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SOLVENT OPTIMIZATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR POLAR ADRENAL STEROIDS USING COMPUTER-PREDICTED RETENTIONS

G. D'AGOSTINO

Institute of Biochemistry, Faculty of Medicine, Policlinico Via Del Vespro, 90127 Palermo (Italy)

F. MITCHELL

Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Sutton, Surrey, SM2 5PX (U.K.)

L. CASTAGNETTA

Institute of Biochemistry, Faculty of Medicine, Policlinico Via del Vespro, 90127 Palermo (Italy)

and

M.J. O'HARE*

Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Sutton, Surrey, SM2 5PX (U.K.)

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SUMMARY

Computer-aided optimization of a mobile phase has been applied to the isocratic reversed-phase separation of ten polar adrenocortical steroids, including aldosterone and reduced metabolites of cortisol and cortisone. A method based on a seven-step procedure for calculation of the Chromatographic Optimization Function (COF) has been used. Logarithmically transformed retention indices were used for computing multiple polynomial regressions for the retention times of compounds as a function of solvent composition, with the resultant COF as a dependent parameter used for selection of the better mobile phase. Peak cross-overs and overlaps are accommodated in this method and the maximum acceptable analysis time factor is incorporated as well as weighting factors for priority separations. The utility of this procedure for complex mixtures of closely eluting compounds is discussed with respect to the Overlapping Resolution Map method and with the COF method of Glajch and Kirkland as used for automated optimization. Its application to aldosterone-containing samples from human adrenocortical tumours is illustrated.

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TABLE I

SYSTEMIC NAMES, TRIVIAL NAMES AND ABBREVIATIONS OF STEROIDS USED

No.	Abbreviation	Trivial name	Systematic name
1	17-isoAldo	Iso-aldosterone	11β,21-Dihydroxy-18-al-(17α)-4-pregnene-3,20-dione
2	18OH-A	18-Hydroxy-11-dehydrocorticosterone	18,21-Dihydroxy-4-pregnene-3,11,20-trione
3	Aldo	Aldosterone	11β,21-Dihydroxy-18-al-4-pregnene-3,20-dione
4	20α-DHE	20α-Dihydrocortisone	17α,20α,21-Trihydroxy-4-pregnene-3,11-dione
5	20α-DHF	20α-Dihydrocortisol	11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one
6	20β-DHE	20β-Dihydrocortisone	17α,20β,21-Trihydroxy-4-pregnene-3,11-dione
7	18OH-B	18-Hydroxycorticosterone	11β,18,21-Trihydroxy-4-pregnene-3,20-dione
8	E	Cortisone	17α,21-Dihydroxy-4-pregnene-3,11,20-trione
9	20β-DHF	20β-Dihydrocortisol	11β,17α,20β,21-Tetrahydroxy-4-pregnen-3-one
10	F	Cortisol	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione

INTRODUCTION

The complete separation of complex naturally occurring mixtures of steroid hormones poses several problems due to the wide range of polarities encountered, and their tendency to cluster in groups of similar polarity, composed of steroids generated by a number of alternative metabolic pathways. An example in point is aldosterone and its congeners, including various 18-hydroxylated steroids. The separation of these using both reversed-phase and normal-phase high-performance liquid chromatography (HPLC) with binary solvent systems has been described [1]. The application of these systems to biological samples containing small quantities of aldosterone has, however, revealed a further requirement to separate aldosterone and 18OH-B from several UV-absorbing metabolites of cortisol and cortisone, a requirement that is not satisfied by the binary solvent systems.

In our original study of steroid hormone separation by HPLC we used gradient elution with binary solvent systems to optimize the separation of typical steroid mixtures corresponding to adrenal and testicular hormones. A dioxane—water gradient was, for example, selected for resolving polar adrenal steroids including mineralocorticoids such as aldosterone [2]. It became apparent from further studies that individual ODS-type packings with different levels of residual accessible silanol groups could show considerable specific selectivities for steroids [3]. The selectivity patterns of a number of such packings have been documented [4] and selected non-maximum coverage packings have been utilized in the separation of certain complex steroid metabolite mixtures [5].

Standardization of bonded-phase technology [6] has, however, increasingly limited the opportunity to exploit mixed mode chromatography for difficult separations. As an alternative strategy we have, therefore, now explored the use of computerized optimization of three and four solvent mobile phases. The general value of such systems for complex separations has long been appreciated [7,8], and systematic statistical procedures for mobile-phase optimization have recently been developed. In this study the general methods described by Glajch and co-workers [9,10] have been modified to take into account the behaviour of the steroids encountered in our biological samples which includes cross-overs using different binary solvents. A satisfactorily optimized mobile phase has been defined using the modified procedure, and its use is illustrated by application to aldosterone-containing samples from human adrenocortical tumours.

