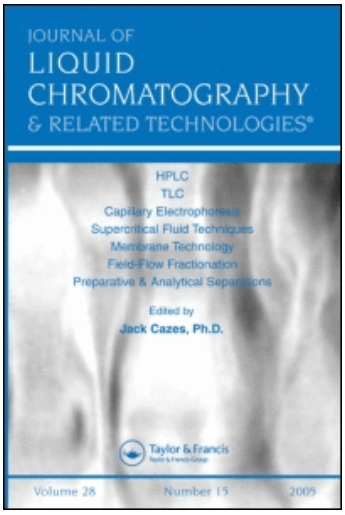

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Optimization of the High-Performance Liquid Chromatographic Separation of Steroids by the Overlapping Resolution Mapping Procedure

Kiao Yuh Chong[a]; Teng Hai Khoo[a]; Fei Sheng Koo[a]; Chye Peng Ong[a]; Sam F. Y. Li[a]; Hian Kee Lee[a]; Byrappa Venkatesh[b]; Cheong Huat Tan[b]
[a] Department of Chemistry, National University of Singapore, Kent Ridge Republic [b] Department of Zoology, National University of Singapore, Kent Ridge Republic

To cite this Article Chong, Kiao Yuh , Khoo, Teng Hai , Koo, Fei Sheng , Ong, Chye Peng , Li, Sam F. Y. , Lee, Hian Kee , Venkatesh, Byrappa and Tan, Cheong Huat(1991) 'Optimization of the High-Performance Liquid Chromatographic Separation of Steroids by the Overlapping Resolution Mapping Procedure', Journal of Liquid Chromatography & Related Technologies, 14: 13, 2445 — 2455
To link to this Article: DOI: 10.1080/01483919108049332
URL: http://dx.doi.org/10.1080/01483919108049332

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(13), 2445-2455 (1991)

OPTIMIZATION OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF STEROIDS BY THE OVERLAPPING RESOLUTION MAPPING PROCEDURE

KIAO YUH CHONG[1], TENG HAI KHOO[1],
FEI SHENG KOO[1], CHYE PENG ONG[1],
SAM F. Y. LI[1], HIAN KEE LEE[1]*,
BYRAPPA VENKATESH[2], AND CHEONG HUAT TAN[2]
[1]*Department of Chemistry*
[2]*Department of Zoology*
National University of Singapore
Kent Ridge
Republic of Singapore 0511

ABSTRACT

The reversed-phase high-performance liquid chromatographic separation of several steroids is described in this paper. The optimization of the solvent composition was achieved by making use of the overlapping resolution mapping scheme. The optimum solvent composition was arrived at from only seven chromatographic experiments. Satisfactory separation was possible for all but one pair of the steroids considered.

INTRODUCTION

The use of systematic procedures for the determination of optimum solvent compositions for isocratic high-performance liquid

* Author to whom correspondence should be addressed

TABLE I

Steroids Studied in this Work.

(a) 11-Ketotestosterone
(b) 5α-Dihydrotestosterone
(c) 11ß-Hydroxytestosterone
(d) Androstenedione
(e) 17α-Hydroxyprogesterone
(f) Testosterone
(g) 17ß-Estradiol
(h) 17α,20ß-Dihydroxyprogesterone
(i) Progesterone

chromatography (HPLC) serves to eliminate time consuming trial-and-error approaches for analyses of this nature. Also, expenditures on expensive HPLC-grade solvents are minimized, apart from the reduction in wear and tear of columns which are more heavily used with conventional methods development procedures. One particular optimization method, the overlapping resolution mapping (ORM) technique (1), has previously been used with success for analytes such as substituted phenols (2), polycyclic aromatic hydrocarbons (3) and phthalates (4). This paper describes the use of the ORM technique in the separation of several steroids found naturally in fish (Table I).

