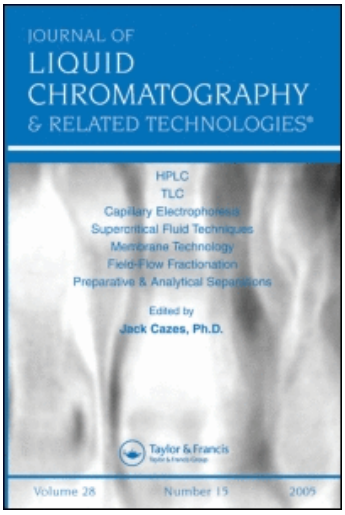

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Optimization of High-Performance Liquid Chromatographic Separation for Progestogenic, Estrogenic, and Andogenic Steroids Using Factorial Design

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OPTIMIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION FOR PROGESTOGENIC, ESTROGENIC, AND ANDOGENIC STEROIDS USING FACTORIAL DESIGN

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ABSTRACT

A 2^3 factorial design has been used in an isocratic HPLC system to study the effect of methanol and acetonitrile composition of the eluents as well as different columns on the retention and resolution of steroids. By factorial design and computer simulation, six steroids of sex hormones and contraceptives were simultaneously separated within 18 min and 28 min using RP-8 column and RP-18 column, respectively.

INTRODUCTION

Norethindrone and mestranol are synthetic steroids showing progestogenic activity. They are usually combined in use as oral concentraceptives. According to therapeutic uses, progesterone, medrogestone, testosterone propionate and fluoxymesterone are categorized in sexual hormones. Various functional group

substitutions in the chemical structures of these six steroids make them show different chromatographic retention characteristics. Many high-performance liquid chromatographic (HPLC) methods have been described for analysis of similar steroids [1-3]. Optimization of separations by a trial and error approach is time-consuming and likely to fail owing to the many variables involved and their interactions. Therefore, there has been much interest in finding a simplified and systematic means of optimizing HPLC separations. Many optimization methods such as sequential simplex method [4], window diagrams [5], overlapping resolution mapping method [6], Plackett-Burman design [7] and factorial design [8], have been employed for the selection of optimal mobile phase compositions in HPLC. In this study, a factorial design and a computer program has been used for the optimization of the retention and separation of the afore mentioned steroids by an isocratic RP-HPLC system. The optimization procedure was focused on selecting the optimum mobile phase composition in the different column.

EXPERIMENTAL

Materials

Norethindrone, progesterone and testosterone propionate were purchased from Sigma (St. Louis, MO, U.S.A.). Mestranol was purchased from Aldrich (Milwaukee, WI, U.S.A.). Fluoxymesterone was from MS Chemicals (Milano, Italy) and Medrogestone was from Diosyth BV (Oss, the Netherlands). Methanol and Acetonitrile (L.C. grade) were purchased from Mallinckrodt (Paris, KY, U.S.A.). The compound numbers, names and concentrations of the steroids are listed in Table 1. Standard solutions were prepared in methanol-water (1:1, v/v).

HPLC

The chromatographic system consisted of a JASCO 880-PO HPLC pump, a JASCO 875-UV variable wavelength detector (Hachioji, Japan) and a SIC Chromatocorder 11 recorder (Tokyo, Japan). UV detection at 230 nm was set for all optimization procedures. Columns used were 250 - 4 mm LichroCART C_{18} (7 mm) and 250 - 4 mm LichroCART C_8 (7 mm) (Merck, Darmstadt, Germany). The

TABLE 1. Compound numbers, names and concentrations of the steroids

No.	Compound name	Concentration
1	Fluoxymesterone	20 mg/ml
2	Norethindrone	20 mg/ml
3	Progesterone	20 mg/ml
4	Mestranol	120 mg/ml
5	Medrogestone	120 mg/ml
6	Testosterone propionate	30 mg/ml

TABLE 2. A 2^3 factorial design : experimental presentation

Variable	Experiment No.							
	1	2	3	4	5	6	7	8
X_1	1	1	1	1	−1	−1	−1	−1
X_2	1	1	−1	−1	1	1	−1	−1
X_3	1	−1	1	−1	1	−1	1	−1
X_1 = Methanol-Water			+1 = 46:54 (v/v)			−1 = 40:60 (v/v)		
X_2 = Acetonitrile-Water			+1 = 38:62 (v/v)			−1 = 32:68 (v/v)		
X_3 = Column			+1 = RP-8			−1 = RP-18		

flow rate of mobile phase was 1 ml min^{-1}, the injection volume was 20 ml. The dead time was measured with the first peak resulting from the injection of 0.5 % NaCl solution (w/v).