MATERIALS AND METHODS

Steroid standards were obtained from Steraloids (Croydon, U.K.), Ikapharm (Ramat-Gan, Israel) and the Medical Research Council Steroid Reference Collection (by courtesy of Professor D. Kirk). Their systematic and trivial names, together with the abbreviations used in this study are given in Table I. Samples of human adrenocortical tumours were obtained at surgery. Freshly disaggregated cell suspensions prepared therefrom were incubated in tissue culture medium (10^6 cells per ml) and the supernatants were stored at −20°C. When

required, aliquots were thawed and extracted as described previously [4] except that ethyl acetate rather than dichloromethane was used, because of the polarities of the compounds involved. For identification of cortisol metabolites, fresh cell suspensions were incubated with [^{3}H-1,2,6,7]cortisol (5 μCi, special activity 90 Ci/mmol, Amersham International, U.K.) for 24 h.

Separations were carried out isocratically on 150 × 5 mm I.D. or 250 × 5 mm I.D. stainless-steel columns slurry packed with ODS-Hypersil (Shandon Southern, Runcorn, U.K.). Chromatographic conditions were controlled using a Spectraphysics SP8000 chromatograph and steroids were eluted at a solvent flow-rate of 1 ml/min at 45°C and detected with a Schoeffel FS770 variable-wavelength spectrophotometer at 240 nm. Organic solvents were obtained from Rathburn Chemicals (Walkerburn, U.K.) and single glass-distilled water was prepared from Milli-Q low-conductivity feedstock. Computations were carried out with an 8K microcomputer (Commodore PET series 2001).

RESULTS AND DISCUSSION

In a preliminary study we examined the feasibility of separating all the steroids in Table I using reversed-phase HPLC with various binary mobile phases. Their retention times were established with four such systems based on different organic solvents compatible with UV detection of 4-en-3-one steroids (240 nm). A maximum-coverage end-capped C_{18}-type packing (ODS-Hypersil) was used. The binary mixtures consisted respectively of 35% methanol, 20% dioxane, 20% acetonitrile, and 12% tetrahydrofuran in water. These organic modifier concentrations were selected because they were of approximately equal solvent strengths in respect of the range of compounds studied giving retention times of 19 ± 1 min for the first steroid eluted, 17-iso-aldosterone using a 25-cm column (Fig. 1). Under the isocratic conditions used this solvent strength was empirically deemed the best practical compromise between resolution and analysis time, and did not significantly impair the accuracy or sensitivity of determinations of cortisol, the least polar compound under investigation, and a major component of adrenal tissue samples. However, none of these binary systems afforded a satisfactory separation of aldosterone and 18OH-B and all of the other polar steroids. Examples of incompletely resolved or co-chromatographing compounds were observed in each case (Fig. 1).

The primary requirement was to separate and measure aldosterone without interference from other, unrelated, steroids (Table I) together with the resolution of the 18-hydroxysteroid congeners of aldosterone, 18OH-B and 18OH-A. These objectives, and the cross-overs noted on the different binary systems (Fig. 1), dictated the approach that was taken to computer-aided optimization of the mobile phase.

Most of the strategies for optimization of solvent composition in HPLC are based on a formula [11] which defines the three independent factors that affect resolution, viz. selectivity, efficiency and retention:

$$R_s = 1/4\,(\alpha-1)\cdot\sqrt{N}\cdot k'/(k'+1) \qquad (1)$$

where R_s = resolution factor, N = plate number, k' = solute capacity factor, and α = selectivity factor (k_2/k_1).