Steroids are important biomolecules, vital as a class of hormones which control the physical growth and reproductive development of animals. The analysis of these compounds in animal tissues is therefore an important undertaking. Of interest in this work were steroids present in the gonads of several species of fish. These steroids are involved with oocyte growth and maturation (5). Preliminary work (6) indicated that satisfactory HPLC separation of these steroids could only be achieved by two separate and sequential isocratic elutions with methanol/water (60:40, %

v/v) and acetonitrile/water (50:50), respectively, on a Waters μBondapak 300 mm x 3.9 mm I.D. (10 μm particle size packing) column. The combined chromatographic runs took 44 min. It was therefore desirable to improve on not only the resolution (with a single eluent mixture) but also the chromatographic run time.

EXPERIMENTAL

Instrumentation

Liquid chromatography was carried out with a Shimadzu (Japan) LC-6A pump and a Shimadzu SPD-6A variable wavelength ultraviolet/visible spectrophotometric detector. The columns used were a Waters (USA) μBondapak C_{18} column (300 mm x 3.9 mm I.D.; 10 μm particle size), and a Shimadzu Shimpack CLC-ODS (C_{18}) column (150 mm x 6 mm I.D.; 5 μm particle size). The Waters column was used in the optimization procedure whereas the Shimadzu column was used after optimum solvent composition(s) had been identified. The detection wavelength was 254 nm. Typically, the mobile phase flow rate was 1 $mLmin^{-1}$, although with *2*-propanol/water and methanol/*2*-propanol/water systems, the flow rates used were 0.6 $mLmin^{-1}$ and 0.8 $mLmin^{-1}$, respectively, due to the excessive pressures obtained with these eluent mixtures. Samples were introduced by using a Rheodyne 7125 injection valve with a 20-μL sample loop. The void time was obtained by using methanol as the unretained substrate. Chromatographic data were collected and analysed on a Shimadzu Chromatopac CR-3A data processor. All chromatographic runs were duplicated; reproducibility of retention times was ±0.5% or better.

Chemicals and solvents

HPLC-grade solvents were used in this work. They were degassed by ultrasonicaton before use. Distilled water was obtained from a

Millipore (USA) water purification system. Mobile phases were prepared by measuring accurately the appropriate volumes of the individual components and mixing them together (A + B addition method (7)).

The steroids were obtained from Aldrich (USA). Individual steroid standard solutions and mixtures were prepared in methanol. All samples were filtered before injection into the column.

RESULTS AND DISCUSSION

The first step in the implementation of the ORM technique is to define the criteria of the analysis. The ultimate aim is the achievement of a satisfactory separation. Thus, a reasonable resolution value (R_S) provided by the eventual solvent composition is specified as the first criterion. Preferably, the value should be at least unity between peaks.

Selection of the second criterion involves the length of the chromatographic analysis. Notwithstanding the importance attached to peak resolution, a reasonable chromatographic run time is also an essential consideration. A capacity factor (k') range of 0 - 20 for all components is usually recommended, although 2 - 10 is considered ideal (8).

Once the criteria of the analysis have been set, the next step is to establish the vertices of the solvent selectivity triangle (9) upon which the ORM technique is based. The first vertex A is selected contingent on the requirement of the k' range. Two or three chromatographic runs may be necessary with (usually) methanol/water as the binary mixture. In our case, 70:30 methanol/water (a common starting composition for most analyses) was selected which satisfied our second criterion. Based on this mixture, the other vertices (B and C) were established by

TABLE II

Solvent Compositions as Percentages of Binary Mixtures Used for the Seven Preliminary Experiments.

