CHROM. 5048

THIN-LAYER AND PAPER CHROMATOGRAPHY OF STEROIDAL β-D-GLUCOPYRANOSIDES, β-D-GLUCOPYRANOSIDURONIC ACIDS, AND DERIVATIVES

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(Received September 7th, 1970)

SUMMARY

A number of related steroidal β-D-glucopyranoside peracetates, free β-D-glucopyranosides, and β-D-glucopyranosiduronic acids, chiefly in the androstane and pregnane series, were chromatographed on thin layers of silica gel. In all three classes R_F differences between members of axial–equatorial pairs were small and, contrary to rule, the equatorially-oriented member frequently was the more mobile. It was not possible to separate mixtures of free glucosides and glucosiduronic acids. Thin-layer chromatography on silica gel remains, however, an excellent method for detecting simple free glucosides.

Partition paper chromatography of the free glucosides and glucosiduronic acids gave far better results: reasonable R_F differences were obtained for all axial–equatorial pairs, the axially-oriented member of a given pair was the more mobile in all cases, and mixtures of free glucosides and glucosiduronic acids were separated without difficulty.

INTRODUCTION

In an earlier study[1] sixteen anomeric pairs of steroidal tri-O-acetyl-D-glucopyranosiduronic methyl esters, seven pairs of D-glucopyranoside tetraacetates, and four pairs of 2′-acetamido-2′-deoxy-tri-O-acetyl-D-glucopyranosides, together with the free steroids (aglycons) from which they were derived, were chromatographed on thin layers of silica gel. It was observed that the α member was more mobile than its anomer in all cases except one, and that the mobility of a given derivative* relative to the corresponding aglycon was variable, but determined in part by the nature of the aglycon and the carbohydrate moiety.

Subsequently an additional fourteen glucoside peracetates and the corresponding free glucosides were prepared[2] as well as the free glucosiduronic acids derived

* The general term "derivative" refers both to β-D-glucopyranoside peracetates and tri-O-acetyl-β-D-glucopyranosiduronic methyl esters. The trivial terms "glucoside" and "glucosiduronic acid" represent in the text conjugates which are formally β-D-glucopyranosides and β-D-glucopyranosiduronic acids, respectively.

from a number of glucosiduronic methyl ester tri-O-acetates synthesized earlier[3]. This completed the preparation of four series of steroidal conjugates, consisting of glucosides, glucosiduronic acids and their respective derivatives, which utilized as far as possible the same group of aglycons (chiefly androstanes and pregnanes) within each series. The present report considers the thin-layer chromatography of the glucoside peracetates, free glucosides and glucosiduronic acids, as well as the partition paper chromatography of the latter two classes.

METHODS

Thin-layer chromatography was carried out at 25 ± 2° by the ascending method on Silica Gel IB-F sheets. Systems (Table I) suitable for the acetylated

TABLE I

COMPOSITION OF CHROMATOGRAPHIC SYSTEMS

System No.	*Composition*
1	Ethyl acetate, 10 ml, diluted to 25 ml with isooctane (2,2,4-trimethylpentane)
2	Ethyl acetate, 12 ml, diluted to 25 ml with isooctane
3	Ethanol, 2.5 ml, diluted to 25 ml with chloroform
4	Ethanol, 3 ml, diluted to 25 ml with chloroform
5	Ethanol, 1.5 ml, acetic acid, 3 ml, diluted to 25 ml with chloroform
6	Ethanol, 2 ml, acetic acid, 3 ml, diluted to 25 ml with chloroform
7	Ethyl acetate–toluene–methanol–water (50:150:70:130)
8	Ethyl acetate–toluene–methanol–water (25:175:80:120)
9	Ethyl acetate–toluene–acetic acid–water (50:150:60:140)
10	Ethyl acetate–toluene–acetic acid–water (30:170:60:140)
11	Isooctane–toluene–methanol–water (170:30:170:30)
12	Isooctane–methanol–water (200:180:20)
13	Toluene–*tert.*-butanol–acetic acid–water (185:30:60:140)

glucosides consist of ethyl acetate–isooctane mixtures[1]; R_F values with such systems usually can be reproduced to within 0.1 R_F unit. Free glucosides are best chromatographed with ethanol–chloroform systems; the glucosides move as well-defined, round components, but R_F values are more difficult to reproduce, due probably to temperature changes within the jar as a result of initiating chromatography too soon after adding the solvent. The 3-component ethanol–chloroform–acetic acid type of system is considered only fairly satisfactory for the chromatography of free glucosiduronic acids; the components are ovoid rather than round, and the solvent front is invariably irregular. In all cases the conjugates were detected by spraying the dried plate with a 20% solution of *p*-toluenesulfonic acid in ethanol followed by heating at *ca.* 120° for 3 to 5 min. This converts them into products which fluoresce moderately to strongly under a lamp emitting maximally at around 360 nm. Compounds bearing the 3β-hydroxy-5-ene system (for example derivative 1) provide visible colors appreciably sooner than their saturated counterparts. Some, but not all, aglycon-conjugate pairs give fluorogens which differ in color.