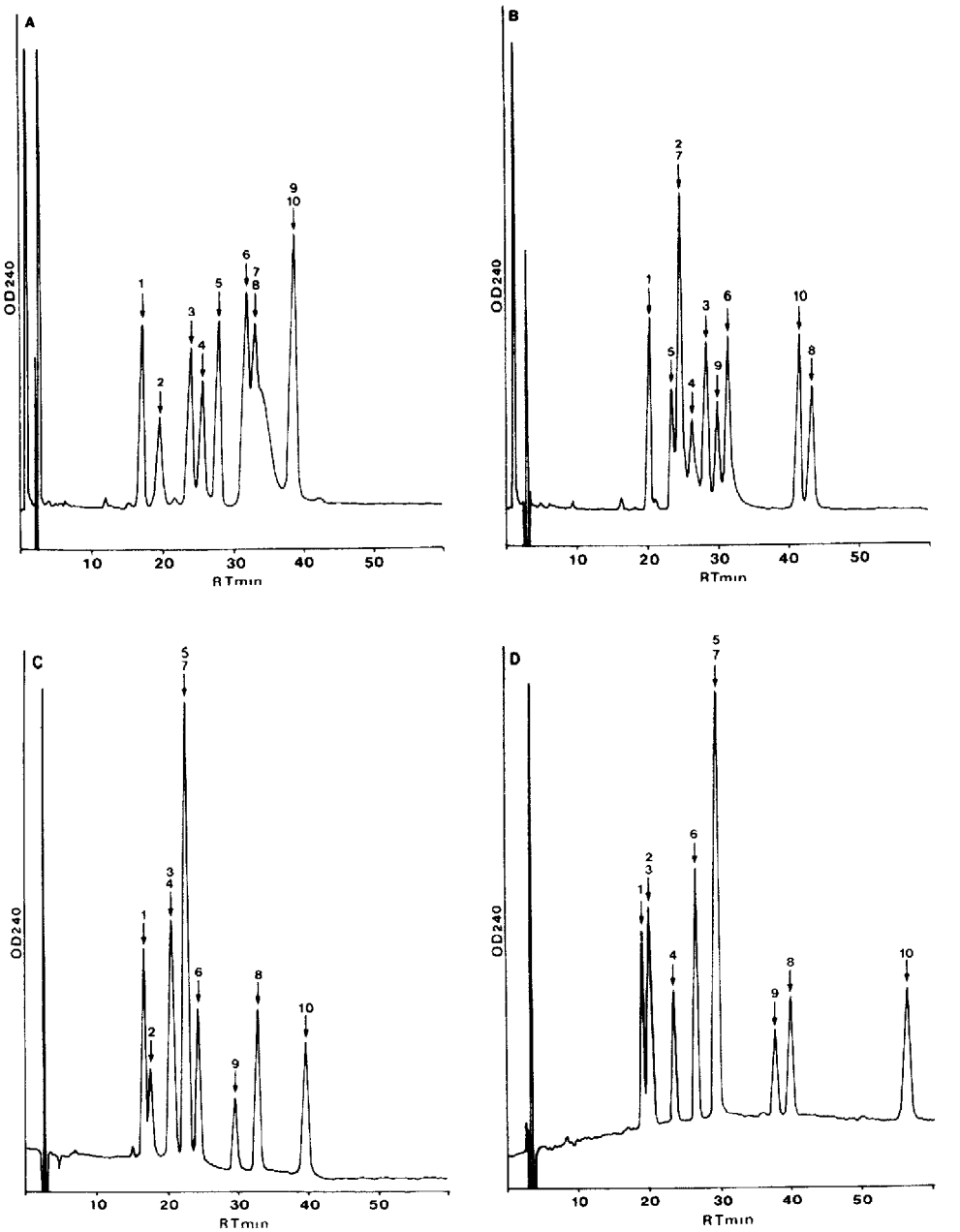

Fig. 1. Isocratic separation of steroids in Table I with binary solvent mixtures. The mobile phase was (A) 35% methanol, (B) 20% acetonitrile, (C) 20% dioxane and (D) 12% tetrahydrofuran, all in water. All separations were carried out with a solvent flow-rate of 1 ml/min at 45°C, using a 250 × 5 mm I.D. ODS-Hypersil column.

Glajch et al. [9] have proposed two methods to optimize complex separations, the Chromatographic Optimization Function (COF), and Overlapping Resolution Mapping (ORM), both based on a seven-step simplex procedure (Fig. 2). The COF is based on a peak resolution parameter and is a modification

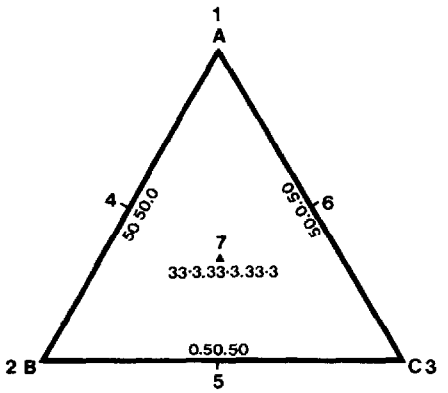

Fig. 2. Simplex design for ternary solvent optimization. This is the seven-point method used by Glajch et al. [9] modified from the ten-point design described by Snee [15].

of the Chromatographic Response Function (CRF) [12]. Unlike the latter it includes a weighting factor for pairs of interest [10]. The COF is calculated on the basis of the formula:

$$\mathrm{COF} = \sum_{i=1}^{k} A_i \ln \frac{R_i}{R_{id}} + B\,(t_m - t_l) \qquad (2)$$

where R_i = resolution between ith pair, R_{id} = desired resolution between that pair, t_l = actual time of analysis, t_m = maximum acceptable analysis time, and A_i and B are arbitrary weighting factors (A may be different for each pair). If $R_i > R_{id}$ then $R_i = R_{id}$; if $t_l < t_m$ then $t_l = t_m$.

Glajch's procedure [9] calculates the COF for each of the seven chromatograms defined in Fig. 2 and derives the best polynomial regression for the four variables, i.e. COF and the amounts of the three solvents. The method was originally designed to optimize the mobile phase without specific identification of individual peaks and is suited for application to a completely automated system. If, however, there is a cross-over or an overlap between any of the peaks, a more complex procedure than that originally described is required [9]. The extension of the method to accommodate cross-overs was deemed cumbersome by Glajch et al. [9] and a procedure based on resolution contour maps for each pair of compounds was, therefore, devised by these workers.