Factorial design [9]

The mobile phase was a ternary system including methanol, acetonitrile and water. The fractions of methanol and acetonitrile in the solvent were assigned as variables X_1 and X_2, and the different column as variable X_3, respectively in the factorial design (Table 2). Two values denoted by +1 (the upper level) and −1 (the lower level) were given for each variable to define the experimental domain. All experiments were performed in a random order.

Computer simulation

A computer program was written in QBASIC to predict chromatograms as systematically changing the three variables X_1, X_2 and X_3 within certain ranges. The maximum t_R was as a measure of analysis time. The predicted t_R was calculated using the following equation:

$$t_R = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_1 X_2 + \alpha_5 X_1 X_3 + \alpha_6 X_2 X_3 + \alpha_7 X_1 X_2 X_3 \quad (1)$$

where t_R is the retention time in minutes of an individual steroid under a given set of conditions and X_1, X_2 and X_3 are values of the three variables as indicated above. The coefficients α_1, α_2, α_3, α_4, α_5, α_6 and α_7 were estimated by Yates algorithm [12]. The predicted t_R were used to predict resolution (Rs) between each pair of successively eluted steroids, using the equation:

$$R\text{s} = 2(t_{R_{i+1}} - t_{R_i})/(W_{i+1} + W_i) \quad (2)$$

where $t_{R_{i+1}}$ and t_{R_i} were the retention times of i+1 and i peaks, and W_{i+1} and W_i were the peak widths of i+1 and i peaks.

RESULTS AND DISCUSSION

In the preliminary studies, tetrahydrofuran was chosen as a mobile phase component for the separation of steroids in the literature [8]. The high column pressure caused by the heavy proportion of tetrahydrofuran rendered its use impraticable. Furthermore, commercially available tetrahydrofuran contains an antioxidant which might cause interference during detection [10]. In the steroid analysis, acetonitrile was claimed to increase the peak sharpness and column efficiency [11]. For these reasons, acetonitrile was used instead of tetrahydrofuran. In the following study, the composition of methanol and acetonitrile in the mobile phase were used as variables. Many papers reported RP-C_{18} column could be used as the stationery phase of steroid separation [1,8,12]. However, it is worthwhile testing other RP columns of shorter hydrocarbon chains to reduce the analysis time while maintaining the same resolution on the basis of a similar retention mechanism.

TABLE 3. Retention times (min) of six steroids while changing the mobile phase according to TABLE 2

Steroid	Experiment No.							
No.[a]	1	2	3	4	5	6	7	8
1	3.29	3.26	3.62	3.86	3.49	3.64	4.10	4.41
2	3.29	3.46	3.95	4.27	3.84	4.07	4.71	5.15
3	4.19	5.51	5.49	8.09	5.27	7.47	7.34	11.23
4	4.19	6.24	5.95	10.45	5.56	9.20	8.40	15.55
5	4.79	7.18	6.91	11.71	6.55	10.58	10.00	17.47
6	5.13	8.47	7.81	14.99	7.36	13.43	11.91	23.91

[a] The corresponding steroids are listed in Table 1

In this study, a RP-C_8 column was tested and the retention times were compared with those of the RP-C18 column.

The retention times of each compound and parts of the chromatograms were shown in Table 3 and Fig. 1 for the eight experiments of the factorial design. In all eight experiments, the elution order of the steroids remained as fluoxymesterone, norethindrone, progesterone, mestranol, medrogestone and testosterone propionate, which is essentially in accordance with the decreasing polarity of these compounds [13]. The peaks of fluoxymesterone and norethindrone, and the peaks of progesterone and mestranol, were overlapped respectively in some experimental conditions (Table 3 and Fig. 1). The nature of organic solvent (the ratio of methanol-acetonitrile) affected the resolution of steroids both on the selecitvity and the column efficiency [11]. In acetonitrile-rich eluents, resolution was mainly dependent on the change in column efficiency better provided by acetonitrile than mathanol (Fig. 1(B)). In acetonitrile-lean eluents, separation was largely influenced by the higher selectivity of methanol compared to acetonitrile; however, the column efficiency decreased with the increase of methanol concentration (Fig. 1(A)).