Paper chromatography employed 19 × 60 cm sheets of Whatman No. 1 paper and was carried out in the descending manner. The considerable polarity of the free

conjugates limited the choice of systems, but two types proved satisfactory, namely one employing ethyl acetate–toluene–aqueous methanol (or aqueous acetic acid) and a second, based on toluene–*tert.*-butanol–aqueous methanol (or aqueous acetic acid). The conjugates were detected by dipping the dried paper in a 10% solution of phosphomolybdic acid in ethanol and heating at *ca.* 90°. However, many of the saturated conjugates give so faint a color as to indicate the need for a more sensitive reagent.

RESULTS AND DISCUSSION

Table II gives R_F values for fourteen glucoside peracetates and the corresponding aglycons when chromatographed on thin layers of silica gel. For purposes of discussion, the compounds may be divided into pairs (2, 3; 4, 5; 7, 8; 9, 10; 11, 12)

TABLE II

THIN-LAYER CHROMATOGRAPHY OF STEROID β-D-GLUCOPYRANOSIDE PERACETATES AND AGLYCONS

Numbers in Tables II–IV are not assigned to individual compounds, but serve as guides between text and tables. Aglycons (free steroids) are given formal names in this and other tables in order better to show steric relationships, and because some lack generally accepted trivial names. The axial (*a*) or equatorial (*e*) orientation of the C-3 hydroxyl group is indicated in parentheses after the name.

No.	*Aglycon*	*System No.*	R_F *values*	
			Derivative	*Aglycon*
1	3β-Hydroxyandrost-5-en-17-one	1	0.16	0.20
2	3α-Hydroxy-5α-androstan-17-one (*a*)	1	0.18	0.24
3	3β-Hydroxy-5α-androstan-17-one (*e*)	1	0.17	0.20
4	3α-Hydroxy-5β-androstan-17-one (*e*)	1	0.18	0.15
5	3β-Hydroxy-5β-androstan-17-one (*a*)	1	0.17	0.24
6	3β-Hydroxypregn-5-en-20-one	1	0.19	0.25
7	3α-Hydroxy-5α-pregnan-20-one (*a*)	1	0.20	0.27
8	3β-Hydroxy-5α-pregnan-20-one (*e*)	1	0.20	0.22
9	3α-Hydroxy-5β-pregnan-20-one (*e*)	1	0.21	0.19
10	3β-Hydroxy-5β-pregnan-20-one (*a*)	1	0.20	0.28
11	3α,17-Dihydroxy-5β-pregnan-20-one (*e*)	2	0.20	0.12
12	3β,17-Dihydroxy-5β-pregnan-20-one (*a*)	2	0.17	0.20
13[a]	Androst-5-ene-3β,17β-diol	1	0.22	0.12
14	Androst-5-ene-3β,17β-diol	1	0.22	0.12

[a] Conjugates 13 and 14 are, respectively, the 3β-yl- and 17β-yl-β-D-glucopyranoside pentaacetates of the diol.

the members of which differ only with respect to the orientation of the C-3 hydroxyl group of the aglycon (equatorial (*e*), in the plane of the ring, or axial (*a*), out of the plane of the ring). As a general (but not invariable) rule in steroid chromatography, that member of a given pair which bears the axially-oriented hydroxyl group is the more mobile[4]. It can be seen from the table that the R_F values derived from the aglycons uniformly adhere to this rule. However, the derivatives do not (one equivocal and two exceptional results in five pairs). Very similar relationships can be seen in Tables II and IV of the earlier study[1] which involved principally the thin-layer chromatography of steroidal glucosiduronic acid derivatives. It is concluded that the