The ORM system [9,10] is based on a graphical representation of the expected overlap between every pair of peaks with four-solvent mobile phases. It is again derived from data generated by the seven experiments denoted in Fig. 2 and is expressed in the form of domains (solvent selectivity areas) superimposed one upon another within the bi-dimensional space triangle (Fig. 2) defined by the three limiting binary compositions. (Such limiting mixtures are sometimes referred to as pseudocomponents, here they can be termed pseudo-solvents.) The ORM can accommodate peak cross-overs and it was considered a significant improvement over the original COF method by Glajch et al. [9]. However, the resulting map only defines the limits of an area corresponding to a range of mobile phases which result in no overlapping, i.e. resolution of all the peaks under consideration; thus, in the original published form [9]

it does not generate a truly unique optimized solution, although recent modifications encompassed in the SENTINEL (DuPont) procedure have rectified this problem. The total analysis time on the other hand is only optimized in the sense that a maximum value is determined by the overall solvent strength selected, and there is no further systematic, interactive optimization of analysis time. The major disadvantage of the ORM is that if it is applied to a large number of compounds with similar retention times it is probable that a complete solution will not result and the overlay intersection of all the solvent selectivity areas will completely cover the overlapping resolution map. In such cases a partial solution can sometimes be obtained by excluding solvent-selectivity areas corresponding to pairs of minor importance. In the present study, however, application of the original ORM method to all ten compounds (Table I) necessitated exclusion of the major compound of interest, aldosterone, in order to generate a solution because priority weighting factors are not available with this technique, or with SENTINEL. An alternative procedure based on a modification of the COF method was, therefore, chosen, with retention data from the seven chromatograms defined in Fig. 2.

In order to minimize bias to the statistical calculations various transformations of the absolute retention values were calculated and their effects on the multiple polynomial regressions examined. Transformations studied included the relative retention time (RT_n/RT_0) in respect of an internal standard, the logarithm of the absolute retention time and the relative logarithm of retention times ($\ln RT_n/\ln RT_0$). 17-Iso-aldosterone was used as the internal standard for calculation of relative retention times and their logarithmic transformations because it showed no cross-overs with other steroids, and a similar retention time with each of the binary systems (Fig. 1). The relative logarithm of retention times gave a ten-fold reduction in the probability of deviation from the calculated regression line when compared with absolute retention values, and was also an improvement on linear retention indices; it was therefore used in all subsequent calculations.

Glajch et al. [9] use a statistical procedure in which the COF is a dependent variable of solvent composition of the mobile phase (solvents A, B, C vs. COF). In reality, however, it is the relationship between retention times and solvent composition that reflects the real chromatographic situation. We have, therefore, calculated all the relative logarithmically transformed retention times, as a function of solvent composition (solvents A, B, C vs. $\ln RT_0/\ln RT_n$). To obtain this result we do not calculate a single polynomial, as in Glajch's procedure [9], but derive instead a separate polynomial regression for the logarithmic retention index of each compound in relation to the internal standard (17-iso-aldosterone). Multiple polynomial regressions (6th degree incomplete) are derived by our program without preselection of the form of the equation. In this case the COF is simply a dependent parameter of the relative retention times calculated according to eqn. 2, and its values used solely to select the better composition. In our first attempt to define an optimized four-solvent mobile phase, appropriately transformed retention data were used from the seven basic chromatograms generated with methanol, dioxane, acetonitrile and water. This combination of solvents, however, did not generate a satisfactory solution to the chromatographic problem when the optimized mobile phase

was calculated and applied to the standards in Table I (data not shown). The results nevertheless provided a good test of the ability of our program to predict the retention times and values of COF and a good correlation between expected and observed values was obtained for the ten compounds ($p < 0.05$).

The data generated with methanol, dioxane, acetonitrile and water also showed that the modified COF procedure used here gives a better fit of predicted-to-actual values when applied to those compounds in Fig. 1 without

