Note

Separation of 7α- and 7β-methoxycarbonylmethyl steroids by preparative high-performance liquid chromatography: comparison with thin-layer chromatography

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The 7α- and 7β-carboxymethyl steroid derivatives have been used either as haptens in radioimmunoassays[1,2] or as ligands in affinity chromatography[3]. We have described[4,5] a general method for introducing a carboxymethyl chain at the C-7 site in the 3-hydroxy-5-ene and 4-en-3-oxo-steroid series (Fig. 1). After protecting the functional groups, bromination by N-bromosuccinimide and condensation with sodium ethyl malonate is followed by saponification and decarboxylation. The subsequent removal of the protective functional groups yields equal proportions of the 7-carboxymethyl derivatives in the two possible orientations. Separation of the epimers before hydrolysis of the 3-ethylene ketal is preferable in order to obtain higher ΔR_F values and to allow easier identification of the epimers through NMR analysis
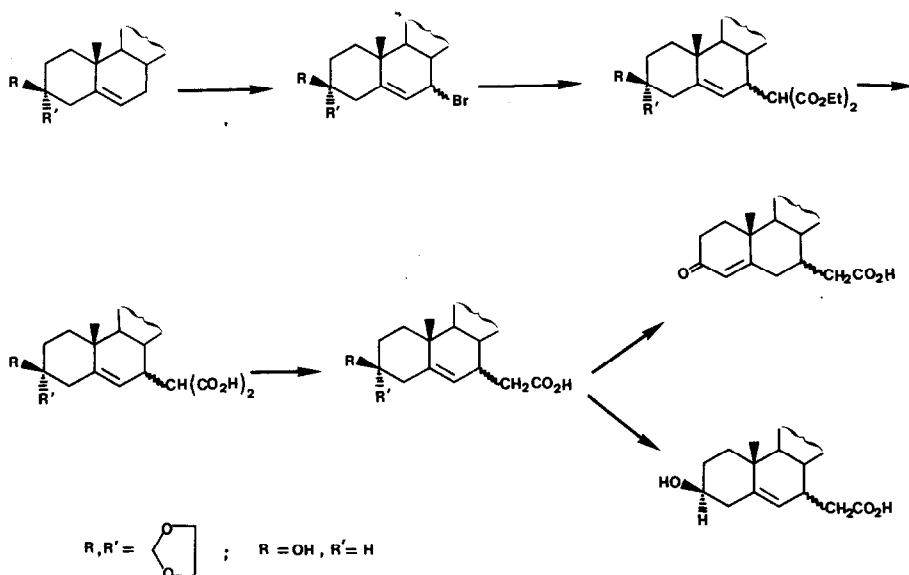

Fig. 1. Flow chart for the preparation of 7-carboxymethyl steroids.

of the proton signal at C-6[4,5]. After preliminary experiments, the separation of the
7α and 7β epimers was found to be more convenient as their methyl ester derivatives
on a normal phase rather than as their acid forms on a C_{18} bonded reversed-phase.
Furthermore, the methyl derivatives, which are conveniently separated using mix-
tures of volatile solvents, require a purification step in the course of their formation.

MATERIALS AND METHODS

Thin-layer chromatography (TLC) was carried out on silica gel plates (60 F_{254};
Merck, Darmstadt, F.R.G.) after activation at 110°C for 30 min, but without pre-
saturation in the solvent tank. A 6000 A liquid chromatograph (Waters Assoc., Mil-
ford, MA, U.S.A.) equipped with a U6-K injector, a Waters UV detector and a
Waters Model 401 difference refractometer was used for analytical and semi-prepar-
ative high-performance liquid chromatography (HPLC). The columns (Waters) were
μPorasil (30 cm × 4 mm I.D.), average particle size 10 μm, or reversed-phase μBon-
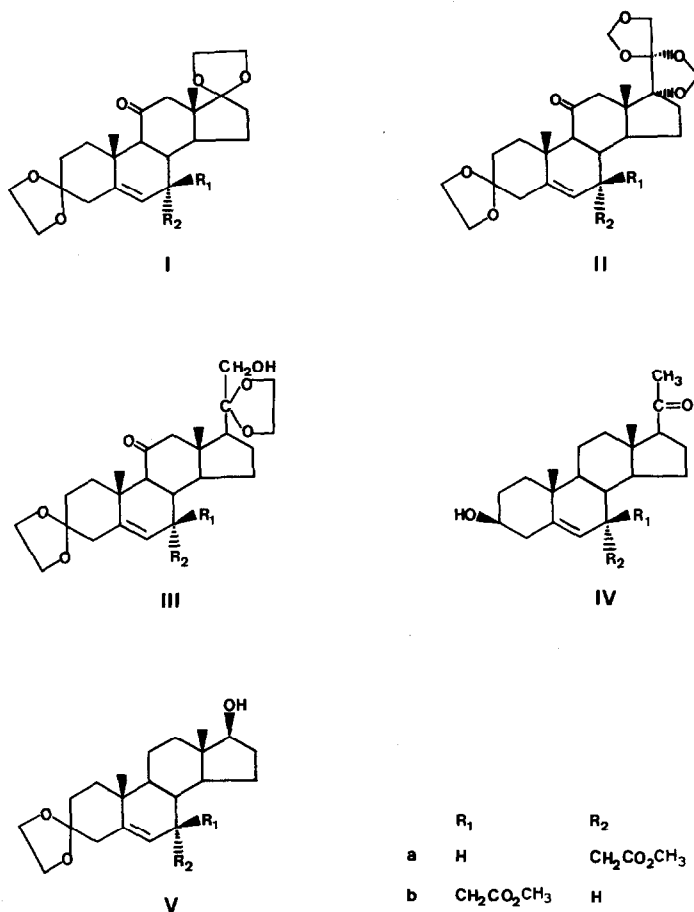

