Journal of Chromatography, 142 (1977) 689–703

CHROM. 10,285

CORRELATION OF RETENTION BEHAVIOUR OF STEROIDAL PHARMACEUTICALS IN POLAR AND BONDED REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY

SHOJI HARA and SUMIE HAYASHI

Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03 (Japan)

SUMMARY

For systematization of the correlation between the chemical structures of solutes and retention behaviour in liquid column chromatography, the retention volumes of 43 modified steroids on silica and chemically bonded reversed-phase columns were examined using various binary solvent systems. Retention parameters for the functional groups of the steroids were calculated according to Martin's additive rule. By comparing these values obtained on normal and reversed-phase columns, characteristic features of both packings with regard to solute structures and the solvent systems were elaborated.

INTRODUCTION

The adaptation of stationary and mobile phase systems for a given set of solutes is particularly important in liquid column chromatographic separations. However, up to now it has been necessary to rely upon empirical and trial-and-error methods. There are various approaches for optimizing packings and solvent systems, and a procedure in which the retention behaviours of a series of compounds are systematically examined and in which the observed correlation between stationary and mobile phases and solute structures may be generalized would be useful.

Synthetic steroids, utilized as pharmaceuticals, contain a wide variety of functional groups and many positional and configurational isomers, and therefore they are very suitable for a systematic investigation of solute–solvent–sorbent relationships. Although abundant liquid column chromatographic data on steroids are already available[1–5], they have been restricted to a limited number of typical steroids that are well known as biologically important constituents. A detailed study in which elution behaviour as a function of chemical structure is particularly stressed has not yet been presented. It would be worthwhile examining systematically the mobilities of modified steroids in various chromatographic systems and formulating the relative retention behaviours of these compounds.

This paper describes studies on the retention data of 43 steroids, obtained by applying two typical stationary phases, *viz.*, polar silica and a non-polar chemically

bondedphase, in high-performance liquid chromatography (HPLC). The quantitative correlation between the retention of the solutes and their chemical structures with regard to the various systems is demonstrated.

EXPERIMENTAL

Apparatus

Chromatographic experiments were performed using a Waters ALC 202 liquid chromatograph with Waters UV and RI detectors. The UV detector operated at 254 nm. Columns were constructed of 2.3 mm I.D. seamless stainless-steel tubing and were 2 ft. long. The columns were filled by the dry tap–fill procedure. On-column septum injections were made with a 10-μl syringe (Kusano Scientific, Tokyo, Japan).

Column media and chemicals

A silica packing (Corasil II, Waters Assoc., Milford, Mass., U.S.A.) and a bonded-phase packing (Bondapak C_{18}/Corasil, Waters Assoc.) were used as polar adsorbent and reversed-phase column media, respectively. Technical-grade solvents (Wako Pure Chemicals, Osaka, Japan) were purified by distillation. Ethyl acetate was distilled after standing over potassium carbonate to remove acetic acid. The steroid samples used were pure crystalline materials obtained from pharmaceutical companies as described in earlier reports[6–8].

Procedures

Flow-rates were 1.0 ml/min for the Corasil II column and 1.5 ml/min for the Bondapak C_{18}/Corasil column with observed pressure drops of 800–1200 psi. An amount of 1–5 mg of sample was dissolved in 1 ml of a mobile phase solvent. For samples that were not soluble in one of these solvents, chloroform or dioxan was used for injection into the polar or reversed-phase column. A volume of 1–5 μl of sample solution was injected into the column. All results were obtained at ambient temperature (15 $\pm$ 5°).

Hold-up volume, V_0

Non-retained solvents such as cyclohexane and benzene were injected into the silica column and were detected by the RI and UV detectors, respectively. The retention volumes of these solvents were considered to be the hold-up volume of the column. Methanol or acetonitrile was used for measuring the hold-up volume of the reversed-phase column, the peaks being detected with an RI detector.

Retention volume, V_R, and capacity factor, k'

The observed retention volume, V_R', is obtained directly in the chromatographic separation, and the net retention volume, V_R, is given by $V_R = V_R' - V_0$. With the bonded-phase column, the observed values were calculated from the relationship V_R' = (distance between injection point and peak maximum/chart speed) $\times$ flow-rate. On the other hand, as reproducible results were not afforded by the polar-phase column, an internal standard was provided for correction of the observed values. A non-retained sample, *e.g.*, benzene, and one of the steroid samples were selected as the internal standards. Firstly, solutions of sample and benzene, then

sample, internal standard and benzene, were injected. When the difference between the retention data of these samples was less than 0.2 ml, the average value was calculated and corrected by using the internal standard data. When the difference was more than 0.2 ml, the sample solution was injected repeatedly in order to obtain a more reliable value. The samples were simply mixed in a microsyringe.

Correction of the data based on the internal standard method

The corrected retention volume is given by

$$V_R = [V_R' - V_R\,(\text{benzene})'] \cdot \frac{V_R\,(\text{IS})}{V_R\,(\text{IS})' - V_R\,(\text{benzene})'}$$

where V_R(IS) is the retention volume of the internal standard which had been measured previously in repeated experiments, the procedure for which is described above, and V_R(IS)′ and V_R(benzene)′ are the observed retention volumes for the internal standard and unretained benzene, respectively. The capacity factor is calculated from $k' = V_R/V_0$.

Sequence of elution of steroids

The elution sequence was determined for a given solvent system, then it was confirmed by simultaneous injection of two samples in instances where their retention volumes were closely similar. The sample number was given by the elution sequence using the silica–*n*-hexane–ethyl acetate system.

