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Note

New paper chromatographic systems for the separation of conjugated corticosteroids

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The final step in the metabolic transformation of almost all steroids is their conjugation with highly polar, ionizable substances, mainly glucuronic or sulphuric acid[1–4]; such conjugation renders the steroids well soluble in water, and is therefore thought to facilitate their urinary excretion[5–7]. Recently, however, it has been shown that certain steroid conjugates (in particular, the sulphates) are synthesized in steroid-producing glands[8–11]. Moreover, evidence has been presented that these conjugates have important biological functions, *viz.*, they can serve as effective precursors in steroid biosynthetic pathways[12–14], they can control rates of steroidogenesis[15], and they may exercise a regulatory role in steroid binding and transport[16].

In our studies on the isolation of a total spectrum of sulphate- and glucuronide-conjugated metabolites of cortisol from urine and plasma[17–20], we have encountered the need for paper chromatographic systems for the separation of individual steroid conjugates, sulphates as well as glucuronides. Most of the solvent systems described by various investigators for the separation of steroid conjugates[21–24] contain either acids or alkali, and frequently cause formation of steroid artefacts, particularly when conjugates of labile corticosteroids are being chromatographed. Moreover, most of these systems are unsuitable for separating spatial isomers of these conjugates, in which minute differences in the relative polarities of the steroid moieties are obliterated by the high polarity of the non-steroidal moieties.

Mattox *et al.*[25] have described the use of a liquid ion exchanger (tetraheptylammonium chloride), in associatoin with conventional chromatographic systems for free steroids, for the separation of steroidal glucosiduronic acids; this is a new and interesting approach to the chromatographic separation of steroid conjugates. However, we have found that satisfactory resolution of a mixture of several pairs of stereoisomers of conjugated corticosteroids, using liquid ion exchangers, required a large number of different solvent systems. The use of thin-layer chromatography (TLC) for the separation of steroid glucuronides was investigated by Schneider[26]. It was concluded that R_F differences between members of axial–equatorial pairs were very small, and that, in general, the suitability of TLC for the separation of steroidal

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β-D-glucopyranosides, and β-D-glucopyranosiduronic acids, as well as their peracetates, was inferior to that of paper chromatography.

We have devised solvent systems (based on isopropyl alcohol) that permit satisfactory separation of individual sulphate- and glucuronide-conjugated metabolites of cortisol, and can be also used for the separation of conjugated metabolites of other steroids. These systems contain neither acids nor alkali and do not cause formation of steroid artefacts even during the prolonged development needed for the separation of various stereoisomers of conjugated metabolites.

ABBREVIATIONS AND TRIVIAL NAMES

The following trivial names and abbreviations are used for steroids and their conjugates: cortisol (F_K) for 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; cortisone (E_K) for 17α,21-dihydroxypregn-4-ene-3,11,20-trione; 20α-dihydrocortisol (*epi*E_R) for 11β,17α,20α,21-tetrahydroxypregn-4-en-3-one; 20β-dihydrocortisol (E_R) for 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one; tetrahydrocortisol (THF) for 3α,11β,17α,21-tetrahydroxypregnan-20-one; *allo*tetrahydrocortisol (5α-THF or *allo*THF) for 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one; tetrahydrocortisone (THE) for 3α,17α,21-trihydroxypregnane-11,20-dione; *allo*tetrahydrocortisone (5α-THE or *allo*THE) for 3α,17α,21-trihydroxy-5α-pregnane-11,20-dione; α-cortol for 3α,11β,17α,20α,21-pentahydroxy-5β-pregnane; β-cortol for 3α,11β,17α,20β,21-pentahydroxy-5β-pregnane; α-*allo*cortol for 3α,11β,17α,20α,21-pentahydroxy-5α-pregnane; β-*allo*cortol for 3α,11β,17α,20β,21-pentahydroxy-5α-pregnane; α-cortolone for 3α,17α,20α,21-tetrahydroxy-5β-pregnan-11-one; β-cortolone for 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one; α-*allo*cortolone for 3α,17α,20α,21-tetrahydroxy-5α-pregnan-11-one; β-*allo*-cortolone for 3α,17α,20β,21-tetrahydroxy-5α-pregnan-11-one; etiocholanolone (Etio) for 3α-hydroxy-5β-androstan-17-one; 11-OH-Etio for 3α,11β-dihydroxy-5β-androstan-17-one; 11-OH-Andro for 3α,11β-dihydroxy-5α-androstan-17-one; 11-oxo-Etio for 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-Andro for 3α-hydroxy-5α-androstane-11,17-dione; dehydroepiandrosterone (DHEA) for 3β-hydroxyandrost-5-en-17-

