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Computer-aided optimization of the experimental conditions for the isocratic reversed-phase high-performance liquid chromatographic separation of hormonal steroids

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ABSTRACT

A computer-aided optimization is described for selecting the optimum conditions for the isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of twenty hormonal steroids. With factorial design and computer simulation, an isocratic RP-HPLC system that separated the twenty steroids simultaneously within 30 min was developed.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a popular technique for hormonal steroid analysis and a wide range of experimental conditions have been used. Reviews of HPLC as applied to steroid analysis have been published by Heftmann and Hunter [1], Kautsky [2] and Robards and Towers [3]. For a complex biological sample containing many hormones, the use of a ternary mobile phase permitted high selectivity [4–6]. However, the selection of the optimum mobile phase composition and other conditions is difficult and complex for multi-component separations by isocratic RP-HPLC. A few optimization procedures have been described, such as solvent triangle [7], window diagram [8,9] and factorial design techniques [10–12] and a multi-criteria decision-making method [13]. In this paper, optimization of an isocratic RP-HPLC system for the separation of twenty steroid hormones by means of factorial design and computer simulation, which has been used to separate fourteen steroids perfectly [14], is described.

EXPERIMENTAL

Chemicals

Methanol was of general-reagent grade. Tetrahydrofuran was analytical-reagent grade and was redistilled before use. The serial numbers, trivial names and abbreviations of the steroid standards used are listed in Table I. Of these samples, 11-hydroxyandrostenedione (11-OHA), 11-hydroxytestosterone (11-OHT), 11-hydroxy-

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TABLE I

SERIAL NUMBERS, TRIVIAL NAMES AND ABBREVIATIONS OF STEROIDS

No.	Trivial name	Abbreviation	No.	Trivial name	Abbreviation
1	Cortisone	E	11	Androstenedione	A
2	Cortisol	F	12	Methandienone	MeA
3	11-Hydroxyandrostenedione	11-OHA	13	11-Deoxycorticosterone	DOC
4	16-Hydroxyprogesterone	16-OHP	14	Testosterone	T
5	11-Hydroxytestosterone	11-OHT	15	11-Hydroxyprogesterone	11-OHP
6	Corticosterone	B	16	17-Methyltestosterone	MeT
7	19-Norandrostenedione	19-NA	17	17-Hydroxyprogesterone	17-OHP
8	11-Deoxycortisol	S	18	Estriol	E_3
9	21-Deoxycortisol	21-DOF	19	Estradiol	E_2
10	19-Nortestosterone	19-NT	20	Estrone	E_1

progesterone (11-OHP), 16-hydroxyprogesterone (16-OHP), 19-norandrostenedione (19-NA) and 21-deoxycortisol (21-DOF) were kindly donated by Dr. Louis Dehennin (Fresngs, France), 19-nortestosterone (19-NT), methandienone and 17-methyltestosterone by Professor Tong-hui Zhou (Beijing, China) and some others by Professor Cheng-yu Ma (Beijing, China) and Professor Xie-liang Su (Tianjin, China).

HPLC

A Model LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) was used, consisting of an LC-6A pump, a Shim-pack CLC-ODS/H column (25 cm × 4.6 mm I.D.) mounted in a CTO-6A column oven, a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop, an SPD-6AV UV–VIS spectrophotometric detector, an RF-535 fluorescence monitor and a Shimadzu C-R4A data processor. The effluent from the analytical column was first monitored at 254 nm by the UV detector and then by the fluorescence detector linked in series, with excitation and emission wavelength of 285 and 310 nm, respectively.

OPTIMIZATION METHOD

The optimization procedure was focused on selecting the optimum mobile phase composition and column temperature. First, the variables were studied in detail by means of a complete two-level factorial design. Then, a computer program was used to simulate chromatograms of these steroids under various experimental conditions. Finally, the theoretically optimum experimental conditions were tested on the HPLC system.