Relative retention value, log $V_R(A)_T$

In order to compare the retention behaviours for whole steroid samples simultaneously, relative retention volumes were calculated using testosterone as a standard. The relative retention value is defined as $\log V_R(A)_T = \log [V_R(A)/V_R(T)]$, where V_R(A) and V_R(T) are the net retention volumes of sample A and testosterone, respectively. When a sample and testosterone could not be separated simultaneously by using the same solvent, the relative retention value of the sample was obtained by extrapolation of the value of another sample whose retention value had been obtained in the same two-component solvent system but with different proportions of the components.

For example, the relative retention value, $\log V_R(A)_T$, of a sample A can be obtained directly by comparison with the value for testosterone which was injected simultaneously as the standard substance when the Corasil II–ethyl acetate–*n*-hexane (1:4) system is applied. On the other hand, with the same solvent system with different proportions of the components, *e.g.*, ethyl acetate–*n*-hexane (1:9), the relative retention value of sample B may be calculated from the value of progesterone (sample 30), where

$$\log [V_R\,(\text{B})/V_R\,(\text{T})] = \log \frac{V_R\,(\text{B})/V_R\,(30)\ [\text{ethyl acetate–}n\text{-hexane (1:9)}]}{V_R\,(30)/V_R\,(\text{T})\ [\text{ethyl acetate–}n\text{-hexane (1:4)}]}$$

When the ethyl acetate–*n*-hexane ratio is changed to 1:19, repeated extrapolation of

the value of sample 4 is required in order to obtain the relative retention value of sample C, where

$$\log [V_R(C)/V_R(T)] = \log \frac{V_R(C)/V_R(4)\ [\text{ethyl acetate–}n\text{-hexane (1:19)}]}{V_R(4)/V_R(30)\ [\text{ethyl acetate–}n\text{-hexane (1:9)}]} \cdot \frac{1}{V_R(30)/V_R(T)\ [\text{ethyl acetate–}n\text{-hexane (1:4)}]}$$

Retention parameter of a functional group of the steroid, $\Delta\log V_R$

For evaluation of the relationship between retention behaviour and the molecular structure of steroids, the contribution of a functional group to the retention value or capacity factor was calculated. Consider a pair of compounds, P and Q, with similar structures but which differ in the presence of one functional group. Their retention volumes and capacity factors are $V_R(P)$, $V_R(Q)$ and $k'(P)$, $k'(Q)$, where

$$\Delta\log V_R = \log V_R(Q) - \log V_R(P) = \log k'(Q) - \log k'(P)$$

A suitable solvent ratio is chosen by controlling the solvent composition so that the peaks of compounds P and Q can be detected simultaneously. By using a given solvent system, retention volumes $V_R(P)$ and $V_R(Q)$ were measured with simultaneous injection of both samples. If the retention characteristics of two samples differ too much, an appropriate eluent cannot always be found for simultaneous separation. In this instance, $\Delta\log V_R$ was calculated from the relative retention values, $\log V_R/V_R(T)$, which were determined previously by using testosterone as the standard.

RESULTS AND DISCUSSION

In order to obtain a systematic evaluation of the correlation between retention behaviour and chemical structure with regard to the stationary and mobile phases, it is essential to establish reproducible experimental conditions that give reliable values for the elution characteristics. The following parameters seem to be the most important factors in establishing the reproducibility of the chromatographic data, and they were therefore examined in detail.

Optimal range of the capacity factor

Prior fundamental considerations of the capacity factor in liquid chromatography with respect to the resolution problem[9] indicated that the allowable range is usually $1 \leqslant k' \leqslant 10$. Examination of the reproducibility of actual steroid samples exhibiting a wide range of capacity factors indicated that the retention data are unreliable when the capacity factor exceeds 8. On the basis of these results, it was decided that the present experiments would be carried out so as to maintain the capacity factor in the range of 1–8. Retention of the sample was controlled and optimized by variation of the solvent component ratio.

Use of internal standards

With a chemically bonded packing, repeated injection of the same sample always gave nearly identical retention volumes. On the other hand, the silica column did

not afford reproducible results but produced scattered retention values. However, when the internal standard was used, the corrected values became almost constant, even in the latter instance. Therefore, a few steroid samples with medium-range capacity factors were selected as internal standards for various solvent systems in order to obtain accurate retention values.

Solvent for sample injection, flow-rate and temperature effects

The solvent used for the mobile phase is commonly preferred for injection of the sample. However, as the steroid sample is sometimes hardly soluble in such solvents, other solvents are needed for the introduction of the sample into the column. In this experiment, dioxan and chloroform were used for the reversed- and polar-phase columns, respectively. Examination of the effects of these solvents on the capacity factors of several steroids did not reveal any significant deviations. The effects of flow-rate and temperature on the retention data were examined and it was found that minor changes in ambient temperature and in the flow-rate (within few ml/min) had negligible effects. These observations suggested that the measurement of reproducible retention data is possible if the experimental conditions described above are adopted.

The various steroidal pharmaceuticals shown in Table I were examined. In this table they are classified in three groups: C_{18}, C_{19} and C_{21} steroids. As all of the steroids studied contain an α,β-unsaturated carbonyl group or an aromatic ring in their nuclei, their peaks are detectable with a UV detector operating at 254 nm.

The initial packing selected was a commercial reversed-phase packing in which the silica surface had an octadecylsilyloxy group bonded to it. This phase is well known as a typical stationary phase applicable to the separation of a wide range of compounds. The mobile phases chosen were methanol–water and acetonitrile–water, which have been widely used with reversed-phase bonded columns.

