CHROM. 6125

STEROIDS

II. THE USE OF MULTIPLE DEVELOPMENT IN THE CORRELATION OF THE CHEMICAL CONSTITUTIONS AND THE CHROMATOGRAPHIC MIGRATIONS OF SOME 17-KETOSTEROIDS AND THEIR 2,4-DINITROPHENYLHYDRAZONES

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SUMMARY

The application of thin-layer one-dimensional chromatography to the separation of thirteen 17-ketosteroids and their 2,4-dinitrophenylhydrazones on silica gel is described. In addition to presenting the R_F and R_M values in two systems, the value of using multiple development for the calculation of ΔR_M and hence ΔR_{M0} and ΔR_{Mr} is discussed for some examples. The comparison of ΔR_M values obtained after the first and second developments and a conversion table for calculating R_M values from the R_F values obtained after the second development are also given.

INTRODUCTION

Thin-layer chromatography (TLC) has been used extensively to study steroids during their synthesis and also in clinical chemistry. The application of TLC to the separation of a large number of steroids of the androstane series and the possible correlation between migration and structure have been discussed by LISBOA[1-3]. The behaviour of the acetates, formates, 2,4-dinitrophenylhydrazones (2,4-DNPHs), bromides and oxidation products of some C_{19}-steroids has been studied by FEHÉR[4].

In this work, the chromatographic behaviour of some 17-ketosteroids (17-KS) and their 2,4-DNPHs was studied by extending the method to the results obtained by multiple development for the calculation of R_M values and hence ΔR_{M0} and ΔR_{Mr}.

EXPERIMENTAL

Materials

Reflexing silica gel sheets of Silufol UV_{254} (Kavalier, Czechoslovakia) with dimensions of 15 × 15 cm and with a 0.1 mm layer of silica gel and an inert inorganic luminiscent indicator were used for TLC. Systematic names, trivial names, abbreviations and sources of the preparations are listed in Table I. The solvents used for the development of the chromatograms were re-distilled; chloroform con-

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TABLE I

SYSTEMATIC NAMES, TRIVIAL NAMES AND SOURCES OF THE 17-KETOSTEROIDS STUDIED

a = Calbiochem, Los Angeles, U.S.A.; b = Sigma Chemical Corp., St. Louis, U.S.A.; c = Koch-Light Laboratories, Colnbrook, Great Britain; d = Schering AG, Berlin, G.F.R.; e = Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czechoslovakia.

No.	*Systematic name*	*Trivial name*	*Abbreviation*	*Source*
1	3β-Hydroxyandrost-5-en-17-one	Dehydroepiandrosterone	3βolΔ^5A17one	a
2	3α-Hydroxy-5α-androstan-17-one	Androsterone	3αol5αA17one	a
3	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone	3αol5βA17one	a
4	3α,11β-Dihydroxy-5α-androstan-17-one	11-Hydroxyandrosterone	3α,11βol5αA17one	b
5	3α,11β-Dihydroxy-5β-androstan-17-one	11-Hydroxyetiocholanolone	3α,11βol5βA17one	c
6	3α-Hydroxy-5α-androstane-11,17-dione	11-Oxoandrosterone	3αol5αA11,17one	b
7	3α-Hydroxy-5β-androstane-11,17-dione	11-Oxoetiocholanolone	3αol5βA11,17one	c
8	3β-Hydroxy-5α-androstane-17-one	Epiandrosterone	3βol5αA17one	a
9	4-Androstene-3,17-dione	Androstenedione	Δ^4A3,17one	a
10	5α-Androstane-3,17-dione	Androstanedione	5αA3,17one	d
11	5α-Androstan-17-one		5αA17one	e
12	5β-Androstan-17-one		5βA17one	e
13	4-Androstene-3,11,17-trione	Adrenosterone	Δ^4A3,11,17one	a

tained 0.6–1.0 % of ethanol. The preparation of the 2,4-DNPHs of 17-KS was carried out by a slightly modified method of TREIBER AND OERTEL[5].

Thin-layer chromatography

The samples were applied as small spots 2.5 cm from the edge of the 15 × 15 cm plate, which was divided into strips with lines. The lines, 0.5 mm broad, were drawn in the layer with a narrow metal spatula or a blunt pencil to a distance of 10 mm. The plates were then heated for 30 min at 60° in a vacuum drying oven, equilibrated for 3 min in the room atmosphere and developed. One-dimensional chromatograms were developed by the ascending technique in two runs. Before the second run, the plates were heated in the same way as above. This procedure gave very good reproducibility of R_F values. For the detection of the spots of 17-KS, the vanillin-sulphuric acid reaction was used[6].