Factorial design

The mobile phase was a ternary system containing tetrahydrofuran, methanol and water. The fractions of tetrahydrofuran and methanol in the solvent were chosen as variables X_1 and X_2, respectively, and the column temperature was chosen as variable X_3 in the factorial design (Table II). The ranges of these variables were set on the basis of prior knowledge from the literature and personal experience. The upper and the lower limits are denoted by 1 and -1, respectively. The retention times (t_R) of

TABLE II

EXPERIMENTAL PRESENTATION AT A COMPLETE TWO-LEVEL FACTORIAL DESIGN

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 = Tetrahydrofuran: +1 = 21% (v/v) −1 = 15% (v/v)
X_2 = Methanol: +1 = 33% (v/v) −1 = 27% (v/v)
X_3 = Column temperature: +1 = 49°C −1 = 41°C

the seventeen steroids with UV absorption were measured by the UV detector in the factorial design experiments, and were then used to estimate the coefficients α_0, α_1, α_2, α_3, α_4, α_5, α_6 and α_7 by Yates' algorithm [15]. The dead time was measured as the first peak resulting from the injection of methanol–water (60:40, v/v).

Computer simulation

A computer program was written in BASIC and run on the C-R4A data processor for simulation of chromatograms while the three variables X_1, X_2 and X_3 changed systematically within certain ranges. The minimum R_s was chosen as a measure of separation and the maximum t_R as a measure of analysis time. The predicted t_R was calculated using the following equation:

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

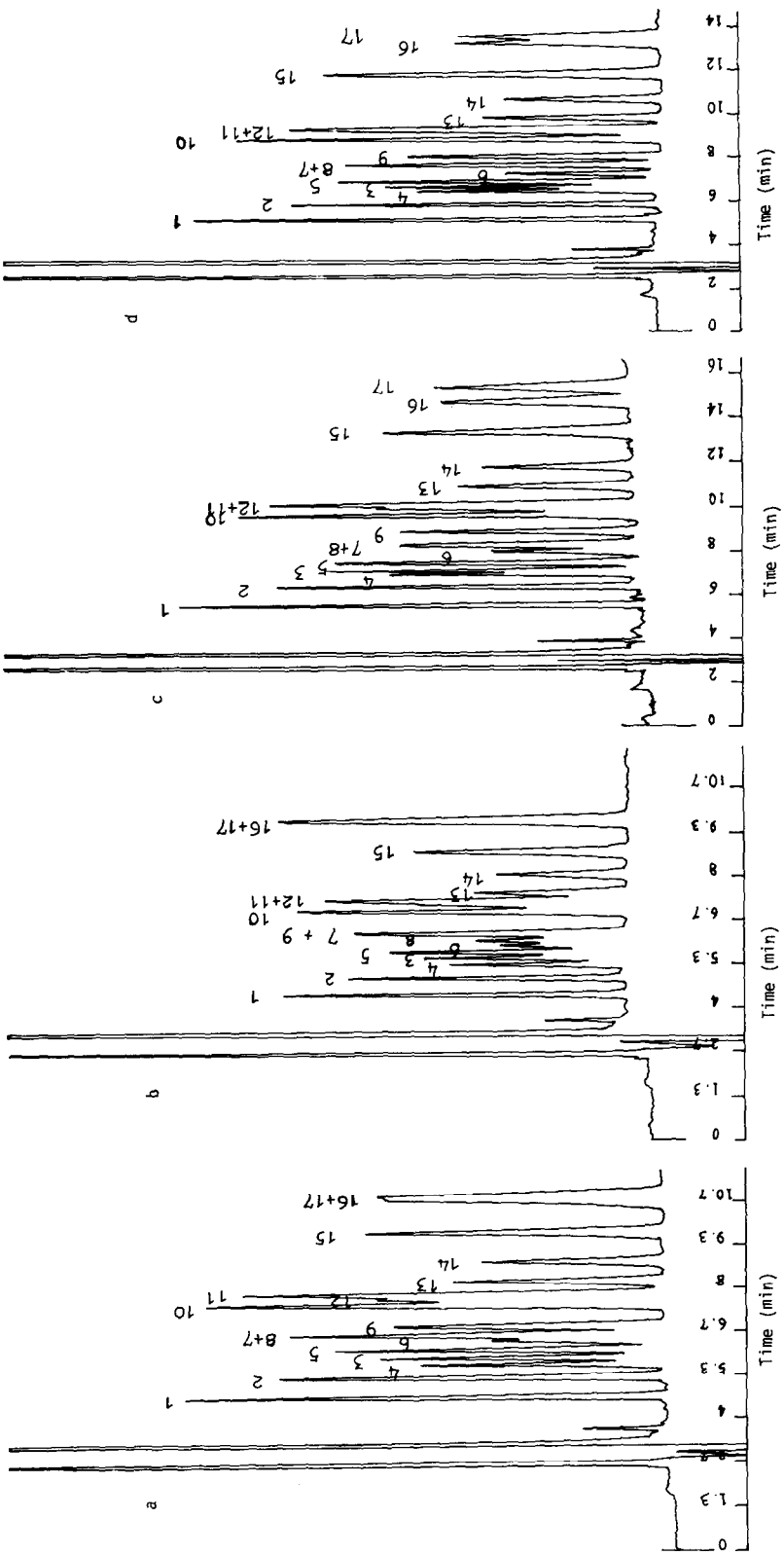
where t_R is the retention time in minutes of an individual steroid under a given set of conditions and X_1, X_2 and X_3 are values of the three variables as indicated above. The predicted t_R values were used to predict R_s by the following equation:

$$R_s = \frac{(t_{R_{i+1}} - t_{R_i})}{A/H_{i+1} + A/H_i} \quad (2)$$

where A/H, provided by the C-R4A automatically, is the area/height ratio of a peak, representing approximately the width at half-height ($W_{\frac{1}{2}}$) of an isosceles triangle ($W_{\frac{1}{2}} = 0.9394A/H$). A linear function describing the relationship between A/H and t_R was obtained on the basis of A/H and t_R values from eight experiments of factorial design. F is a correct coefficient of R_s calculated by A/H instead of peak width (W) by the classical equation

$$R_s = 2(t_{R_{i+1}} - t_{R_i})/(W_{i+1} + W_i) \quad (3)$$

A/H was easier to obtain than W.


a
b
c
d
Time (min)
1
2
3
4
5
6
7+8
9
10
11
12+13
14
15
16+17
16
17

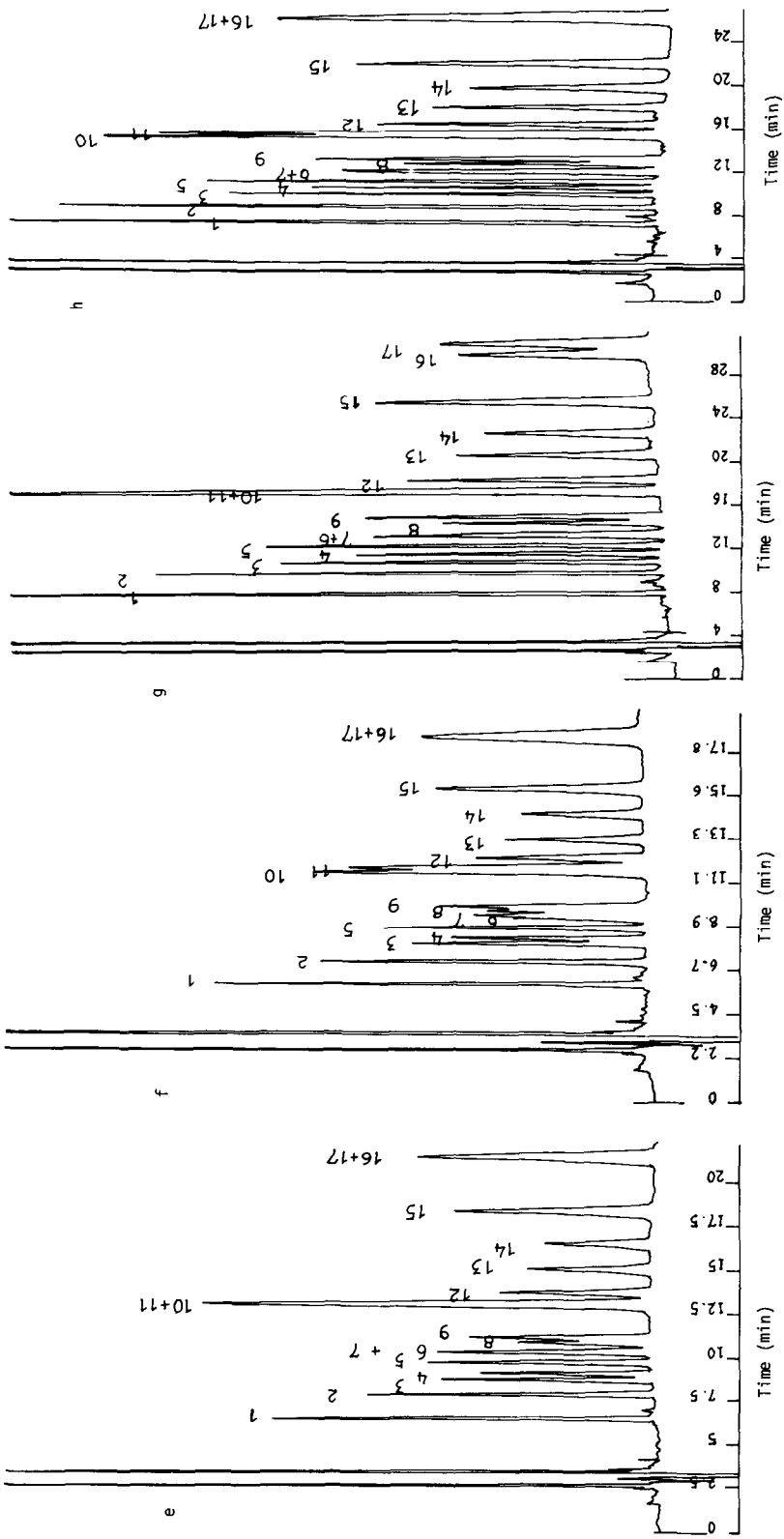


Fig. 1. Chromatograms of a mixture of twenty steroids. Mobile phase composition and column temperature in (a)–(h) as in Table II, Expt. Nos. 1–8, respectively; column, Shim-pack ODS (25 cm × 4.6 mm I.D.); detector, UV (254 nm). Peak numbers represent steroid numbers as in Table I.

TABLE III