The other packing was also a commercial silica which is the most common adsorbent for liquid chromatography. Binary solvent mixtures containing non-polar and polar solvents are usually adopted for the polar silica column. The non-polar solvent used here was *n*-hexane and the polar solvents were ethyl acetate, diethyl ether and 2-propanol.

As the elution behaviour of the steroids in a liquid–solid chromatographic system corresponds to their polarities, and as a systematic correlation in a silica thin-layer chromatographic system had already been accomplished[6–8], it seemed convenient to select one of the adsorption systems as the basic standard for examination of the elution behaviour in various chromatographic systems. Therefore, the steroid samples in each column in Table I were arranged and numbered according to the elution sequence using silica–*n*-hexane–ethyl acetate, one of the liquid–solid systems.

Table II gives examples of systematic elution data showing the retention volumes of steroid samples in one adsorption and two typical reversed-phase systems. Because the ratio of the components in the binary solvent system was varied stepwise to cover the elution characteristics of all steroid samples, the retention data were divided into several columns corresponding to the particular solvent compositions. Internal standards selected for a specific solvent system were also assigned in this table.

For the comparison of the elution characteristics of all samples on the same scale, the data obtained by using different solvent ratios were combined. As testosterone

TABLE I

STEROIDAL PHARMACEUTICALS USED FOR LIQUID CHROMATOGRAPHY

No.	*Steroid**	*No.*	*Steroid*
1	3,17β-Dipropionyloxy-E-1,3,5(10)-triene	23	17β-Hydroxy-17α-Me-A-4-en-3-one
2	3-Methoxy-17α-Etin-E-1,3,5(10)-trien-17β-ol	24	17β-Hydroxy-A-4-en-3-one (testosterone)
3	17β-Valeryloxy-E-1,3,5(10)-trien-3-ol	25	17β-Hydroxy-17α-Me-A-1,4-dien-3-one
4	3-Benzoyloxy-16α,17β-diacetoxy-E-1,3,5(10)-triene	26	14α-Hydroxy-A-4-ene-3,17-dione
5	17α-Etin-E-1,3,5(10)-triene-3,17β-diol	27	6β,17β-Dihydroxy-A-4-en-3-one
6	3-Hydroxy-E-1,3,5(10)-trien-17-one (estrone)	28	14α,17β-Dihydroxy-A-4-en-3-one
7	17β-[β-(Phenylpropionyloxy)]-E-4-en-3-one		
8	17β-[β-(2-Furylpropionyloxy)]-E-4-en-3-one	29	17α-Caproyloxy-P-4-ene-3,20-dione
9	3-Benzoyloxy-E-1,3,5(10)-trien-17β-ol	30	P-4-ene-3,20-dione (progesterone)
10	E-1,3,5(10)-triene-3,17β-diol (estradiol)	31	9β,10α-P-4,6-diene-3,20-dione
11	17β-Hydroxy-17α-Etin-E-4-en-3-one	32	17α-Acetoxy-6-Me-P-4,6-diene-3,20-dione
12	17β-Hydroxy-17α-Et-E-4-en-3-one	33	17α-Acetoxy-6-Cl-P-4,6-diene-3,20-dione
13	17β-Hydroxy-17α-Me-E-4-en-3-one	34	17α-Hydroxy-21-acetoxy-P-4-ene-3,11,20-trione (cortisone acetate)
14	17β-Hydroxy-E-4-en-3-one (19-nortestosterone)	35	11β,17α-Dihydroxy-21-acetoxy-16α-Me-6α,9α-di-F-P-1,4-diene-3,20-dione
15	E-1,3,5(10)-triene-3,16α,17β-triol (estriol)	36	11β,17α-Dihydroxy-21-acetoxy-P-4-ene-3,20-dione (hydrocortisone acetate)
		37	11β,17α,21-Trihydroxy-16α-Me-9α-F-P-1,4-diene-3,20-dione
16	17β-Propionyloxy-4-Cl-A-4-en-3-one	38	11β,17α,21-Trihydroxy-16β-Me-9α-F-P-1,4-diene-3,20-dione
17	17β-Acetoxy-4-Cl-A-4-en-3-one	39	17α,21-Dihydroxy-P-4-ene-3,11,20-trione (cortisone)
18	17β-Propionyloxy-A-4-en-3-one	40	11β,17α,21-Trihydroxy-P-4-ene-3,20-dione (hydrocortisone)
19	17β-Acetoxy-A-4-en-3-one	41	11β,17α,21-Trihydroxy-6β-Me-P-1,4-diene-3,20-dione
20	17β-Acetoxy-1-Me-A-1-en-3-one	42	11β,17α,21-Trihydroxy-P-1,4-diene-3,20-dione (prednisolone)
21	17β-Hydroxy-6α-Me-17α-(1-propynyl)-A-4-en-3-one	43	11β,16α,17α,21-Tetrahydroxy-9α-F-P-1,4-diene-3,20-dione
22	17β-Hydroxy-17α-Etin-A-4-en-3-one		

* Parent compounds and substituents: E = estrane; A = androstane; P = pregnane; Me = methyl; Et = ethyl; Etin = ethynyl; F = fluoro; Cl = chloro.

lies in the middle of the elution sequence, it is a desirable standard compound and relative retention data were calculated using testosterone as the standard. Because the logarithm of the capacity factor or retention volume is considered to be a thermodynamic parameter of a migrant for a given column and as these values are available from Martin's additive rule, it was convenient to calculate these values for a precise examination of the relationship between chromatographic data and steroid structure in order to evaluate the contribution of the functional groups to the retention behaviour. Therefore logarithms of the retention volumes corrected with testosterone as the standard, were calculated and were defined as relative retention values, log $V_R(A)_T$.