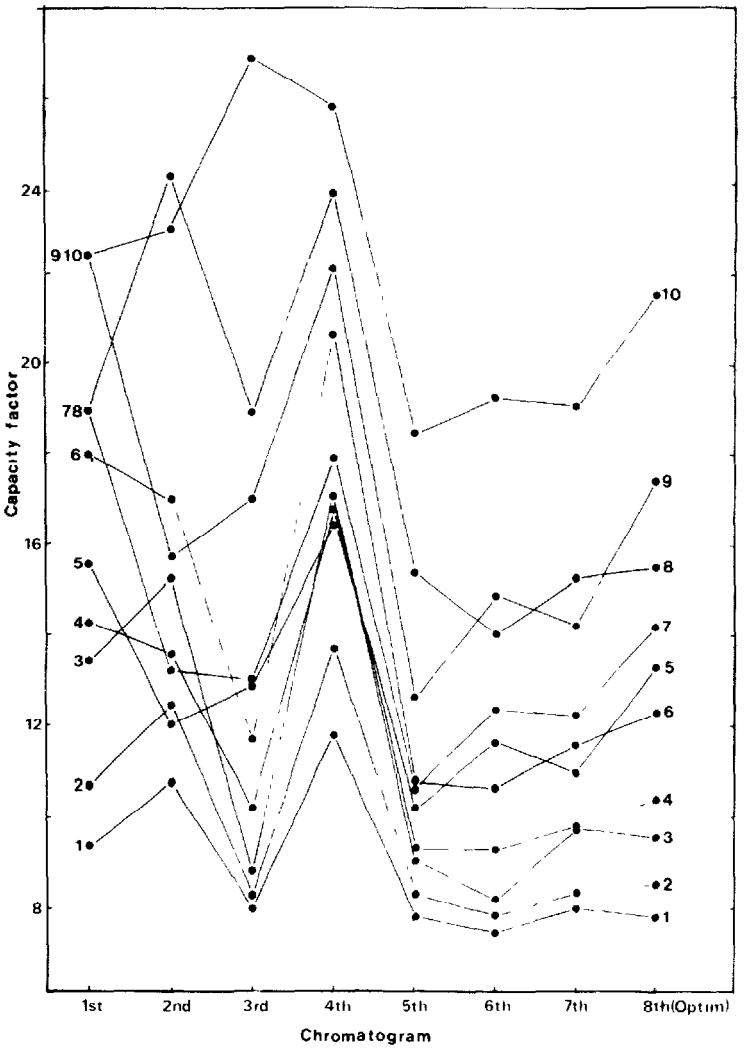

Fig. 3. Solvent selectivity data for steroids in Table I, obtained for the seven different mobile phases denoted by Fig. 2. The 1st, 2nd and 3rd chromatograms correspond to pseudo-solvents A, B and C and comprise, respectively, the binary mixtures methanol—water (35:65), acetonitrile—water (20:80), and tetrahydrofuran—water (12:88). The mobile phases for chromatograms 4, 5 and 6 were three-component mixtures denoted by the corresponding points on Fig. 2, and comprising the mixtures A—B, B—C and C—A; the 7th was obtained with a four-component mixture (A—B—C). The 8th chromatogram illustrates the actual retentions observed using the optimized mobile phase methanol—tetrahydrofuran—water (22.4:4.3:73.3). All separations were carried out with a solvent flow-rate of 1 ml/min at 45°C, using a 150 × 5 mm I.D. ODS-Hypersil column.

cross-overs, to which the original COF method of Glajch et al. [9] (as distinct from their ORM procedure) can also be applied. Thus, values of $p < 0.01$ were obtained for solvents (A, B, C) vs. $\ln RT_0/\ln RT_n$ compared with $p = 0.05$ for solvents (A, B, C) vs. COF. Furthermore, this method will cope with overlaps and cross-overs, unlike, for example, the CRF-based method of Berridge for unattended optimization [13].

Despite observed differences between dioxane—water, methanol—water and acetonitrile—water binary mixtures (Fig. 1), the failure of the computed ternary system for these particular solvents is not altogether surprising. Better resolution is to be expected when the three organic solvents that are selected are well separated in respect of their proton acceptor, proton donor and dipole interaction parameters [14]. Dioxane and acetonitrile both belong to Group VI as defined by Snyder [14], methanol to group II and tetrahydrofuran to group III. These considerations have been extensively discussed by Glajch et al. [9].

A second set of experiments was therefore carried out to provide the data for optimizing the mobile phase in respect of methanol, tetrahydrofuran, acetonitrile and water, as illustrated in Fig. 2. These three organic modifiers are well separated in terms of their solubility parameters and have been recommended as generally preferred solvents for reversed-phase ternary systems [10]. Results are shown in Fig. 3, and the initial values of COF calculated therefrom in Table II. The desired resolution (R_{id}) was set at 1 min and a maximum analysis time (t_m) of 22 min was chosen (see eqn. 2), based on the performance of the columns used in these experiments. The goodness of fit of the model was controlled by the *F*-ratio, as discussed by Snee [15], calculated for each compound prior to the insertion of the weighting factors and computation of the optimized mobile phase yielding the maximum COF value. This was done by sequential calculation of the COF for all possible combinations, initially at 4% steps in pseudosolvents A, B and C (Fig. 2) followed by 1% steps, once appropriate weighting factors had been defined, and their effects on the predicted separations determined.