RETENTION TIMES (min) OF SEVENTEEN STEROIDS ON CHANGING THE VARIABLES ACCORDING TO TABLE II

Steroid No.[a]	Experiment No.							
	1	2	3	4	5	6	7	8
1	4.67	4.43	5.53	5.16	6.49	5.98	7.91	7.18
2	5.28	4.94	6.39	5.87	7.88	7.11	9.82	8.73
3	5.87	5.55	7.16	6.68	8.73	8.02	10.84	9.85
4	5.65	5.34	6.97	6.48	9.10	8.32	11.62	10.42
5	6.11	5.72	7.51	6.92	9.80	8.83	12.35	10.99
6	6.40	5.92	8.02	7.28	10.46[b]	9.32	13.48[b]	11.81[b]
7	6.59[b]	6.22[b]	8.27[b]	7.71[b]	10.35[b]	9.51	13.24[b]	12.01[b]
8	6.54[b]	6.07	8.40[b]	7.63[b]	10.93	9.75	14.44	12.65
9	6.84	6.28[b]	8.92	8.06	11.25	9.96	15.02	13.07
10	7.47	6.93	9.66	8.85	13.03[b]	11.67	17.24[b]	15.22
11	7.80[b]	7.30[b]	10.16[b]	9.35[b]	13.16[b]	11.94	17.41[b]	15.59
12	7.65[b]	7.15[b]	9.96[b]	9.18[b]	13.76	12.47	18.34	16.34
13	8.20	7.54	10.95	9.86	15.13	13.36	20.69	17.95
14	8.81	8.11	11.82	10.69	16.55	14.64	22.64	19.73
15	9.74	8.82	13.37	11.87	18.40	15.97	25.55	21.83
16	10.68[b]	9.74[b]	14.77	13.27	21.36[b]	18.90[b]	29.98	25.94[b]
17	10.88[b]	9.78[b]	15.47	13.60	21.54[b]	18.56[b]	30.95	26.22[b]

[a] Steroid numbers as in Table I.