Initial values for a sample which was eluted together with testosterone in a given solvent system were calculated by direct comparison with the retention volume of testosterone. Other values were derived successively from the initial value by using another internal standard selected for each solvent system. Adjustment of retention data using data obtained for an internal standard in two solvent systems adjacent in the table provided relative retention values for all samples.

To examine the correlation between elution behaviour and the chemical structure of the steroids, pairs of compounds having the same basic structure but differing in one functional group were selected. Relative retention values for these pairs of steroids are illustrated in Fig. 1, where sample numbers that were given by the elution sequence in the silica–*n*-hexane–ethyl acetate system (Table I) are listed on the abscissa and relative retention values obtained by using two reversed phases and three polar phases are plotted on the ordinate.

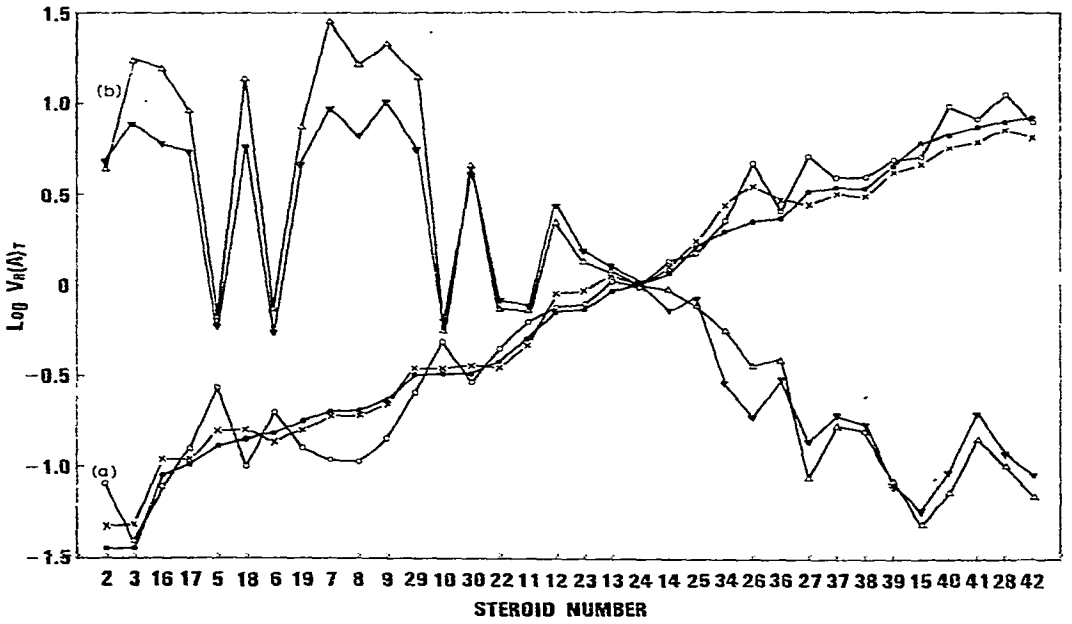

Fig. 1. Relative retention values of steroidal pharmaceuticals. (a) Packing: Corasil II. Solvents: ●, *n*-hexane–ethyl acetate; ×, *n*-hexane–diethyl ether; ○, *n*-hexane–2-propanol. (b) Packing: Bondapak C_{18}/Corasil. Solvents: ▼, methanol–water; △, acetonitrile–water.

TABLE II

RETENTION VOLUMES OF STEROIDAL PHARMACEUTICALS

The numbers of the steroids correspond to those given in Table I.

No. of steroid	*Silica (Corasil II) packing*				*No. of steroid*	*Reversed-phase (Bondapak C_{18}/Corasil) packing*							
	n-Hexane–ethyl acetate (v/v)					*Methanol–water (v/v)*			*Acetonitrile–water (v/v)*				
	19:1	*9:1*	*4:1*	*3:2*		*2:3*	*1:1*	*4:1*	*1:9*	*1:4*	*3:7*	*2:3*	*1:1*
1	0.85	0.26			43	0.60			1.50	0.60			
2	2.79	0.78			15	1.40			4.65	1.65			
3	2.79	0.82			39	2.00*			7.10*	2.70*			
16	6.83	1.87			42	2.20			6.30	2.20			
4	7.30*	2.00*	0.39		40	2.30			6.80	2.30			
17	8.95	2.31	0.55		28	3.10				3.50			
5	9.98	2.77	0.69		27	3.40				2.90			
18	10.83	3.00	0.80		38	4.30				5.30			
6	11.84	3.23	0.92		37	4.60				5.60			
19		3.70	0.96		26	4.60				12.10			
20		3.71	0.96		41	4.90				4.80			
7		4.21	1.12		34	7.40	2.20			19.40	3.65		
8		4.21	1.12		36	7.80*	2.30*			13.20*	2.50*		
9		4.58	1.28		6		3.90				4.80		
21		6.85	1.52		5		4.00				4.60		
29		6.85	1.53		35		4.10			22.40	4.18		

31	10.25	2.60		25	6.00			1.35	
32		3.30		24	7.40*			1.70	0.75
12		3.77		13	9.50			2.02	0.95
23		3.92		23	11.50			2.30	1.02
13		5.10	0.96	21	20.40			4.70	1.60
33		5.10	1.02	12	20.80			4.00	1.35
24		5.20*	1.10*	31	22.10			5.00	1.65
14		6.20	1.15	32	24.60			5.60	1.70
25		8.10	1.68	33	27.80*	1.15*		5.73*	1.75*
34			2.20	30		1.25			2.58
35			2.45	19		1.40			4.00
26			2.45	2		1.50			2.20
36			2.50	17		1.55			4.90
27			3.55	29		1.65			8.10
37			3.65	20		1.70			5.10
38			3.65	18		1.75			7.60
39			4.71*	16		1.85			8.30
15			6.60	8		2.00			8.52
40			6.90	3		2.35			9.35
41			7.80	7		2.95			15.30
28			8.20	9		3.10			11.15
42			8.70	4		3.30			14.92
43			9.05	1		3.50			19.27

* Internal standards.