TABLE III

THIN-LAYER CHROMATOGRAPHY OF STEROID β-D-GLUCOPYRANOSIDES AND β-D-GLUCOPYRANOSIDURONIC ACIDS

No.	*Aglycon*	*Glucoside*		*Glucosiduronic acid*	
		System No.	R_F	*System No.*	R_F
15	3β-Hydroxyandrost-5-en-17-one	3	0.17	5	0.15
16	3α-Hydroxy-5α-androstan-17-one (*a*)	3	0.19	5	0.18
17	3β-Hydroxy-5α-androstan-17-one (*e*)	3	0.16	5	0.15
18	3α-Hydroxy-5β-androstan-17-one (*e*)	3	0.18	5	0.14
19	3β-Hydroxy-5β-androstan-17-one (*a*)	3	0.17	5	0.16
20	3β-Hydroxypregn-5-en-20-one	3	0.13	5	0.16
21	3α-Hydroxy-5α-pregnan-20-one (*a*)	3	0.18	5	0.23
22	3β-Hydroxy-5α-pregnan-20-one (*e*)	3	0.13	5	0.18
23	3α-Hydroxy-5β-pregnan-20-one (*e*)	3	0.15	5	0.19
24	3β-Hydroxy-5β-pregnan-20-one (*a*)	3	0.14	—	—
25	3α,17-Dihydroxy-5β-pregnan-20-one (*e*)	4	0.14	6	0.13
26	3β,17-Dihydroxy-5β-pregnan-20-one (*a*)	4	0.20	—	—
27	(22S,25S)-Spirost-5-en-3β-ol	3	0.17	5	0.23
28	(22S,25S)-5α-Spirostan-3β-ol	3	0.17	5	0.23
29	Cholest-5-en-3β-ol	3	0.17	5	0.23
30	5α-Cholestan-3β-ol	3	0.17	5	0.23
31[a]	Androst-5-ene-3β,17β-diol	4	0.15	6	0.17
32	Androst-5-ene-3β,17β-diol	4	0.15	6	0.17

[a] Conjugates 31 and 32 are, respectively, the 3β-yl- and 17β-yl-β-D-glucopyranosides (or the 3β-yl- and 17β-yl-β-D-glucopyranosiduronic acids) of the diol.

axial–equatorial rule cannot be extended to compounds bearing large, polyfunctional groups at C-3 under the conditions prevailing in adsorption chromatography.

It is also to be noted that R_F differences in axial–equatorial pairs are distinctly smaller in derivatives than in the corresponding aglycons. Although these differences can be reproduced (as opposed to R_F values), it is clear that it would be very difficult to separate, as on a column, any of the pairs in this table.

The mobility of a given aglycon relative to the corresponding derivative is variable and unpredictable except in those cases where the aglycon bears an additional functional hydroxyl group (as in pairs 13 and 14), or where the carbohydrate moiety is inherently more polar, as in 2′-acetamido-2′-deoxy-tri-O-acetyl-α(and β)-D-glucopyranosides (see Table V in ref. 1). This is a point of practical importance because such aglycon–derivative mixtures are encountered in the synthesis of glycosides by the Koenigs-Knorr method. It cannot be assumed that substrates bearing a single functional hydroxyl group will conveniently precede or follow the product in the course of column chromatography*.

The R_F values in Table III were derived from eighteen free glucosides and, in most cases, the corresponding glucosiduronic acids. These results roughly parallel those noted in Table II and demonstrate that separations between axial–equatorial or other pairs are not improved by substituting free conjugates for their derivatives.

* As indicated earlier[1] inseparable aglycon–derivative mixtures can be resolved by acetylation followed by re-chromatography. However, it is inadvisable to acetylate the primary reaction mixture prior to its initial column chromatography.

TABLE IV

PAPER CHROMATOGRAPHY OF STEROID β-D-GLUCOPYRANOSIDES, β-D-GLUCOPYRANOSIDURONIC ACIDS AND AGLYCONS

No.	Aglycon	Glucoside		Glucosiduronic acid		Aglycon	
		System No.	R_F	System No.	R_F	System No.	R_F
33	3β-Hydroxyandrost-5-en-17-one	7	0.14	9	0.12	11	0.21
34	3α-Hydroxy-5α-androstan-17-one (*a*)	7	0.25	9	0.21	11	0.38
35	3β-Hydroxy-5α-androstan-17-one (*e*)	7	0.17	9	0.15	11	0.29
36	3α-Hydroxy-5β-androstan-17-one (*e*)	7	0.18	9	0.17	11	0.33
37	3β-Hydroxy-5β-androstan-17-one (*a*)	7	0.23	9	0.21	11	0.40
38	3β-Hydroxypregn-5-en-20-one	8	0.11	10	0.11	12	0.25
39	3α-Hydroxy-5α-pregnan-20-one (*a*)	8	0.22	10	0.22	12	0.40
40	3β-Hydroxy-5α-pregnan-20-one (*e*)	8	0.14	10	0.12	12	0.26
41	3α-Hydroxy-5β-pregnan-20-one (*e*)	8	0.16	10	0.14	12	0.26
42	3β-Hydroxy-5β-pregnan-20-one (*a*)	8	0.19	—	—	12	0.31
43	(22S,25S)-Spirost-5-en-3β-ol	8	0.17	10	0.17		
44	(22S,25S)-5α-Spirostan-3β-ol	8	0.18	10	0.19		
45	Cholest-5-en-3β-ol	8	0.21	10	0.21		
46	5α-Cholestan-3β-ol	8	0.21	10	0.24		