[b] The peak of the steroid overlapped the peak of another steroid.

TABLE IV

CALCULATED MEAN EFFECTS (α VALUES) ON THE RETENTION TIMES OF SEVENTEEN STEROIDS ON CHANGING THE VARIABLES ACCORDING TO TABLE II

Steroid No.[a]	α_0	α_1	α_2	α_3	α_4	α_5	α_6	α_7
1	5.92	−0.97	−0.53	−0.23	0.13	0.08	0.04	−0.01
2	7.00	−1.38	−0.70	−0.34	0.19	0.13	0.06	−0.02
3	7.84	−1.52	−0.79	−0.31	0.19	0.11	0.05	−0.01
4	7.99	−1.88	−0.88	−0.35	0.27	0.15	0.08	−0.03
5	8.53	−1.96	−0.91	−0.41	0.26	0.17	0.07	−0.02
6	9.09	−2.18	−1.06	−0.50	0.32	0.20	0.10	−0.03
7	9.24	−2.04	−1.07	−0.38	0.28	0.14	0.07	−0.03
8	9.55	−2.39	−1.23	−0.53	0.37	0.22	0.11	−0.04
9	9.92	−2.40	−1.34	−0.58	0.38	0.23	0.12	−0.04
10	11.26	−3.03	−1.48	−0.59	0.46	0.25	0.12	−0.05
11	11.86	−3.37	−1.60	−0.57	0.51	0.25	0.12	−0.05
12	11.59	−2.94	−1.54	−0.54	0.44	0.22	0.11	−0.04
13	12.96	−3.82	−1.90	−0.78	0.63	0.34	0.18	−0.07
14	14.12	−4.27	−2.10	−0.83	0.70	0.37	0.18	−0.07
15	15.69	−4.74	−2.46	−1.07	0.79	0.47	0.23	−0.09
16	18.08	−5.96	−2.91	−1.12	1.01	0.51	0.27	−0.13
17	18.37	−5.94	−3.18	−1.33	1.08	0.59	0.32	−0.12

[a] Steroid numbers as in Table I.

RESULTS AND DISCUSSION

The seventeen steroids with UV absorption were not separated simultaneously in the eight factorial design experiments (Fig. 1, Table III). The elution order of some steroids varied with the experimental conditions, such as 16-OHP and 11-OHA, and S and 19-NA, which made the separation complex.

The individual 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 IV). Negative α_1, α_2 and α_3 indicated that there was a negative relationship between variables X_1, X_2 and X_4 and t_R. The α_0 values are average retention times of individual steroids, including the dead time. In eight experiments, the dead time was 2.62 $\pm$ 0.02 min (mean $\pm$ S.D.) with a relative standard deviation of 0.7%.

With the computer program, no satisfactory HPLC conditions were found between -1 and $+1$, so the domain of variables searched was extended to -3 and $+3$. Optimum theoretical HPLC conditions were found where X_1, X_2 and X_3 were -0.333, -2 and $+1$, respectively, representing the mobile phase composition tetrahydrofuran–methanol–water (17:24:59, v/v/v) and a column temperature of 49°C. When the optimum theoretical HPLC conditions were tested with the HPLC system, a satisfactory separation was obtained. The actual t_R and R_s values corresponded closely to the predicted values (Table V, Fig. 2). The twenty hormonal steroids, including three natural fluorescent materials, E_1, E_2 and E_3, were simultaneously separated within 30 min and progesterone could be eluted within 45 min. The results suggested that the optimization procedure for isocratic RP-HPLC conditions is simple, accurate and rapid.

TABLE V

COMPARISON BETWEEN ACTUAL AND PREDICTED RETENTION TIMES (t_R)

Mobile phase, tetrahydrofuran–methanol–water (17:24:59, v/v/v); flow-rate, 1 ml/min; column, Shim-pack ODS (25 cm × 4.6 mm I.D.); column temperature, 49°C.