From Fig. 1, the following conclusions can be drawn.

(1) Elution sequences are mainly dependent upon the stationary phase rather than the mobile phase. In general, the sequences in the reversed-phase separations are opposite to those in the polar-phase separations, in accordance with widely accepted concepts.

(2) However, there are several inversions of the sequences in the reversed-phase system compared with those in the polar-phase system. The relative retentions of steroids 2, 5, 6, 10, 11, 15 and 22 are much smaller than expected if the order would be reversed from the polar-phase system. Examination of the structures of these samples revealed that the steroids which showed unusual behaviour were phenols or phenol ether and ethynyl derivatives. These results suggest that π-bonding electrons in estratrienes or ethynyl compounds decrease the retention values in reversed-phase systems.

(3) With the reversed-phase packing, the difference in the retention sequences with different mobile phases is very small; methanol–water and acetonitrile–water systems gave parallel retention values.

(4) On the other hand, with the adsorption packing, a difference in retention sequences with different solvent systems was clearly observed. Although the sequences in diethyl ether- and ethyl acetate-containing solvent systems were similar, the sequence in the 2-propanol-containing system is sometimes different from the others. For example, the retention values of steroids 5, 6, 10, 26 and 28 are increased in the last solvent system compared with the first two systems. Steroids 5, 6 and 10 are phenolic estratrienes and steroids 26 and 28 contain a 14α-hydroxyl group. Similar solvent effects were also observed in the thin-layer chromatography of steroidal pharmaceuticals[8]. It was suggested that interactions between these hydroxyl groups and basic solvents that are classified as B-type solvents[10], such as diethyl ether and ethyl acetate, in mobile phases were so large that the adsorptivities in these solvents were decreased compared with those in a system containing a protic solvent which is classified as an AB-type solvent[10]. It has been assumed that the steroid molecule is usually localized with its α-side on the silica surface. Therefore, a 14α-hydroxyl group would contribute significantly towards the adsorptivity of the steroid. These considerations allow us to interpret the results as follows: desorption, provided by the considerable interaction between these steroids and B-type solvents, increases the relative retention of the solutes in AB-type solvent systems.

By using the relative retention values shown in Fig. 1, parameters for various functional groups can be readily derived. However, it should be recalled that these pairs of relative retention values were usually obtained indirectly from the values originally measured by using a variety of solvent compositions, as described above. If the retention data for a pair of steroids were measured directly, using the same solvent system, more reliable data could be obtained. Therefore, a given pair of steroids was injected simultaneously into a column using a suitable solvent system in which both steroid compounds afforded optimal retention values. Retention data for a pair of compounds were thus afforded with respect to two stationary phases and several mobile phases that were selected as described above. Parameters for various functional groups and also changing functional groups were obtained directly following this procedure and are given in Table III. The first two columns include data for a reversed-phase packing and of the last three columns for a polar-packing,

respectively. However, the retention differences of some pairs of steroids were extremely large and therefore a suitable solvent system for simultaneous analysis could not be prepared. In this instance, the calculated values given by the successive approximation procedure as described earlier were included in this table. These values are indicated by the designation "(calcd.)" in the table.

Examination of the parameters in this table revealed the following facts.

(1) The 6β-, 14α- and 16α-hydroxyl groups afford large absolute values of the parameters for either stationary phase. In reversed-phase systems, negative parameters with little differences among these hydroxyl groups are obtained. Conversely, in polar-phase systems, positive parameters are afforded. Parameters of the 14α- and 16α-hydroxyl groups are larger than parameters of 6β-hydroxyl group, which is interpreted as follows: the 6β-hydroxyl group is sterically hindered by the 10β- and 13β-methyl groups in a 1,3-diaxial configuration. The polar system will thus be preferred to the reversed-phase system for the separation of the positional or configurational isomers such as the hydroxyl derivatives described above.

(2) Acylation of alcohol hydroxyl groups also affords large positive and negative values for the reversed and polar phases, respectively. With the reversed-phase systems, the parameter for acylation of the 17β-hydroxyl group increases with the size of the alkyl group in the acyl derivative in the order Me $<$ Et $<$ Bu. The values for β-phenylpropionyloxy and β-2-furylpropionyloxy groups are approximately similar to the values for the valeryloxy group. Acylation of 21- and 17β-hydroxyl groups gives a similar contribution to the mobilities of steroids. With the polar-phase systems, the differences in the parameters among various acyloxy groups are interpreted in terms of the differences in the steric hindrances of these groups. Acylation of the 21-hydroxyl group affords smaller negative parameters than that of the 17β-hydroxyl group. In general, absolute values in polar- and reversed-phase systems for given functional groups vary in a parallel manner. However, net values in reversed-phase systems are always larger than those in polar-phase systems. Therefore, the former systems are more advantageous than the latter for the separation of the hydroxy steroids and their acyl derivatives and the corresponding acyloxy steroids. Exchange of a 17β-hydroxyl group with an acetyl group results in parameters smaller than those obtained from the exchange of a 17β-hydroxyl group with an acyloxy group.

(3) Alkylation and acylation of a phenolic hydroxyl group in reversed-phase systems afford especially large positive values. The parameters of the benzoyl group are always larger than those of the methyl group in those conversions. Conversely, in polar-phase systems, negative parameters whose absolute values are smaller than those from the former systems are obtained.