In this study, the following two systems described earlier[7] were used: system B, chloroform–acetone (96:4); and system E, diethyl ether–light petroleum (70:30).

The developed chromatogram was left in the solvent after the front had reached the top of the layer so as to avoid a decreasing ratio of liquid-to-solid phase. This "over-running" technique has many advantages[8]. The time required was 35 min with system B and 25 min with system E.

RESULTS AND DISCUSSION

The contribution of any given functional group to the chromatographic mobility of a molecule will depend mainly on the nature of the substituent, its position and the nature of the chromatographic system. The calculation of R_M value used for the analysis of a possible correlation between migration of the steroid and its structure from $R_F < 0.05$ or $R_F > 0.90$ values is of little use. R_F values of over 0.75 and less than 0.15 are also approximations.

The R_M values were used according to the definition of BATE-SMITH AND WESTALL[9]:

$$R_M = \log\left(\frac{1}{R_F} - 1\right) \qquad (1)$$

To obtain a better separation of the substances with the lowest values multiple development can be recommended, based on the assumption that re-chromatography in the same system can be considered as being a separation corresponding to a longer running distance.

This method not only gave parallel movement of spots, but also a new distribution. However, for spots with $R_F > 0.45$, the results obtained after the first development are the most valuable[10]. In multiple development, as discussed by LENK[11], the spots become flattened and hence the resolution is improved, as opposed to a longer running distance.

In the following section, the possibility of using R_F values obtained from the first and second developments for the calculation of R_M values is demonstrated. This procedure allowed the use of a higher value, ${}^nR_F$ (obtained from the multiple development), which was out of the range of the greatest experimental errors.

${}^nR_F$ values were introduced in order to enable the positions of the separated

substances for n developments to be obtained[12]. These can be calculated from the relationship

$$^nR_F = 1 - (1 - R_F)^n \quad (2)$$

The evaluation of R_F and ^nR_F values for the calculation of R_M values, and hence for use in structural analysis, has the following theoretical basis.

From eqn. 2,

$$R_F = 1 - \sqrt[n]{1 - {}^nR_F} \quad (3)$$

Eqns. 1 and 3 can then be combined to give

$$R_M = \log\left(\frac{\sqrt[n]{1 - {}^nR_F}}{1 - \sqrt[n]{1 - {}^nR_F}}\right) \quad (4)$$

and eqn. 4 can be written as

$$R_M = \log\left(\frac{a}{1 - a}\right) \quad (5)$$

where $a = \sqrt[n]{1-{}^nR_F}$. Table II can be used for converting ^2R_F into R_M values.

Generally, it can be considered that compound A has the value

$$\Delta R_{M(A)} = m\Delta R_{M(x)} + n\Delta R_{M(y)} + o\Delta R_{M(z)} + \ldots K \quad (6)$$

where $\Delta R_{M(x)}$, $\Delta R_{M(y)}$ and $\Delta R_{M(z)}$ are the R_M values for functional groups x, y, z; m, n, o are the numbers of particular functional groups; and K is a constant corresponding to the fundamental skeleton.

Because it is not possible to ascertain the constant K for the steroid fundamental skeleton experimentally, the R_M values obtained from 5αA17one and 5βA17one are used as a basis.

Hence, for the calculation, there are used new constants, K_0 and K_0', from eqns. 7 and 8:

$$K + \Delta R_{M(17\text{-CO})} + \Delta R_{M(5\alpha A)} = K_0 \quad (7)$$

$$K + \Delta R_{M(17\text{-CO})} + \Delta R_{M(5\beta A)} = K_0' \quad (8)$$

The R_M values of the derived compounds are then calculated as the total sum of K_0 or K_0' and the constants for the functional groups. For practical purposes, it is important to note that from the above it appears that

$$K_0 = R_{M(5\alpha A17\text{one})}$$

$$K_0' = R_{M(5\beta A17\text{one})}$$

The chromatographic results for thirteen 17-KS and their 2,4-DNPHs, always separated in two runs and in the same system, are summarised in Tables III and IV. The mobilities of the 2,4-dinitrophenylhydrazone derivatives of 17-KS were greater in all instances than those of the corresponding parent substances.

The symbols ΔR_{Mg} and ΔR_{Mr} are used here with the same meanings as those used by BUSH[13]. The ΔR_{Mg} value of a radical is the ΔR_M value resulting from the replacement of a hydrogen atom in the molecule with this radical. The ΔR_{Mr} value is the ΔR_M value for the substance before and after chemical reaction.