Steroid No.[a]	Actual t_R (min)	Predicted t_R (min)	Steroid No.[a]	Actual t_R (min)	Predicted t_R (min)
1	7.05	7.04	2	8.45	8.46
3	9.66	9.59	4	9.98	9.97
5	10.50	10.55	6	11.27	11.35
7	11.75	11.65	8	12.20	12.19
9	12.85	12.72	10	14.61	14.58
11	15.18	15.07	12	15.63	15.58
13	17.04	17.14	14	18.81	18.83
15	20.96	20.95	16	24.66	24.62
17	25.23	25.26			

[a] Steroid numbers as in Table I.

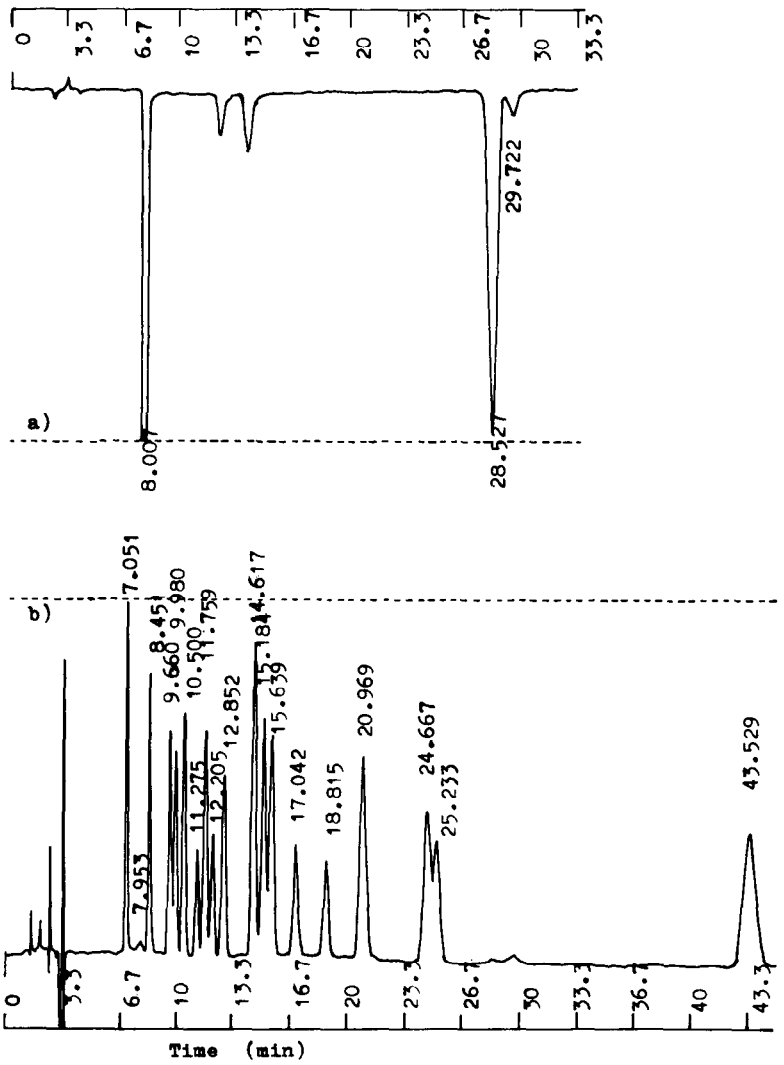


Fig. 2. Optimized isocratic separation of steroid mixture. Column, Shim-pack ODS (25 cm × 4.6 mm I.D.); column temperature, 49°C; mobile phase, tetrahydrofuran–methanol–water (17:24:59, v/v/v); flow-rate, 1 ml/min; detector, (a) fluorescence ($\lambda_{ex.}$ 285 nm, $\lambda_{em.}$ 310 nm); (b) UV (254 nm). Peaks: (a) 8.007 = estriol; 28.527 = estradiol; 29.722 = estrone; (b) 7.051 = E; 7.953 = estriol; 8.451 = F; 9.66 = 11-OHA; 9.98 = 16-OHP; 10.50 = 11-OHT; 11.275 = B; 11.759 = 19-NA; 12.205 = S; 12.852 = 21-DOF; 14.617 = 19-NT; 15.184 = A; 15.639 = MeA; 17.042 = DOC; 18.815 = T; 20.969 = 11-OHP; 24.667 = MeT; 25.233 = 17-OHP; 43.529 = progesterone.

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