(4) The introduction of an alkyl group into the 17α-position of a steroid with a β-hydroxyl group always affords positive parameters, which increase from methyl to ethyl in the reversed-phase systems. The introduction of an acyloxy group into the 17α-position also gives positive values. These results are explained by the increase in the lipophilicity of the steroid. However, the effect of the ethynyl group, giving extremely small values of the parameters, is unusual. This characteristic of the ethynyl group affords large positive parameters for the conversion of a 17α-ethynyl group into an ethyl group. This behaviour is remarkable in reversed-phase systems compared with polar-phase systems. With the polar phase, the negative parameters increase in order of the groups Me $<$ Et $<$ Etin. These values run parallel with the

TABLE III

RETENTION PARAMETERS OF CONVERTED FUNCTIONAL GROUPS, ΔLOG V_R

*Functional group**		*Steroid*		*Reversed-phase (Bondapak C_{18}/Corasil) packing*					
From	*To*	*From*	*To*	*Methanol–water (v/v)***			*Acetonitrile–water (v/v)***		
				Δ log V_R	*Retention volume (ml)*		*Δ log V_R*	*Retention volume (ml)*	
					P	*Q*		*P*	*Q*
6β-H	OH	24	27	−0.83 (1:1)	7.40	1.10	−1.08 (calcd.)	1.70 (2:3)	2.90 (1:4)
14α-H	OH	24	28	−0.91 (calcd.)	7.40 (1:1)	3.10 (2:3)	−0.99 (calcd.)	1.70 (2:3)	3.50 (1:4)
16α-H	OH	10	15	−1.03 (calcd.)	4.40 (1:1)	1.40 (2:3)	−1.05 (1:4)	18.60	1.65
17β-OH	OCCOMe	24	19	0.66 (calcd.)	7.40 (1:1)	1.40 (4:1)	0.69 (2:3)	1.70	8.40
	OCOEt	24	18	0.76 (calcd.)	7.40 (1:1)	1.75 (4:1)	1.01 (1:1)	0.75	7.60
	$OCO(CH_2)_3CH_3$	10	3	1.11 (calcd.)	4.40 (1:1)	2.35 (4:1)	1.54 (calcd.)	3.40 (3:7)	9.35 (1:1)
	$OCO(CH_2)_2C_6H_5$	14	7	1.12 (calcd.)	5.45 (1:1)	2.95 (4:1)	1.48 (calcd.)	1.65 (2:3)	15.30 (1:1)
	$OCO(CH_2)_2C_4H_4O$	14	8	0.95 (calcd.)	5.45 (1:1)	2.00 (4:1)	1.23 (calcd.)	1.65 (2:3)	8.52 (1:1)
21-OH	OCOMe	39	34	0.57 (calcd.)	2.00 (2:3)	2.20 (1:1)	0.86 (1:4)	2.70	19.40
		40	36	0.53 (2:3)	2.30	7.80	0.76 (1:4)	2.30	13.20
17β-OH	COMe	24	30	0.61 (calcd.)	7.40 (1:1)	1.25 (4:1)	0.54 (1:1)	0.75	2.58
3-OH	OMe	5	2	0.96 (calcd.)	4.00 (1:1)	1.50 (4:1)	0.78 (calcd.)	4.60 (3:7)	2.20 (1:1)
	$OCOC_6H_5$	10	9	1.23 (calcd.)	4.40 (1:1)	3.10 (4:1)	1.62 (calcd.)	3.40 (3:7)	11.15 (1:1)
17α-H	Me	24	23	0.19 (1:1)	7.40	11.50	0.13 (2:3)	1.70	2.30
		14	13	0.24 (1:1)	5.45	9.50	0.09 (2:3)	1.65	2.02
	Et	14	12	0.58 (1:1)	5.45	20.80	0.39 (2:3)	1.65	4.00
	Etin	10	5	−0.04 (1:1)	4.40	4.00	0.13 (3:7)	3.40	4.60
		24	22	−0.09 (1:1)	7.40	6.00	−0.12 (2:3)	1.70	1.30
		14	11	0.00 (1:1)	5.45	5.50	−0.12 (2:3)	1.65	1.25
	$OCO(CH_2)_4CH_3$	30	29	0.12 (4:1)	1.25	1.65	0.50 (1:1)	2.58	8.10
17α-Etin	Et	11	12	0.58 (1:1)	5.50	20.80	0.51 (2:3)	1.25	4.00
C_{11}=O	11β-OH	39	40	0.06 (2:3)	2.00	2.30	−0.07 (1:4)	2.70	2.30
		34	36	0.02 (1:1)	2.20	2.30	−0.17 (1:4)	19.40	13.20
C_{17}=O	17β-OH	6	10	0.05 (1:1)	3.90	4.40	−0.15 (3:7)	4.80	3.40
		26	28	−0.17 (2:3)	4.60	3.10	−0.54 (1:4)	12.10	3.50
4-H	Cl	19	17	0.04 (4:1)	1.40	1.55	0.09 (1:1)	4.00	4.90
		18	16	0.02 (4:1)	1.75	1.85	0.04 (1:1)	7.60	8.30
sat.[1,2]	$\Delta^{1,2}$	23	25	−0.28 (1:1)	11.50	6.00	−0.23 (2:3)	2.30	1.35
		40	42	−0.02 (2:3)	2.30	2.20	−0.02 (1:4)	2.30	2.20
6β-H	Me	42	41	0.35 (2:3)	2.20	4.90	0.34 (1:4)	2.20	4.80
10β-H	Me	14	24	0.13 (1:1)	5.45	7.40	0.01 (2:3)	1.65	1.70
		13	23	0.08 (1:1)	9.50	11.50	0.03 (1:1)	0.95	1.02
		11	22	0.04 (1:1)	5.50	6.00	0.02 (2:3)	1.25	1.30
16α-Me	β-Me	37	38	−0.03 (2:3)	4.60	4.30	−0.02 (1:4)	5.60	5.30

* Abbreviations as in Table I.