The optimum calculated mobile phase under these conditions was methanol—tetrahydrofuran—water (22.4:4.3:73.3) corresponding to pseudo-

TABLE II

VALUES OF COF IN SEVEN BASIC CHROMATOGRAMS DENOTED BY FIG. 2 FOR STEROIDS IN TABLE I, RESULTS OF WHICH ARE ILLUSTRATED IN FIG. 3

Chromatogram	*COF**
1	−11.26
2	−4.59
3	−8.77
4	−6.67
5	−6.49
6	−1.92
7	−3.88

*Computed with weighting factor *A* set at 1 for all compounds, a desired separation factor of 1 min and a maximum analysis time of 22 min (see eqn. 2); weighting factor *B* was 0.1.

TABLE III

EXPECTED AND OBTAINED RELATIVE RETENTION TIMES AND COF VALUES* FOR POLAR STEROIDS USING OPTIMIZED TERNARY MOBILE PHASE**

	Compound***										COF
	1	2	3	4	5	6	7	8	9	10	
RT_n/RT_1 (expected)	1.0	1.06	1.16	1.25	1.51	1.44	1.62	1.77	1.92	2.30	−0.85
RT_n/RT_1 (obtained)	1.0	1.07	1.18	1.28	1.59	1.48	1.69	1.84	2.0	2.11	−0.50

*Calculated as in Table II.
**Methanol—tetrahydrofuran—water (22.4 : 4.3 : 73.3).
***See Table I.

solvent A—B—C (64 : 0 : 36) (Fig. 2), giving a predicted COF of −0.85. When this system was used to separate the steroids in Table I not only was the predicted retention order fulfilled, but observed retentions were close to predicted values (Table III), giving an effective COF value of −0.50. A value of

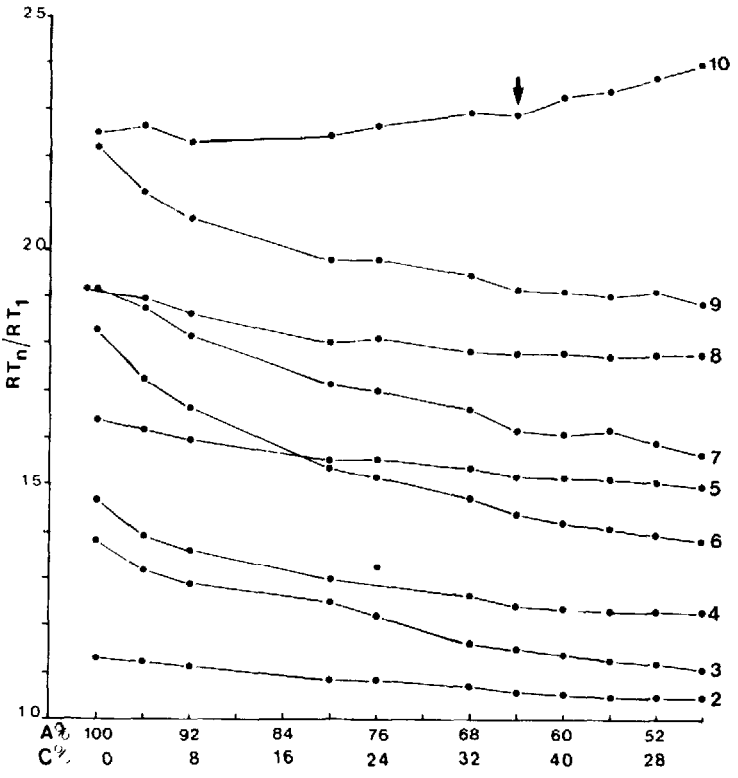

Fig. 4. Computer-predicted retentions for selected three-component mobile-phase mixtures (methanol—tetrahydrofuran—water). The input data were from chromatograms 1—7 in Fig. 3. A denotes methanol—water (35 : 65), C tetrahydrofuran—water (12 : 88). The arrow indicates the retentions predicted for the mobile phase identified by the program as optimal, corresponding to A—B—C (64 : 0 : 36).

0.1 was assigned to weighting factor B. As is evident from Fig. 3 there was also a significant reduction in total analysis time, reflecting the fact that a full version of eqn. 2 was used. This is important because any reduction in total analysis time without compromising resolution, over and above that simply achieved by prior solvent strength selection in respect of the pseudosolvents A, B and C, results in improved precision in quantitative analysis when using isocratic conditions. Berridge [13] has used a version of the CRF formula that involves a t_m component but his TERNOPT programme does not accommodate peak overlaps or cross-overs, being designed for fully automated optimization. As mentioned above the CRF procedure does not include priority weightings for compounds of specific importance.