Eluent Mixture	Methanol/water (A)	ACN/water* (B)	*2*-Pro/water# (C)
1	100 %	0 %	0 %
2	0	100	0
3	0	0	100
4	50	50	0
5	0	50	50
6	50	0	50
7	33.3	33.3	33.3

* ACN = Acetonitrile; # *2*-Pro = *2*-Propanol

considering two other binary mixtures (acetonitrile/water and 2-propanol/water) which possess similar solvent strengths. The latter can be calculated by equation (1) (9).

$$ST = S_a\phi_a + S_b\phi_b + \ldots\ldots \qquad (1)$$

where ST = solvent strength of the mixture, S_i = individual solvent strengths of the organic modifiers and ϕ_i = volume fractions of the solvents.

To complete the series of seven eluent mixtures required for the ORM scheme, the solvent compositions represented by the midpoints of A-B, B-C and A-C, and the centre of the selectivity triangle were selected (1). Table II shows the solvent compositions of the eluent mixtures as percentages of the binary mixtures. In terms of percentages of the pure solvents in the eluent mixture, the solvent compositions are as shown in Table III.

TABLE III

Solvent Composition as Percentages of Pure Solvents in the Mobile Phase Used for the Seven Preliminary Experiments.

Eluent Mixture	Methanol	Acetonitrile	2-Propanol	Water
		%		
1	70.00	0.00	0.00	30.00
2	0.00	67.70	0.00	32.30
3	0.00	0.00	50.00	50.00
4	35.00	33.85	0.00	31.15
5	0.00	33.85	35.00	41.15
6	35.00	0.00	25.00	40.00
7	23.31	22.54	16.65	37.50

Retention time data from the seven chromatographic runs are shown in Table IV. By using equation (2), the resolution (R_s) for every pair of adjacent peaks may be calculated,

$$R_s = \frac{2(t_2 - t_1)}{(w_1 + w_2)} \qquad (2)$$

where t_i = retention time of the i^{th} peak and w_i = width (taken at the baseline) of the i^{th} peak. Table V shows the R_s values calculated for the seven preliminary experiments.

By substituting the R_s values into a second-order polynomial surface response equation, equation (3) (9),

$$R_s = a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{123}x_1x_2x_3 \qquad (3)$$

TABLE IV

Retention Times of Steroids Obtained from the Seven Preliminary Experiments.

	Eluent mixture						
Steroid*	1	2	3	4	5	6	7
a	4.383	3.483	5.667	3.952	4.750	3.633	3.825
b	4.420	4.442	6.675	4.467	5.383	4.258	3.432
c	5.017	3.425	5.850	4.250	5.250	3.775	4.150
d	5.558	4.658	6.900	5.352	6.117	4.700	5.050
e	6.525	4.533	6.942	5.642	6.642	4.792	5.408
f	6.675	4.308	7.108	5.500	6.867	4.775	5.358
g	6.683	3.925	6.858	5.067	6.833	4.500	5.033
h	6.758	4.225	7.175	5.625	6.817	4.792	5.442
i	9.250	6.317	8.983	8.592	9.375	6.717	7.808

* Identities of steroids as in Table I.

TABLE V

Resolution (Rs) Between Adjacent Steroid Peak-Pairs Calculated for the Seven Preliminary Experiments.

Peak-	Eluent Mixture						
Pair	1	2	3	4	5	6	7
A	0.080	0.094	0.207	0.692	0.483	0.256	0.476
B	1.205	0.815	0.906	0.405	0.116	0.786	0.256
C	1.220	0.608	0.199	0.937	0.712	0.369	0.950
D	1.630	0.164	0.047	0.371	0.609	0.306	0.024
E	0.195	0.211	0.043	0.247	0.203	0.113	0.442
F	0.006	0.142	0.178	0.184	0.016	0.025	0.070
G	0.050	0.191	0.074	0.025	0.031	0.082	0.045
H	2.660	2.266	1.854	3.363	2.267	2.391	2.637