The main effects and interaction effects of variables X_1, X_2 and X_3 on the t_R of an individual steroid are indicated by α_1, α_2, α_3, α_4, α_5, α_6 and α_7 (Table 4).

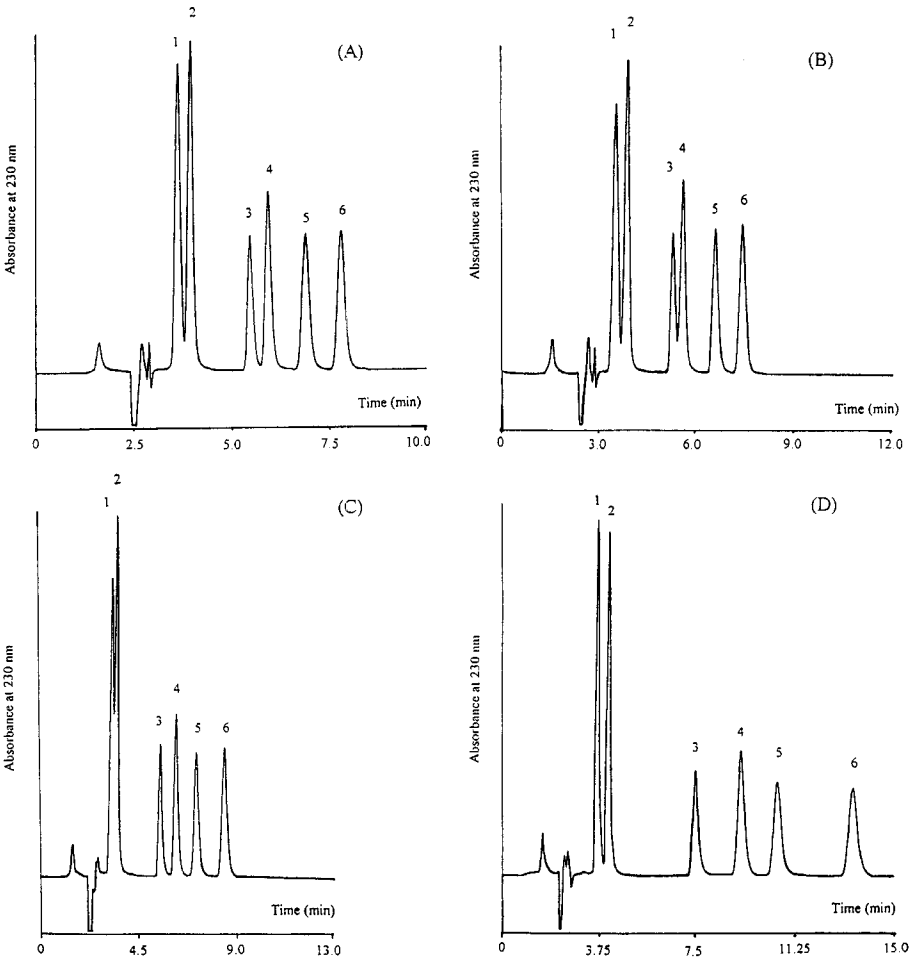

FIGURE 1.

Chromatograms of the steroid mixture in different chromatographic conditions. (A) column : LichroCART RP-8 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (46:32:22, v/v/v). (B) column : LichroCART RP-8 (250 - 4 mm , 7 mm); mobile phase : methanol-acetonitrile-water (40:38:22, v/v/v). (C) column : LichroCART RP-18 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (46:38:16, v/v/v). (D) column : LichroCART RP-18 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (40:38:22, v/v/v). For compound numbers, see Table 1.

TABLE 4. Calculated mean effects (α values) on the retention times of the substances while changing the variables according to TABLE 2

Steroid No.[a]	α_0	α_1	α_2	α_3	α_4	α_5	α_6	α_7
1	3.71	-0.20	-0.29	-0.08	0.06	0.03	0.05	0.01
2	4.09	-0.35	-0.43	-0.15	0.06	0.02	0.05	-0.01
3	6.82	-1.00	-1.21	-1.25	0.24	0.27	0.37	-0.05
4	8.19	-1.49	-1.90	-2.17	0.40	0.53	0.75	-0.13
5	9.40	-1.75	-2.12	-2.34	0.46	0.54	0.73	-0.13
6	11.63	-2.53	-3.03	-3.58	0.73	0.94	1.22	-0.26

