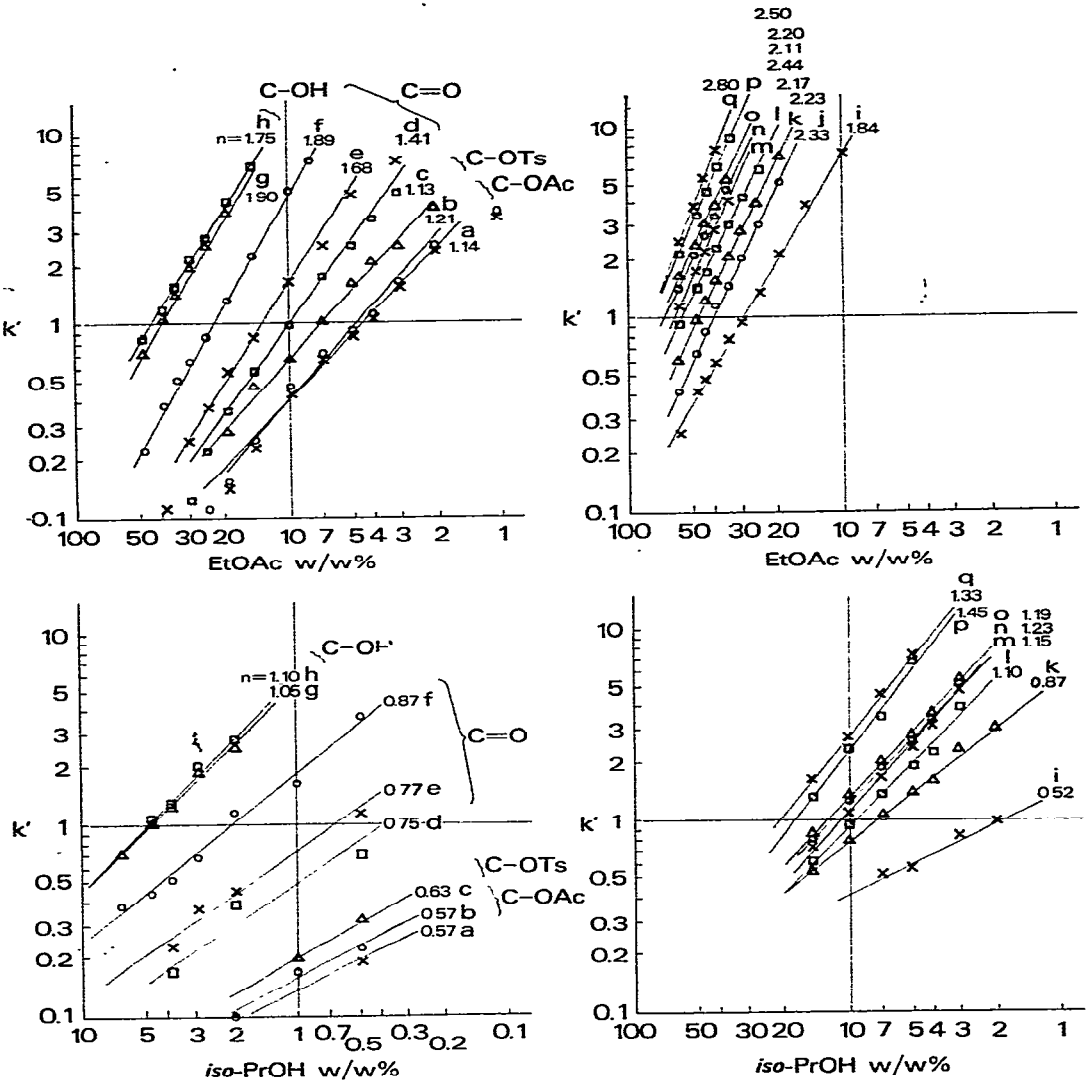

Fig. 4. Correlation between capacity factor and polar solvent composition using a silica column. Packing: particle size 10 μm, 4.4 g. CIG column, 300 × 5 mm I.D. Solvent systems: *n*-hexane–ethyl acetate and *n*-hexane–2-propanol. Flow-rate: 1 ml/min. Void volume, V_0: 6.5 ml (cyclohexane). Samples: 0.1 mg in 5 μl of dichloromethane. Steroids: a = 3β-acetoxy-5α-cholestane; b = 3β-acetoxy-5-cholestene; c = 3β-tosyloxy-5-cholestene; d = 5β-cholestan-3-one; e = 5α-cholestan-3-one; f = 4-cholesten-3-one; g = 5α-cholestan-3β-ol; h = 5-cholesten-3β-ol; i = 3β-acetoxy-5α-androstan-17-one; j = 3-hydroxy-1,3,5(10)-estratrien-17-one; k = 17β-acetoxy-4-androsten-3-one; l = 17β-hydroxy-17α-methyl-5α-androstan-3-one; m = 17β-hydroxy-5α-androstan-3-one; n = 3β-hydroxy-5-androsten-17-one; o = 3β-hydroxy-5α-androstan-17-one; p = 17β-hydroxy-4-androsten-3-one; q = 17β-hydroxy-19-nor-4-androsten-3-one.

was packed by a mechanical dry-tapping procedure. The results in Table I indicate that the R/R_F ratio fell into the range 1.18–1.81 with an average of *ca.* 1.5, except for the methanol-containing solvent (system VII), which gave extremely high values. In

TABLE I

R/R_F RATIO OBSERVED IN TLC AND HPLC OF STEROIDS BY EMPLOYING BINARY SOLVENTS

The R/R_F values were obtained by using as steroid samples 17β-hydroxy-4-estren-3-one, 17β-hydroxy-17α-methyl-4-estren-3-one, 17β-hydroxy-17α-ethyl-4-estren-3-one, 17β-hydroxy-17α-ethynyl-4-estren-3-one and 17β-hydroxy-4-androsten-3-one.

No.	*Solvent system*	R/R_F
I	*n*-Hexane–ethyl acetate (2:8, v/v)	1.27, 1.27, 1.28, 1.30, 1.43
II	Benzene–ethyl acetate (3:7, v/v)	1.46, 1.50, 1.51, 1.55, 1.58
III	Diethyl ether	1.18, 1.44, 1.50, 1.51, 1.53
IV	Benzene–acetone (4:1, v/v)	1.29, 1.47, 1.51, 1.53, 1.55
V	Chloroform–acetone (3:1, v/v)	1.46, 1.65, 1.66, 1.76, 1.81