TABLE I

SOLVENT SYSTEMS FOR PAPER CHROMATOGRAPHIC SEPARATION OF INDIVIDUAL STEROID SULPHATES AND STEROID GLUCURONIDES

System designation	*Solvents (v/v)*	*Whatman paper No.*	*Running time to front (h)*
K_1	Light petroleum–isopropanol–water (100:80:20)	2	2.5–3
K_2	Light petroleum–isopropanol–water (90:80:20)	3 MM	3.5–4
Boric K_2*	Same as K_2	3 MM	3
K_3	Light petroleum–benzene–isopropanol–water (50:50:80:20)	3 MM	5
Boric K_3*	Same as K_3	3 MM	4
K_4	Benzene–isopropanol–water (100:70:30)	3 MM	6
Boric K_4*	Same as K_4	3 MM	5
K_5	Light petroleum–ethyl acetate–isopropanol–water (70:30:85:20)	3 MM	8

* Before application, the paper was dipped in 5% boric acid and dried in a hood (see text).

one; sulphate (-S) for (steroid)-yl-sulphate; -glucuronide (-G) for (steroid)-yl-β-D-glucopyranosiduronide.

MATERIALS AND METHODS

All solvents used were J. T. Baker (Phillipsburgh, N.J., U.S.A.) "analyzed reagent" grade, and were twice distilled before use. Tetrahydrocortisol 3-glucuronide, tetrahydrocortisone 3-glucuronide and β-cortol 3-glucuronide were synthesized and generously donated by Dr. Vernon R. Mattox of the Mayo Clinic and Mayo Foundation (Rochester, Minn., U.S.A.); cortisol 21-glucuronide and cortisone 21-glucuronide were synthesized by us, using the method of Mattox *et al.*[24], and cortisol 21-sulphate was synthesized as previously described by us[27]; dehydroepiandrosterone glucuronide, etiocholanolone glucuronide and dehydroepiandrosterone sulphate

TABLE II

CHROMATOGRAPHIC MOBILITY OF VARIOUS SULPHATE-CONJUGATED STEROID METABOLITES IN THE NEW SYSTEMS

*Steroid conjugate**	*System*							
	K_1		K_2		K_3		K_4	
	T**	R_F***	T	R_F	T	R_F	T	R_F
DHEA-S			× 1 × 7	0.04 0.14	× 1 × 2	0.25 0.37	× 1	0.33
11-oxo-Andro-S	× 30	0.76	× 10 × 30	0.50 R.O.§			× 6	0.82
11-oxo-Etio-S	× 30	0.68	× 10 × 30	0.50 R.O.			× 6	0.82
11-OH-Andro-S	× 30	0.50	× 30	0.86			× 6	0.74
11-OH-Etio-S	× 30	0.43	× 30	0.86			× 6	0.74
E_K-21-S			× 30 × 50	0.75 R.O.			× 6	0.70
F_K-21-S			× 7 × 50	0.10 0.79	× 1 × 2	0.12 0.17	× 6	0.56
*allo*THE-3-S			× 50	0.65	× 15	0.75	× 6	0.38
THE-3-S			× 50	0.57			× 6	0.48
E_R-21-S			× 50	0.51	× 15	0.75	× 6	0.38
*allo*THF-3-S			× 50	0.47	× 15	0.58	× 6	0.29
THF-3-S			× 50	0.39	× 15	0.58	× 6	0.29
β-*allo*Cortolone-3-S			× 50	0.28	× 10	0.78	× 6	0.24
β-Cortolone-3-S					× 10	0.70	× 6	0.24
α-*allo*Cortolone-3-S					× 10	0.62	× 6	0.24
α-Cortolone-3-S					× 10	0.51	× 6	0.24
β-*allo*Cortol-3-S					× 25	0.86	× 6	0.15
α-*allo*Cortol-3-S					× 25	0.78	× 6	0.15
β-Cortol-3-S					× 25	0.69	× 6	0.15
α-Cortol-3-S					× 25	0.59	× 6	0.15

* For abbreviations see text.

** T = length of run: *e.g.*, × 1 = single-length run; × 10 = over-run 9 times.

*** R_F = mobility relative to solvent front, or, when systems over-run, to the length of chromatogram.

§ R.O. = run-off.

were purchased from Ikapharm (Ramat-Gan, Israel). All other steroid conjugates used in this study were isolated by us from human urine, and were identified as described previously[17,19].

The chromatographic systems devised are listed in Table I; these are Bush-type systems[28], in which the paper is impregnated by the vapour of the stationary phase placed at the bottom of a paper-lined chromatography jar. The systems are listed in the order of increasing polarity. Systems K_2, K_3, and K_4 were also used, with paper impregnated with 5% boric acid, for more efficient separation of the conjugates of stereoisomers of certain steroid metabolites. System K_5 is a monophasic system; however, it requires saturation of the atmosphere within the jar with the solvent, which is therefore placed in the bottom of the jar, just as with the biphasic systems. The chromatographic jars were lined with Whatman No. 3 MM paper. Whenever overrunning of the chromatograms was required, the paper strip was cut at the bottom so as to give an obtuse angle of 120°, and the run-off was collected in a small beaker placed at the bottom of the chromatographic jar.