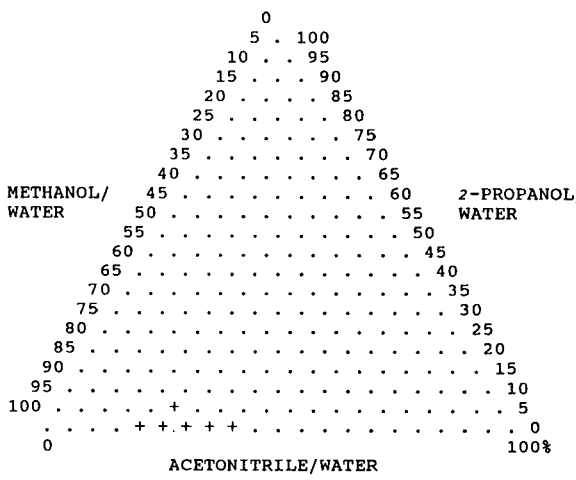

Figure 1: Overlapping resolution mapping diagram for steroid peak-pairs. Symbol "+" shows the region corresponding to optimized solvent compositions where resolution (R_s) = 1.

(where a_i = coefficients and x_i = volume fractions of the binary mixtures A, B and C), the coefficients for each peak pair were determined. A computer program was used for the calculations (9). By returning the coefficients to equation (3), the R_s values for any composition within the selectivity triangle could be determined. The result is a resolution contour plot (or Venn diagram (1)) for every peak-pair in the mixture of steroids under study. The region on the map corresponding to a previously chosen value (unity in our case, our first selection criterion) would establish the area of interest. By overlapping all the contour maps (one for each pair of peaks), the region on the resultant plot where resolution is unity could subsequently be obtained. Solvent compositions corresponding to the points in this region are the optimized eluent mixtures which should permit at least reasonable separation of all the components in the steroid mixture. For the present work, such an overlapping resolution map is shown in Figure 1. The symbol "+" represents the area where R_s is unity.

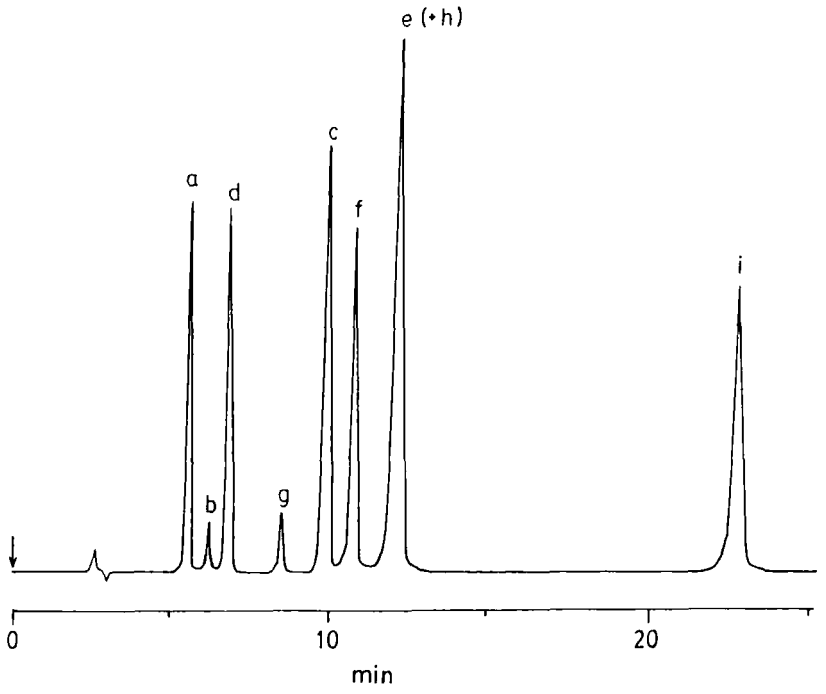

Figure 2: Isocratic liquid chromatogram of steroids obtained by using methanol/acetonitrile/water, 49.00:20.31:30.69 (% v/v) as mobile phase. Column: Shimadzu Shimpack CLS-ODS (150 mm x 6 mm I.D.; 5 μm particle size). Flow rate: 1 $mLmin^{-1}$. Detection wavelength: 254 nm. Peak identities : (a) 11-ketotestosterone; (b) 5α-dihydrotestosterone; (c) 11ß-hydroxytestosterone; (d) androstenedione; (e) 17α-hydroxyprogesterone; (f) testosterone; (g) 17ß-estradiol; (h) 17α,20ß-dihydroxyprogesterone; (i) progesterone.