TABLE II

TABLE FOR CONVERSION OF ^2R_F INTO R_M VALUES

$R_M = \log\left(\frac{a}{1-a}\right)$; $a = 1 - {}^2R_F$.

^2R_F	R_M	^2R_F	R_M	^2R_F	R_M	^2R_F	R_M
0.01	2.298	0.26	0.788	0.51	0.367	0.76	−0.020
0.02	1.995	0.27	0.767	0.52	0.351	0.77	−0.037
0.03	1.817	0.28	0.746	0.53	0.337	0.78	−0.054
0.04	1.668	0.29	0.726	0.54	0.323	0.79	−0.074
0.05	1.573	0.30	0.707	0.55	0.307	0.80	−0.093
0.06	1.494	0.31	0.688	0.56	0.293	0.81	−0.114
0.07	1.427	0.32	0.670	0.57	0.278	0.82	−0.134
0.08	1.369	0.33	0.652	0.58	0.264	0.83	−0.155
0.09	1.306	0.34	0.635	0.59	0.249	0.84	−0.177
0.10	1.260	0.35	0.618	0.60	0.234	0.85	−0.200
0.11	1.218	0.36	0.602	0.61	0.219	0.86	−0.224
0.12	1.179	0.37	0.583	0.62	0.205	0.87	−0.250
0.13	1.136	0.38	0.567	0.63	0.190	0.88	−0.277
0.14	1.103	0.39	0.552	0.64	0.176	0.89	−0.306
0.15	1.066	0.40	0.534	0.65	0.159	0.90	−0.336
0.16	1.037	0.41	0.519	0.66	0.145	0.91	−0.369
0.17	1.009	0.42	0.502	0.67	0.129	0.92	−0.406
0.18	0.978	0.43	0.486	0.68	0.113	0.93	−0.446
0.19	0.954	0.44	0.472	0.69	0.097	0.94	−0.492
0.20	0.925	0.45	0.456	0.70	0.081	0.95	−0.543
0.21	0.899	0.46	0.440	0.71	0.065	0.96	−0.603
0.22	0.877	0.47	0.426	0.72	0.050	0.97	−0.680
0.23	0.853	0.48	0.412	0.73	0.033	0.98	−0.785
0.24	0.829	0.49	0.397	0.74	0.015	0.99	−0.955
0.25	0.810	0.50	0.382	0.75	0.000		

ΔR_{M0} values calculated from the R_F values after the first and second developments show corresponding results, as can be seen from Table V, in which ΔR_{M0} values of some hydroxyl and ketonic groups in pure steroids and their 2,4-DNPHs after the first (a) and second (b) developments are compared.

It can be seen that the greatest influence on the chromatographic mobility for both systems and for both the parent steroids and their 2,4-DNPH derivatives is given by the 3-hydroxyl group. For isomers the separation depends on the spatial configuration, and the equatorial (*e*) position is appreciably more reactive than the axial (*a*) position, as usual.

The mobility of 3-OH steroids also depends on the configuration of the 5-H group. The R_{M0} values in both systems for the parent 3-OH steroids and their 2,4-DNPH derivatives remained identical or very similar. The introduction of one isolated unsaturated bond altered the mobility of the steroids in both systems only slightly, as can be seen by comparison of the R_F values of dehydroepiandrosterone and androsterone.

A greater difference is seen between 17-KS with an 11-OH and an 11-CO group and their 2,4-DNPHs. The 11β-hydroxyl group with rings A/B in the *cis* configuration is slightly less polar in system B. In system E there is no difference.

Mono- and bis-2,4-DNPH derivatives were formed during the production of 2,4-DNPHs if the molecule contained one or two keto groups in addition to the