VII	Chloroform–methanol (97:3, v/v)	2.60, 2.66, 3.00, 3.03, 3.25

this instance, the R/R_F ratio can be adjusted when the methanol content in HPLC eluents is decreased[15].

These results and other relevant data can be summarized as follows:

(i) with O + O or O + B class binary solvents,

$$R_F \text{ (TLC)} \times 1.5 \approx R \text{ (HPLC)}$$

$$R \text{ (mobility)} = \frac{1}{k' + 1}$$

where k' = capacity factor;

(ii) with O + AB class binary solvents, the AB (*e.g.*, methanol) proportion in TLC should be decreased from 1/5 to 1/10 on transfer to HPLC systems.

In the first relationship, the approximate coefficient 1.5 can be interpreted as the ratio of the volumes of mobile and stationary phases within a column or thin-layer bed. This ratio seems to be derived from the difference in the mobile phase profile on the stationary phase between the TLC and HPLC systems[22].

The decrease in the proportion of the AB solvent from 1/5 to 1/10 on transfer to HPLC systems signifies the ratio of the strong component in the HPLC wet column eluent to the original TLC solvent in the chamber; this is so because AB solvent on the thin-layer plate should be pre-adsorbed if de-mixing occurs.

Although the simple scaling procedure described above seems to be an extremely rough approximation, it shows unusually good agreement with several independent data, as follows:

	R_F*	k'**	$k'_{calc.}$**
(i) *Solute*			
Cholesteryl benzoate	0.36	0.82	0.85
Cholesteryl phenylacetate	0.21	2.06	2.17
(ii) *Solute*	R_F***	k'§	
15-Epiprostaglandin $F_{2\alpha}$	0.38	3.0	
Prostaglandin $F_{2\alpha}$	0.22	6.6	

* Waters Assoc. data given by Prep LC/System 500. Solvent: benzene–hexane (1:1).

** $k'_{calc.} = \dfrac{1}{R_F \cdot 1.5} - 1.$

*** Solvent: ethyl acetate–acetic acid (98:2).

§ Solvent: ethyl acetate–acetic acid (98.8:0.2). Data were provided by Waters Assoc., brochure AN 146.