However, thin-layer chromatography in ethanol–chloroform systems remains an excellent method for detecting glucosides (as in extracts from biological systems), and the chief utility of Table III is therefore to record representative R_F values in this type of system*.

It was noted in Table II that the epimeric diol glucoside pentaacetates 13 and 14 were not separated, and a recent attempt to distinguish between the corresponding glucosiduronic acid derivatives was equally unsuccessful. The values in Table III (31 and 32) show, not surprisingly, that the free conjugates are equally difficult to separate. It seems reasonable to conclude in this case that a given carbohydrate moiety provides the same contribution to polarity at either the C-3 or C-17 sites.

The complete lack of separation between members of pairs 27, 28 and 29, 30 is difficult to understand when it is considered that the structural difference in each case (the 3β-hydroxy-5-ene system *vs.* the 3β-hydroxy-A/B-*trans* (5α) arrangement) is common also to pairs 15, 16 and 20, 21 which are to a degree separated. These results suggest that the large side chains of the spirostan(ene) and cholestan(ene) conjugates interfere with those adsorption/elution processes on which separation depends.

Finally, it was not possible to separate mixtures of free glucosides and glucosiduronic acids by thin-layer chromatography in ethanol–chloroform–acetic acid systems. It was observed earlier[1] that their derivatives have virtually identical R_F values in neutral systems.

* As an aid in detecting glucosides under circumstances where only the aglycon is available, each pair was simultaneously chromatographed in ethanol–chloroform systems so adjusted as to provide R_F (glucoside) values of about 0.1. $\Delta R_{M(\text{glucosidation})}$ values calculated from these data varied from 0.095 to 1.16 and averaged 1.08.

It is to be noted that ethanol–chloroform systems are limited in application to weakly or moderately polar glucosides, that is monosides in which the aglycon bears at most three hydroxyl groups. Our experience to date relative to adsorption chromatography suggests that, once detected, the glucoside is best recovered by acetylation followed by chromatography on silica gel using an ethyl acetate–isooctane or similar system. Ref. 5 furnishes an example.

Table IV presents the results of a study in which suitable* free glucosides, glucosiduronic acids and the corresponding aglycons were chromatographed on paper using similar systems. The results are superior to those obtained using thin-layer chromatography. Considered in terms of axial–equatorial pairs within the conjugates proper, R_F differences average 0.06; this does not approach the averaged value of 0.09 obtained in the case of the aglycons, but is sufficiently large to assure separations on partition-type columns in most cases. Secondly, the axial–equatorial relative mobility rule is adhered to by conjugates and aglycons alike. Finally, true separations of glucoside–glucosiduronic acid mixtures can be obtained. For example, R_F values for the 20-ketopregnanes 38–41 in system 13 are: 0.15, 0.08; 0.28, 0.16; 0.19, 0.10; 0.19, 0.10 (R_F values for the uronic acids are the second in each pair).

It was stated over a decade ago (and in rather lofty terms considering the limited evidence available) that "the polar conjugating group does not cancel those influences which the (remaining) functional groups in the steroid nucleus or side chain have on partitioning processes. Thus in appropriate systems dehydroisoandrosterone, etiocholanolone and androsterone glucuronides move in the same order and are separated about as well as the corresponding free compounds"[6]. This generalization expresses the net observed effect of various factors, including the configuration of the substituent at C-3 of the steroid moiety, on relative mobilities within a given series. The paper chromatographic results in Table IV substantiate this proposition, but it is equally clear, from the relative R_F values in Tables II and III, that it is invalid under the conditions prevailing in adsorption chromatography.

ACKNOWLEDGEMENT

This work was made possible by a research grant, AM 01255, from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service. We are grateful to this Institute for its continued and generous support of our research.

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* The few compounds which tended to streak are not included.