TABLE III

R_F AND R_M VALUES OF 17-KETOSTEROIDS IN TWO SOLVENT SYSTEMS AFTER THE FIRST DEVELOPMENT

No.	Steroid	*Parent substance*				*2,4-DNPH derivative*			
		System B		*System E*		*System B*		*System E*	
		R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
1	3βolΔ^5A17one	0.16	0.720	0.12	0.865	0.27	0.432	0.18	0.659
2	3αol5αA17one	0.18	0.659	0.13	0.826	0.39	0.194	0.23	0.525
3	3αol5βA17one	0.11	0.908	0.08	1.061	0.32	0.327	0.18	0.659
4	3α11βol5αA17one	0.03	1.510	0.05	1.279	0.15	0.753	0.13	0.826
5	3α11βol5βA17one	0.02	1.690	0.03	1.510	0.14	0.788	0.10	0.954
6	3αol5αA11,17one	0.05	1.279	0.03	1.510	0.20	0.600	0.07	1.128
7	3αol5βA11,17one	0.04	1.380	0.01	1.996	0.18	0.659	0.05	1.279
8	3βol5αA17one	0.15	0.753	0.11	0.908	0.26	0.454	0.16	0.720
9	Δ^4A3,17one	0.31	0.347	0.11	0.908	0.82	—0.660	0.49	0.017
10	5αA3,17one	0.45	0.087	0.23	0.528	0.82	—0.660	0.52	—0.034
11	5αA17one	0.74	—0.456	0.67	—0.308	0.85	—0.754	0.76	—0.500
12	5βA17one	0.74	—0.456	0.67	—0.308	0.85	—0.754	0.76	—0.500
13	Δ^4A3,11,17one	0.16	0.720	0.03	1.510	0.52	—0.034	0.15	0.753
	No. of experiments	6		6		10		8	

TABLE IV

^2R_F AND R_M VALUES OF 17-KETOSTEROIDS IN TWO SOLVENT SYSTEMS AFTER THE SECOND DEVELOPMENT

No.	Steroid	Parent substance				2,4-DNPH derivative			
		System B		System E		System B		System E	
		^2R_F	R_M	^2R_F	R_M	^2R_F	R_M	^2R_F	R_M
1	3βolΔ^5A17one	0.27	0.767	0.23	0.853	0.45	0.456	0.32	0.670
2	3αol5αA17one	0.29	0.726	0.25	0.810	0.63	0.190	0.40	0.534
3	3αol5βA17one	0.19	0.954	0.15	1.066	0.53	0.337	0.31	0.688
4	3α11βol5αA17one	0.05	1.573	0.09	1.306	0.26	0.788	0.24	0.829
5	3α11βol5βA17one	0.04	1.668	0.05	1.573	0.24	0.829	0.19	0.954
6	3αol5αA11,17one	0.09	1.306	0.06	1.494	0.34	0.635	0.14	1.103
7	3αol5βA11,17one	0.07	1.427	0.03	1.817	0.30	0.707	0.10	1.260
8	3βol5αA17one	0.26	0.788	0.20	0.925	0.43	0.486	0.28	0.746
9	Δ^4A3,17one	0.47	0.426	0.19	0.954	0.95	—0.543	0.71	0.065
10	5αA3,17one	0.70	0.081	0.39	0.552	0.96	—0.603	0.75	0.000
11	5αA17one	0.93	—0.446	0.88	—0.277	0.96	—0.603	0.92	—0.406
12	5βA17one	0.93	—0.446	0.88	—0.277	0.96	—0.603	0.92	—0.406
13	Δ^4A3,17one	0.26	0.788	0.06	1.494	0.77	—0.037	0.26	0.788
	No. of experiments	6		6		10		8	

TABLE V

ΔR_{Mg} VALUES OF SOME HYDROXYL AND KETONIC GROUPS FOR PURE STEROIDS AND THEIR 2,4-DINITROPHENYLHYDRAZONES IN THE ANDROSTANE SERIES AFTER THE FIRST (a) AND SECOND (b) DEVELOPMENTS

Group	*Compound in which radical is substituted*	*Ring conformation*	*System B*				*System E*			
			Parent substance		*2,4-DNPH*		*Parent substance*		*2,4-DNPH*	
3-Oxo	5α-A17one	A/B-*trans*	(a)	0.54	(a)[b]	0.09	(a)	0.84	(a)[b]	0.47
			(b)[c]	0.53	(b)	—	(b)[b]	0.83	(b)[c]	0.41
3β-OH	5α-A17one	A/B-*trans*-3β(*e*)	(a)	1.21	(a)[b]	1.21	(a)[a]	1.22	(a)[b]	1.22
			(b)[c]	1.23	(b)	—	(b)[b]	1.20	(b)	1.15
3α-OH	5α-A17one	A/B-*trans*-3α(*a*)	(a)	1.11	(a)[b]	0.95	(a)[a]	1.13	(a)[b]	1.02
			(b)[c]	1.17	(b)	—	(b)[b]	1.09	(b)[c]	0.94
	5β-A17one	A/B-*cis*-3α(*e*)	(a)	1.36	(a)[b]	1.08	(a)[a]	1.37	(a)[b]	1.16
			(b)[c]	1.40	(b)	—	(b)[b]	1.34	(b)[c]	1.09
11β-OH	3α-ol-5α-A17one	A/B-*trans*-3α(*a*)	(a)[c]	0.85	(a)	0.56	(a)[a]	0.45	(a)[a]	0.30
	3α-ol-5β-A17one	A/B-*cis*-3α(*e*)	(b)[a]	0.85	(b)	0.60	(b)[a]	0.50	(b)	0.29
			(a)	—	(a)	0.46	(a)	0.45	(a)[a]	0.29
			(b)[c]	0.71	(b)	0.49	(b)[a]	0.51	(b)	0.27
11-Oxo	3α-ol-5α-A17one	A/B-*trans*-3α(*a*)	(a)[a]	0.62	(a)	0.41	(a)[c]	0.68	(a)[a]	0.60
			(b)[a]	0.58	(b)	0.44	(b)[a]	0.68	(b)	0.57
	3α-ol-5α-A17one	A/B-*cis*-3α(*e*)	(a)[c]	0.47	(a)	0.33	(a)	—	(a)[a]	0.62
			(b)[a]	0.47	(b)	0.37	(b)[c]	0.75	(b)[a]	0.57
	Δ^4-A3,17one		(a)	0.37	(a)[b]	0.63	(a)[c]	0.60	(a)	0.74
			(b)	0.36	(b)	—	(b)[a]	0.54	(b)	0.72