The separations predicted and achieved under optimized conditions using our programme can be best visualized by reference to Fig. 4. By inserting a variety of weighting factors (A_i) for priority separations of compounds 1–10 into the computation we have used the program to generate predicted retentions for a large number of three- and four-solvent mobile phases. Those corre-

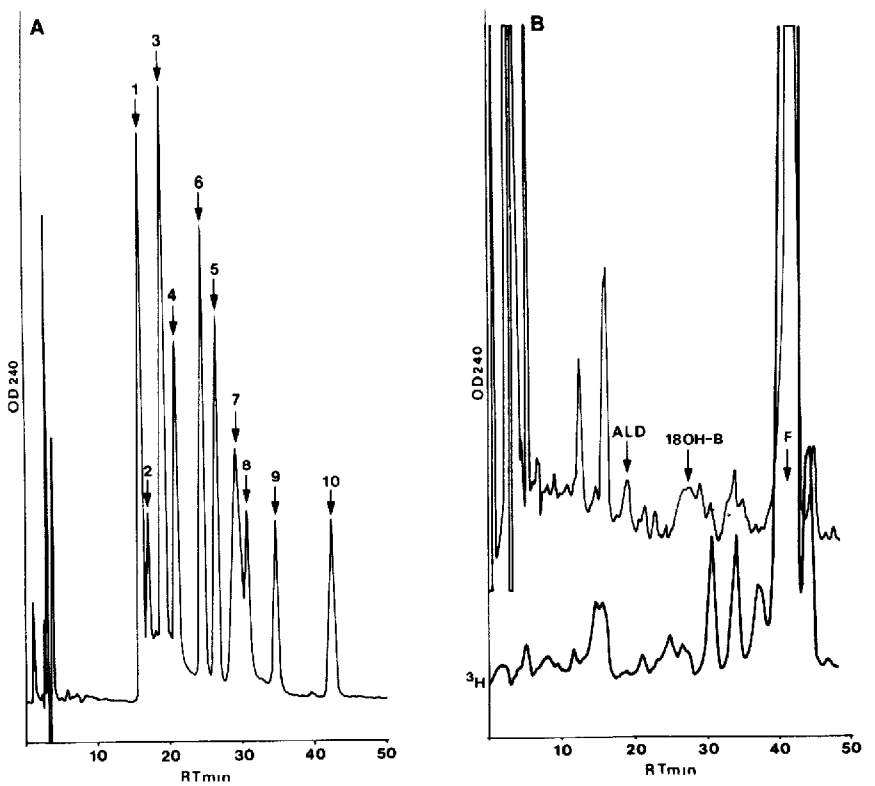

Fig. 5. Separation of steroid standards (A) and a sample (B) using the optimized ternary mobile phase. The identity of cortisol and cortisone metabolites in the sample from a cell suspension of human adrenocortical tumour cells is demonstrated by the concurrent profile of [^{3}H]radiometabolites generated from [^{3}H]cortisol. Chromatographic conditions comprised a mobile phase of methanol–tetrahydrofuran–water (22.4 : 4.3 : 73.3) at a flow-rate of 1 ml/min at 45°C with a 250 × 5 mm I.D. ODS-Hypersil column. See Table I for key to identity of all steroid standards.

sponding to the three-component methanol tetrahydrofuran water mixtures on either side of the optimum conditions have been plotted in Fig. 4. They demonstrate the capability of the program to control the predicted retentions in a systematic manner as well as illustrating the manner in which the optimized separation differs from its neighbours.

As a final test of its utility, the optimized ternary mobile phase was applied to an aldosterone-containing sample from a human adrenocortical tumour. The results are illustrated in Fig. 5 and demonstrate the solution to our original problem, viz., the separation of aldosterone and 18OH-B from UV-absorbing metabolites of cortisol and cortisone.

This separation is not, of course, necessarily the best obtainable under any isocratic conditions, but simply the best in relation to the original choice of solvents comprising the mobile phase and the choice of the ratio between water and each organic solvent that defines the mobile phase at 1, 2 and 3 in Fig. 2, i.e. pseudosolvents A, B and C. The range of potential solvent mixtures explored in this study can best be visualized as a plane intersecting a pyramid (Fig. 6). The results obtained do not, therefore, rule out a better isocratic solution with a different choice of A, B and C, particularly as the addition of an organic solvent to water may change its bulk properties with effects on retention that are not intuitively obvious [9].