[a] The corresponding steroids are listed in Table 1

TABLE 5. Comparison between actual and predicted retention times(t_R)

Steroid	RP - 8[a]		RP - 18[b]	
No.[c]	Actual t_R (min)	Predicted t_R (min)	Actual t_R (min)	Predicted t_R (min)
1	4.49	4.35	4.40	4.40
2	5.35	5.08	5.22	5.19
3	8.98	8.25	11.58	11.33
4	10.68	9.63	15.95	15.64
5	12.83	11.53	18.09	17.66
6	15.82	13.98	24.92	24.22

[a] Mobile Phase : methanol-acetonitrile-water(37:32:31,v/v/v)
[b] Mobile Phase : methanol-acetonitrile-water(37:34:31,v/v/v)
[c] The corresponding steroids are listed in Table 1

Negative α_1, α_2, and α_3 indicated that there was a negative relationship between main effects and t_R. In the eight experiments, the mean dead time was 2.21 min. With the computer simulation, satisfactory HPLC conditions were not found between the +1 and −1 range of the experimental variables, so we extended the searched intervals to between +2 and −2. When X_1, X_2 and X_3 were (−2, −1, +1) and (−2, −0.333, −1), optimum theoretical HPLC conditions were found. These represent the mobile phase composition methanol-acetonitrile-water (37:32:31, v/v/v) with RP-8 column used and the mobile phase composition methanol-

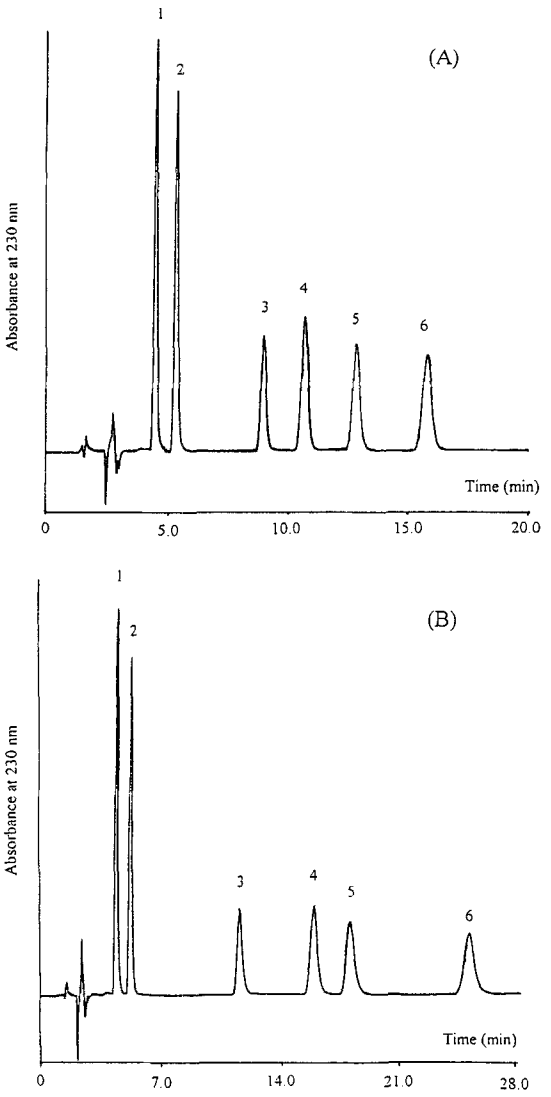

FIGURE 2.
Chromatograms of optimized isocratic separation of a steroid mixture. (A) column : LichroCART RP-8 (250 - 4 mm, 7 mm); mobile phase : methanol-acetonitrile-water (37:32:31, v/v/v). (B) column : LichroCART RP-18 (25 0 - 4 mm., 7 mm); mobile phase : methanol-acetonitrile-water (37:34:29, v/v/v). For compound number, see Table 1.

acetonitrile-water (37:34:29 v/v/v) with RP-18 column used, respectively. On testing these optimum theoretical conditions with the HPLC system, satisfactory separations were obtained. The actual t_R and *R*s values were closely corresponding to the predicted values (Table 5 and Fig. 2). With the same resolution of chromatograms, analysis time required was shorter in a RP-8 column and longer in a RP-18 column.

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