Following chromatography, sulphate-conjugated steroids were located on paper by the barium–rhodizonate colour spot-test[23], and steroid glucuronides by the 1-(2-pyridylazo)2-naphthol–cobalt nitrate[29] and naphthoresorcinol[30] colour spot-tests, as modified by us[19]. When conjugated metabolites of radioisotopically labelled tracer-steroids (administered by intravenous injection before collection of the blood or urine) were chromatographed, they were first located on paper with the aid of a radiochromatogram-scanner[19], then the colour spot-test was applied. For the characterization and quantitation of various steroid conjugates isolated from urine or blood, an aliquot of the solution containing the steroid mixture to be chromatographed was applied to a pilot strip, to which, after chromatography, the spot-tests were applied; from the remaining part of the paper chromatogram, the separated steroid conjugates were eluted with *n*-butanol–70% aqueous methanol as previously described[31].

RESULTS AND DISCUSSION

Tables II and III list the mobilities of various conjugated steroids, most of them metabolites of cortisol, in the systems described. It will be seen that, for satisfactory separation of the conjugates of steroids closely related structurally, prolonged development was used in a system of a relatively low polarity with regard to the group of steroids being separated. Because of the absence of acids or alkali in the systems used, no artefact formation was observed, even when chromatograms were over-run up to 50 times a single-length run. The separation of the conjugates of certain steroid isomers required, in addition, pre-impregnation of the paper with boric acid. This expedient was used by Schneider and Lewbart[32] in the separation of α- and β-isomers of 20-hydroxy-steroids. The separation of these isomers presumably involves formation of complexes of boric acid[32].

We have used the chromatographic systems described here, in conjunction with systems for conjugated steroids described by others[21,22], for the isolation and characterization of a whole spectrum of sulphate- as well as glucuronide-conjugated metabolites of cortisol in human urine and plasma[17–20,33,34]. Our more recent experience (unpublished data) indicates that the systems described are also suitable for the

TABLE III

CHROMATOGRAPHIC MOBILITY OF VARIOUS GLUCURONIDE-CONJUGATED STEROID METABOLITES IN THE NEW SYSTEMS*

Steroid conjugate	*System*													
	K_2				K_3				K_4				K_5	
	*Reg.***		*Boric*		*Reg.*		*Boric*		*Reg.*		*Boric*		*Reg.*	
	T	R_F	*T*	R_F	*T*	R_F	*T*	R_F	*T*	R_F	*T*	R_F	*T*	R_F
Etio-G	×12	0.21			× 6	0.30			×3	0.32				
DHEA-G	×12	0.14			× 6	0.20			×3	0.24				
11-oxo-Andro-G	×50	0.31												
11-oxo-Etio-G	×50	0.25												
11-OH-Andro-G	×50	0.17												
11-OH-Etio-G	×50	0.10												
E_K-21-G					× 8	0.93							× 5	0.65
*allo*THE-3-G					× 8	0.80							× 5	0.65
F_K-21-G					× 8	0.74							× 5	0.65
THE-3-G					× 8	0.68							× 5	0.54
*allo*THF-3-G					× 8	0.58							× 5	0.38
THF-21-G					× 8	0.52							× 5	0.48
THF-3-G					× 8	0.46							× 5	0.38
E_R-21-G					× 8 ×12	0.38 0.81			×6	0.52				
β-*allo*Cortolone-3-G					×12	0.63			×6	0.33				
β-Cortolone-3-G					×12	0.57					×10	0.53		
α-*allo*Cortolone-3-G					×12	0.49					×10	0.26		
α-Cortolone-3-G					×12 ×20	0.43 0.75	×6	0.48			× 8	0.22	×15	0.68
β-*allo*Cortol-3-G					×20	0.68	×6	0.76			× 8	0.46	×15	0.68
β-Cortol-3-G			×20	0.64	×20	0.62	×6	0.68			× 8	0.60	×15	0.68
α-*allo*Cortol-3-G					×20	0.57	×6	0.39			×20	0.50	×15	0.42
α-Cortol-3-G			×20	0.28	×20	0.41	×6	0.30			×20	0.42	×15	0.42

* For abbreviations and symbols, see text and footnotes to Table II, respectively.
** Reg. = regular run, *i.e.*, without impregnation of paper with boric acid.

chromatographic separation of individual glucuronide- and sulphate-conjugated metabolites of various other steroids.

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