TABLE II

RETENTION DATA GIVEN BY SYNTHETIC REACTION (a)

Solute	*n-Hexane–ethyl acetate (4:1)*			*n-Hexane–ethyl acetate (20:1) : HPLC*	
	TLC		*HPLC: k'_{found}*	$k'_{pred.}$*	k'_{found}
	$R_{F\ pred.}$*	$R_{F\ found}$			
Tosylate	0.57	0.68		1.60	1.45
Pyridine			2.68		
Cholestanol	0.18	0.23		ca. 60	—

* Predicted values from the data for cholesterol derivatives.

Examples of applications

The scheme described above was applied to several synthetic reactions, as described under Experimental.

(a) 5α-Cholestan-3β-ol + TsCl $\xrightarrow[C_5H_5N]{}$ Tosylate + TsOH

5α-Cholestan-3β-ol as a substrate and its tosylate as a product were characterized on the basis of their structures as monohydroxy and monoacyloxy derivatives of cholestane, respectively. When the silica *n*-hexane–ethyl acetate system is chosen, the correct ethyl acetate composition can be found directly by referring to Fig. 4. Optimization of the TLC solvent was then easily accomplished. The observed R_F values are shown in Table II.

The eluotropic behaviour of pyridine as the solvent was examined by using a silica HPLC column. Pyridine was eluted from the column long after the tosylate.

The crude reaction mixture was injected directly on to the silica column fitted with a short pre-column for retaining *p*-toluenesulphonic acid, which was derived from tosyl chloride. The tosylate fraction was collected with the aid of a differential refractometer (RI detector). Unreacted cholestanol and pyridine were removed by stepwise elution.

(b) Cholesterol + α-Phenylpropionic acid + DCC $\xrightarrow[Et_3N,\ THF]{}$ Propionate + + Dicyclohexylurea

TLC binary solvent systems were formulated and utilized in pilot separations, making an initial guess concerning the chromatographic behaviour of the expected products in this reaction mixture. Some of the results are shown in Table III. The O + O system was preferred for HPLC isolation of the phenylpropionate fraction because there was no overlapping with the DCC peak. In contrast, the O + B system did result in some overlapping.

The propionate fraction was collected and DCC and phenylpropionic acid were removed by stepwise elution. Cholesterol and dicyclohexylurea remained on the pre-column.

(c) Cholesteryl acetate $\xrightarrow[HOAc]{H_2/Pt}$ Dihydrocholesteryl acetate (5α- and 5β-)

$\xrightarrow[H_2O]{NaOH}$ Dihydrocholesterol (5α- and 5β-)

TABLE III

RETENTION DATA GIVEN BY SYNTHETIC REACTION (b)

Solute	*TLC R_F value*		*HPLC k′ value*	
	O + B: n-hexane–ethyl acetate (5:1)	*O + O: n-hexane–benzene (1:1)*	*O + O: n-hexane–benzene (1:1)*	
			$k'_{calc.}$	k'_{found}
Cholesteryl phenylpropionate	0.56	0.44	0.51	0.70
DCC	0.54	0.29 (t*)		
Phenylpropionic acid	0.28 (t*)	0.21 (t*)		
Cholesterol	0.15	0.05		
Dicyclohexylurea	0	0		

* Tailing.

TABLE IV

RETENTION DATA GIVEN BY SYNTHETIC REACTION (c)

Solute	*n-Hexane–ethyl acetate (4:1)*		
	TLC R_F value	*HPLC*	
		$k_{calc.}$	k_{found}
5β-Cholestan-3β-ol	0.35	0.91	0.94
5α-Cholestan-3β-ol	0.27	1.47	1.71

Dihydrocholesteryl acetate, which was obtained from cholesteryl acetate by catalytic reduction, was hydrolyzed in the presence of alkali.

The eluotropic behaviour of the pair of stereoisomeric dihydrocholesterols was then studied by TLC and the retention data are shown in Table IV. When a silica column was used, both stereoisomers were quantitatively separated.

According to the scheme described above, single-step isolation and purification of the synthetic products without any pre-treatment was effectively accomplished.

ACKNOWLEDGEMENTS

I thank Prof. Shigenobu Okuda of Tokyo University, Dr. Jack Cazes and Dr. James A. Prendergast of Waters Associates for their helpful suggestions, Dr. Kitaro Oka of this laboratory for his cordial support, Misses Y. Fujii, N. Shimura, K. Ito, M. Yanagawa, M. Hirasawa, S. Hamano and K. Kawabata of this college for their experimental cooperation, and Mr. Sakae Kusano, Kusano Scientific Co., for his technical assistance.

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