To evaluate the ORM scheme, a point was picked from the optimized region in Figure 1 and the corresponding solvent composition prepared (methanol/acetonitrile/water, 49.00:20.31:30.69, % v/v). Using this mobile phase and the Shimpack CLC-ODS column (150 mm x 6 mm I.D.; 5 μm particle size packing), the chromatogram shown in Figure 2 was obtained. The separation is generally satisfactory. It should be noted that the total chromatographic run time was

under 23 minutes. The sequential analysis referred to earlier (6), involving two different mobile phase systems, took nearly forty-five minutes for the chromatography alone. In addition, more time was required for the HPLC system to equilibrate upon changeover of mobile phase.

The advantage of a systematic solvent optimization technique is thus clear; based on just a small number of chromatographic runs, an optimum solvent composition (it is not uncommon to have more than one such composition, as Figure 1 shows) is identified and subsequently used successfully for isocratic HPLC separations. The tedium and inconvenience of conventional and non-systematic procedures as well as extra expenditures on solvents and columns, as mentioned previously, are therefore largely minimized, if not eliminated.

It should, however, be pointed out that the ORM method is not infallible. It was found that in the present work, the consideration of an additional steroid, 17α,20ß-dihydroxyprogesterone, showed up the procedure's limitation; this steroid co-eluted with 17α-hydroxyprogesterone, even when the optimized solvent composition was used. While this result does not in any way detract from the overall success and satisfactoriness of the ORM scheme, it suggests that a different approach may be required to specifically resolve these two compounds, without at the same time degrading the separation of the others.

ACKNOWLEDGEMENTS

The authors thank the National University of Singapore for financial support, and are grateful to Madam Frances Lim for technical assistance.

REFERENCES

1. Glajch, J.L., Kirkland, J.J., Squire, K.M. and Minor, J.M., Optimization of Solvent Strength and Selectivity for Reversed-Phase Liquid Chromatography Using an Interactive Mixture-Design Statistical Technique, J. Chromatogr., 199, 57, 1980.

2. Ong, C.P., Lee, H.K. and Li, S.F.Y., Optimization of Mobile Phase Composition for HPLC Analysis of Eleven Priority Substituted Phenols, J. Chromatogr., 464, 405, 1989.

3. Li, S.F.Y., Lee, H.K. and Ong, C.P., Optimization of Mobile Phase Composition for HPLC Separation Using the Overlapping Resolution Scheme, J. Chromatogr., 506, 245, 1990.

4. Khan, M.R., Ong, C.P., Li, S.F.Y. and Lee, H.K., Optimization of Isocratic HPLC Separation of Selected Phthalates Using the Overlapping Resolution Mapping Technique, J. Chromatogr., 513, 360, 1990.

5. Fostier, A., Jalabert, B., Billard, R., Breton, B. and Zohar, Y., The Gonadal Steroids in "Fish Physiology," Hoar, W.S., Randall, D.J. and Donaldson, E.M., eds., Academic Press, New York/London, 1983, Vol. 9A, p. 277.

6. Venkatesh, B. and Tan, C.H., unpublished results.

7. Runser, D.J. Maintaining and Troubleshooting HPLC Systems, User's Guide, Wiley, New York, 1981, p.19.

8. Dolan, J.W., Peak Problems, LC-GC Intern., 2, 20, 1989.

9. Berridge, J.C., Techniques for the Automated Optimization of HPLC Separations, Wiley, Chichester, 1985, Ch. 4.