[a] Calculated with one or both R_F values in the region 0.15–0.05.
[b] Calculated with one or both R_F values in the region 0.75–0.90.
[c] Calculated with an R_F value of 0.03, 0.04 or 0.91–0.93.

11-keto group[3]. The 11-keto group is known to be unreactive towards all carbonyl reagents.

The following order of decreasing polarity was found for the functional hydroxyl groups studied:

Parent substance:	3αOH(*e*)
	3βOH(*e*)
	3αOH(*a*)
	11βOH
2,4-DNPH derivative:	3βOH(*e*)
	3αOH(*e*)
	3αOH(*a*)
	11βOH

The orders of decreasing polarity in both systems are similar.

TABLE VI

ΔR_{Mr} VALUES FOR 2,4-DINITROPHENYLHYDRAZONES OF 17-KETOSTEROIDS AFTER THE FIRST (a) AND SECOND (b) DEVELOPMENTS

Group that is converted	*Parent substance*	ΔR_{Mr}	
		System B	*System E*
17-keto	5αA17one	(a)[b] −0.30	(a)[b] −0.19
		(b) —	(b)[c] −0.13
	5βA17one	(a)[b] −0.30	(a)[b] −0.19
		(b) —	(b)[c] −0.13
	3βolΔ^5A17one	(a) −0.29	(a)[a] −0.21
		(b) −0.31	(b) −0.18
	3αol5αA17one	(a) −0.46	(a)[a] −0.32
		(b) −0.54	(b) −0.28
	3αol5βA17one	(a)[a] −0.58	(a)[a] −0.40
		(b) −0.62	(b) −0.38
	3α,11βol5αA17one	(a)[c] −0.76	(a)[a] −0.45
		(b)[a] −0.78	(b)[a] −0.48
	3α,11βol5βA17one	(a) —	(a)[c] −0.56
		(b)[c] −0.84	(b)[a] −0.62
	3αol5αA11,17one	(a)[a] −0.68	(a)[c] −0.38
		(b)[a] −0.67	(b)[a] −0.39
	3αol5βA11,17one	(a)[c] −0.72	(a) —
		(b)[a] −0.72	(b)[c] −0.56
	3βol5αA17one	(a) −0.27	(a)[a] −0.19
		(b) −0.30	(b) −0.18
3,17-diketo	5αA3,17one	(a)[b] −0.75	(a) −0.56
		(b) —	(b) −0.55
$\Delta^4$3,17-diketo	Δ^4A3,17one	(a)[b] −1.01	(a)[a] −0.92
		(b) —	(b) −0.89
	Δ^4A3,11,17one	(a) −0.75	(a)[c] −0.76
		(b)[b] −0.82	(b)[a] −0.71

[a] Calculated with one or both R_F values in the region 0.15–0.05.
[b] Calculated with one or both R_F values in the region 0.75–0.90.
[c] Calculated with an R_F value of 0.03, 0.04 or 0.91–0.93.

Table VI shows ΔR_{Mr} values for the conversion of 17-oxo, 3,17-dioxo and $\Delta^4$3,17-dioxo groups to their mono- or bis-2,4-DNPHs. The ΔR_{Mr} values for 5αA17one and 5βA17one are equal in both systems. In all other instances the β-androstane derivatives have a lower value than the corresponding α-androstane derivatives. This observation supports the earlier results obtained by FEHÉR[4]. The increase in ΔR_{Mr} values in both solvent systems is not parallel; the greatest differences were observed in this work for the 11-substituted derivatives.

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