** Ratios of components of solvent systems are given in parentheses.

Functional group*		Steroid		Silica (Corasil II) packing								
				n-Hexane–ethyl acetate (v/v)**			n-Hexane–diethyl ether (v/v)**			n-Hexane–2-propanol (v/v)**		
From	To	From	To	Δ log V_R	Retention volume (ml)		Δ log V_R	Retention volume (ml)		Δ log V_R	Retention volume (ml)	
					P	Q		P	Q		P	Q
6β-H	OH	24	27	0.51 (3:2)	1.10	3.55	0.44 (1:1)	2.10	5.78	0.73 (15:1)	0.62	3.29
14α-H	OH	24	28	0.87 (3:2)	1.10	8.20	0.87 (0:1)	0.35	2.60	1.06 (15:1)	0.62	7.10
16α-H	OH	10	15	1.30 (calcd.)	1.57 (4:1)	6.60 (3:2)	1.21 (calcd.)	1.06 (3:2)	9.55 (1:1)	0.85 (15:1)	0.50	3.50
17β-OH	OCOMe	24	19	−0.73 (4:1)	5.20	0.96	−0.72 (calcd.)	3.78 (3:2)	1.67 (4:1)	−0.89 (50:1)	6.99	0.90
	OCOEt	24	18	−0.81 (4:1)	5.20	0.80	−0.79 (calcd.)	3.78 (3:2)	1.63 (4:1)	−1.00 (50:1)	6.99	0.70
	$OCO(CH_2)_3CH_3$	10	3	−0.92 (9:1)	6.88	0.82	−0.87 (4:1)	3.10	0.42	−1.08 (50:1)	3.40	0.28
	$OCO(CH_2)_2C_6H_5$	14	7	−0.74 (4:1)	6.20	1.12	−0.82 (3:2)	4.80	0.73	−1.06 (50:1)	9.15	0.80
	$OCO(CH_2)_2C_4H_4O$	14	8	−0.74 (4:1)	6.20	1.12	−0.82 (3:2)	4.80	0.73	−1.06 (50:1)	9.15	0.80
21-OH	OCOMe	39	34	−0.33 (3:2)	4.71	2.20	−0.40 (1:3)	9.10	3.60	−0.36 (15:1)	3.10	1.35
		40	36	−0.44 (3:2)	6.90	2.50	−0.49 (1:3)	11.90	3.85	−0.62 (15:1)	6.10	1.45
17β-OH	COMe	24	30	−0.48 (4:1)	5.20	1.73	−0.45 (3:2)	3.78	1.35	−0.52 (50:1)	6.99	2.10
3-OH	OMe	5	2	−0.55 (9:1)	2.77	0.78	−0.51 (4:1)	1.35	0.42	−0.42 (50:1)	1.50	0.57
	$OCOC_6H_5$	10	9	−0.18 (9:1)	6.88	4.58	−0.21 (4:1)	3.10	1.91	−0.53 (50:1)	3.40	1.00
17α-H	Me	24	23	−0.12 (4:1)	5.20	3.92	−0.03 (1:1)	2.10	1.96	−0.12 (50:1)	6.99	5.37
		14	13	−0.09 (4:1)	6.20	5.10	−0.01 (1:1)	2.50	2.47	−0.10 (50:1)	9.15	7.32
	Et	14	12	−0.22 (4:1)	6.20	3.77	−0.15 (3:2)	4.80	3.44	−0.25 (50:1)	9.15	5.19
	Etin	10	5	−0.40 (9:1)	6.88	2.77	−0.36 (4:1)	3.10	1.35	−0.24 (50:1)	3.40	1.96
		24	22	−0.42 (4:1)	5.20	2.00	−0.42 (1:1)	2.10	0.80	−0.35 (50:1)	6.99	3.12
		14	11	−0.38 (4:1)	6.20	2.60	−0.44 (3:2)	4.80	1.76	−0.31 (50:1)	9.15	4.50
	$OCO(CH_2)_4CH_3$	30	29	−0.05 (4:1)	1.73	1.53	−0.02 (3:2)	1.35	1.30	−0.06 (50:1)	2.10	1.84
17α-Etin	Et	11	12	0.16 (4:1)	2.60	3.77	0.29 (3:2)	1.75	3.44	0.06 (50:1)	4.50	5.19
C_{11}=O	11β-OH	39	40	0.17 (3:2)	4.71	6.90	0.12 (1:3)	9.10	11.90	0.29 (15:1)	3.10	6.10
		34	36	0.06 (3:2)	2.20	2.50	0.03 (1:3)	3.60	3.85	0.03 (15:1)	1.35	1.45
C_{17}=O	17β-OH	6	10	0.33 (9:1)	3.23	6.88	0.39 (4:1)	1.27	3.10	0.39 (50:1)	1.40	3.40
		26	28	0.53 (3:2)	2.45	8.20	0.39 (0:1)	1.05	2.60	0.38 (15:1)	2.95	7.12
4-H	Cl	19	17	−0.21 (9:1)	3.70	2.31	−0.22 (4:1)	1.67	1.00	−0.11 (50:1)	0.90	0.70
		18	16	−0.21 (9:1)	3.00	1.87	−0.20 (4:1)	1.63	1.03	−0.11 (50:1)	0.70	0.54
Sat.[1,2]	$\Delta^{1,2}$	23	25	0.32 (4:1)	3.92	8.10	0.26 (1:1)	1.96	3.53	0.34 (50:1)	5.37	11.69
		40	42	0.12 (3:2)	6.90	8.70	0.17 (0:1)	1.38	2.05	−0.08 (15:1)	6.10	5.10
6β-H	Me	42	41	−0.05 (3:2)	8.70	7.80	−0.04 (0:1)	2.05	1.86	0.00 (15:1)	5.10	5.10
10β-H	Me	14	24	−0.08 (4:1)	6.20	5.20	−0.10 (3:2)	4.80	3.78	−0.12 (50:1)	9.10	6.99
		13	23	−0.11 (4:1)	5.10	3.92	−0.10 (1:1)	2.47	1.96	−0.14 (50:1)	7.36	5.39
		11	22	−0.11 (4:1)	2.60	2.00	−0.13 (3:2)	1.76	1.31	−0.16 (50:1)	4.50	3.12
16α-Me	β-Me	37	38	0.00 (3:2)	3.65	3.65	0.00 (0:1)	0.96	0.96	0.00 (15:1)	2.17	2.17