To select a completely optimized isocratic mobile phase without preselection

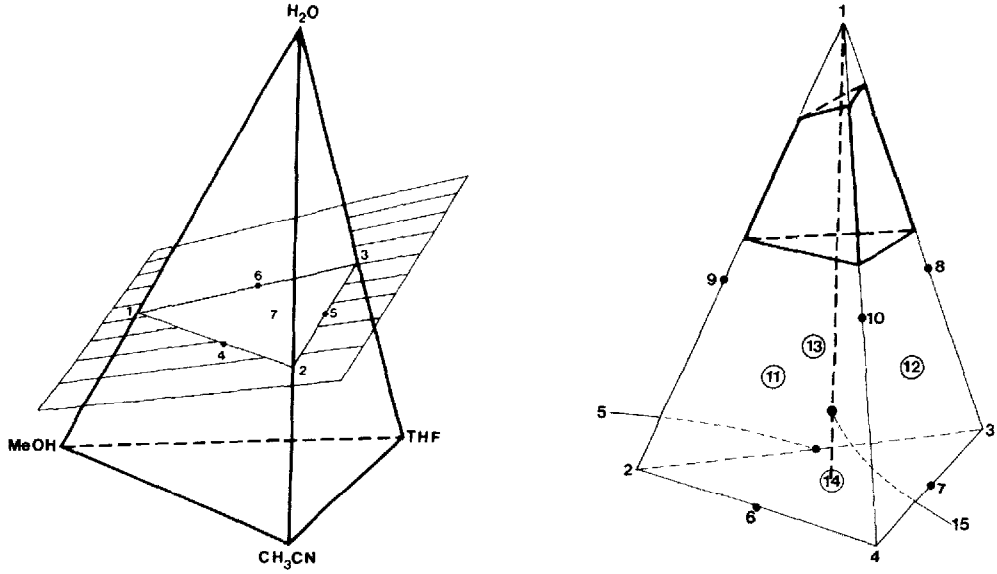

Fig. 6. Three-dimensional representation of the range of mobile-phase mixtures available with four solvents. The conditions searched by computer-aided statistical methods in the present study correspond to a plane intersecting the tetrahedron, as illustrated.

Fig. 7. Statistical design for searching the complete range of mobile phases afforded by four solvents. The numbers indicate individual mobile phases for which retention data for all compounds must be generated. In isocratic reversed-phase separations, some water-rich and water-poor phases can be eliminated; a twelve-point search design is then required to control the behaviour of compounds within the mobile-phase conditions represented by the resultant truncated pyramid.

of solvent strength, as distinct from optimizing gradient elution conditions, it is, therefore, necessary in principle to search the full quaternary system represented in three-dimensional space by a tetrahedron (Fig. 7). The seven-point statistical approach illustrated in Figs. 2 and 6 must consequently be modified into a more complex one. To search the full range of four solvent phases, such as, for example, might be employed in a normal-phase separation, a fifteen-step design is necessary because no preselected equation is used for calculation of polynomial regressions (Fig. 7). Given the nature of the solvents available for a normal-phase separation and their widely different solubility parameters [10, 14], it might be expected that this would be likely to give a more effective separation of a complex group of compounds of similar polarity such as we have been concerned with in this study. Antle [16] has described the use of ORM and COF type procedures to optimize normal-phase separation of simple steroid hormone mixtures. The computational COF method was not precisely described but was probably of the form described by Glajch et al. [9]; better results were, however, obtained with the ORM method and by visual optimization, reflecting to some extent the limitations of the original COF method.

However, despite these advantages of normal- as opposed to reversed-phase systems, it must be borne in mind that the separations illustrated here are only one facet of a more general analytical problem that simultaneously involves other groups of natural steroids of widely different polarities. Although flow-programming can be used to facilitate the elution of individual strongly retained compounds, for the general problem gradient elution still probably provides the best solution [2]. For this purpose normal-phase systems present certain practical problems due, in part, to difficulties in ensuring reproducibility of re-equilibration. If, on the other hand, isocratic reversed-phase systems are chosen the tetrahedron in Fig. 6 can be searched by a simplified procedure. As one component of the system is inevitably water, the parts of the tetrahedron corresponding to extremely water-rich and water-poor phases can be eliminated as they will lead to solutions with unacceptably long and short analysis times, respectively. The resultant truncated pyramid requires a total of twelve experiments to generate the requisite data for statistical optimization. The seven-step method described here required a total calculation time of approximately 1 h to search all combinations of mobile phases with ten compounds and 4% steps between A, B and C once the retention values for the seven chromatograms had been inserted and the multiple polynomial regressions calculated and controlled for each compound, a step which itself required approximately 1 h. It took 14 h to identify the optimum conditions when 1% steps were programmed, corresponding to a precision of ±0.1—0.7% in the actual organic modifier and water concentrations defined. While the computation time and memory capacity required to run a twelve- or fifteen-step programme are obviously considerably greater, they are nevertheless, within the scope of the current generation of personal computers. Further aspects of the interactive program which we have outlined here and which we have termed the Chromatographic Optimization Coefficient (COC) procedure, are detailed elsewhere [17]. These include the development of a program for full quaternary reversed-phase isocratic optimization. The same statistical approach can be used for optimizing gradient elution with advantages

of enhanced precision in prediction of retention times compared with the ORM-based seven-step, isoselective multisolvent gradient elution procedure (IMGE) and the semi-empirical step-selective multisolvent gradient elution (SMGE) procedure developed by Kirkland and Glajch [18,19]. Ultimately, a fully systematic procedure for multisolvent gradient elution (i.e. without constraints on the relative proportions of the different organic modifiers and water) should be possible.

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