size of the group introduced and can be rationalized in terms of the steric hindrance of the functional group interacting with the 17β-hydroxyl group and the silanol group on the adsorbent surface. The introduction of an acyloxy group into the 17-position gives small negative values.

(5) As the reduction of C-11 and C-17 carbonyl groups to a β-hydroxyl group affords small parameters in the reversed-phase systems, the separation of a pair of these compounds is difficult. On the contrary, in the polar-phase systems, large positive parameters are obtained on forming the 17β-hydroxyl group. The conversion of a C-11-carbonyl group into a C-11-hydroxyl group gives rather small parameters. This result is interpreted by the steric hindrance of the 11β-hydroxyl group interacting with the 10β- and 13β-methyl groups located in a 1,3-diaxial relation.

(6) Introduction of chlorine into the 4-position of the 3-oxo-4-ene steroids affords positive and negative parameters in the reversed-phase and polar-phase systems, respectively. The absolute values of the latter are larger than the former.

(7) Derivation of the conjugated diene from the 3-oxo-4-ene steroids affords negative and positive values in the reversed-phase and polar-phase systems, respectively.

(8) The introduction of a methyl group on to the 6β- and 10β-carbon atoms in the steroid nuclei affords positive and negative parameters in the reversed-phase and polar-phase systems, respectively. The absolute values of the parameters of the 6β-methyl group in the former systems are larger than in the latter. However, the absolute values of the parameters of a 10β-methyl group in the former systems are smaller than in the latter. Therefore, the latter systems are favourable for the separation of a pair of these compounds. The negative parameters obtained for the 10β-methyl group in the polar-phase systems are rationalized by the steric hindrance that occurs in the interaction between the silanol group on the adsorbent surface and the active group of the steroids. However, the difference in the mobilities of 16α-methyl and 16β-methyl steroids is very small. The separation of a pair of configurational isomers is extremely difficult in any system.

Parameters of various functional groups in the polar-phase systems obtained here were compared with ΔR_m values of the same groups in steroidal pharmaceuticals given by thin-layer chromatography, in which similar solvent systems such as *n*-hexane–ethyl acetate, benzene–ethyl acetate, diethyl ether and benzene–methanol were used[8]. Although the ΔR_m value for a particular group was always slightly larger than the HPLC parameters, both values changed in parallel. This shows that the parameters obtained here are universally applicable even if the type of chromatography and the activity of the packing materials are changed, provided appropriate correction to the parameters is made.

Using chemically bonded reversed-phase and normal polar-phase packings which are widely accepted as typical stationary phases, the elution behaviours of steroids were correlated by calculating the retention parameters of various functional groups on the rigid steroid molecules. The characteristics of both phases with respect to the chemical structures of the steroid compounds in various solvent systems were determined.

The results obtained here can be used widely for the optimization of the elution conditions for various compounds that have similar functional groups, *e.g.*, the steroid pharmaceuticals considered here. They are also useful for the prediction of the chemical structures of unknown compounds by examining their elution behaviour.

ACKNOWLEDGEMENTS

We thank Professor Masayuki Ishikawa, Director of the Research Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, for his cordial support and Dr. Jack Cazes, Waters Associates, and Dr. Victor Fung, Stanford Research Institute, for their helpful suggestions.

REFERENCES

1 E. Heftmann, *Chromatography of Steroids*, Elsevier, Amsterdam, 1976, p. 10.
2 Ž. Procházka, in Z. Deyl, K. Macek and J. Janák (Editors), *Liquid Column Chromatography*, Elsevier, Amsterdam, 1975, p. 593.
3 N. A. Parris, *Instrumental Liquid Chromatography*, Elsevier, Amsterdam, 1976, p. 281.
4 S. Siggia and R. A. Dishman, *Anal. Chem.*, 42 (1970) 1223.
5 M. J. O'Hare, E. C. Nice, R. Magee-Brown and H. Bullman, *J. Chromatogr.*, 125 (1976) 357.
6 S. Hara and K. Mibe, *Chem. Pharm. Bull.*, 15 (1967) 1036.
7 S. Hara and K. Mibe, *Anal. Chem.*, 40 (1968) 1605.
8 S. Hara and K. Mibe, *Chem. Pharm. Bull.*, 23 (1975) 2850.
9 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, London, Sydney, Toronto, 1974.
10 G. C. Pimentel and A. L. McClellan, *The Hydrogen Bond*, Freeman, San Francisco, 1960.