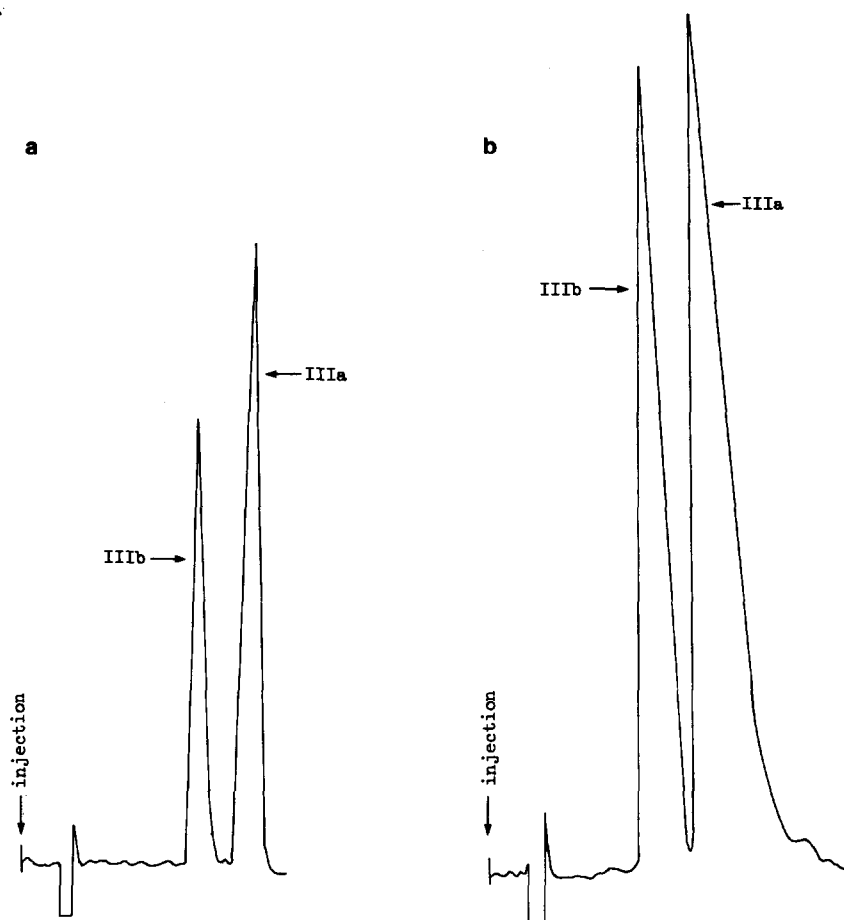Fig. 2. The pairs of epimers studied.

Fig. 3. Separation of IIIa and IIIb. (a) Analytical HPLC: flow-rate 2 ml/min, t_0 = 1.6 min. (b) Semi-preparative HPLC: flow-rate 8 ml/min, t_0 = 1.6 min.

TABLE I

TLC R_F VALUES AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN VARIOUS SOLVENT SYSTEMS

Capacity factor, $k' = (1 - R_F)/R_F$.

Sample	Hexane–ethyl acetate (30:70)			Ethyl acetate			Benzene–diethyl ether (60:40)			Benzene–diethyl ether (30:70)		
	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β
I	0.44	0.40	1.18	0.54	0.50	1.18	0.35	0.30	1.26	0.39	0.34	1.24
II	0.46	0.43	1.13	0.58	0.56	1.09	0.42	0.37	1.23	0.44	0.40	1.18
III	0.14	0.13	1.09	0.34	0.33	1.05	0.07	0.06	1.18	0.07	0.06	1.18
IV	0.37	0.36	1.04	0.50	0.48	1.08	0.27	0.26	1.06	0.27	0.26	1.05
V	0.39	0.36	1.14	0.49	0.45	1.17	0.28	0.24	1.23	0.29	0.26	1.16

dapak C_{18} (30 cm × 4 mm I.D.), average particle size 10 μm, in the case of analytical separation, μPorasil (30 cm × 7.8 mm I.D.), average particle size 10 μm, in the case of semi-preparative separations and PrePak (30 cm × 5.7 cm I.D.), average particle size 35–75 μm, in the case of preparative separations. Solvents for analytical and semi-preparative separations (Carlo Erba, RS HPLC) were filtered through Millipore filters (0.45 μm) and degassed by ultrasonication for 10 min, while those for preparative separations were pure grade, distilled and filtered through Millipore filters (0.45 μm) before use.

The amounts injected were usually up to 25 μg in 25 μl of solvent in analytical HPLC, at a flow-rate of 2 ml/min, up to 50 mg in 500 μl in semi-preparative HPLC, at a flow-rate of 8 ml/min and up to 2 g in 10 ml for preparative HPLC, at a flow-rate of 200 ml/min.

The following epimer pairs were studied (Fig. 2): 7α-methoxycarbonyl-5-pregnene-3,11,17-trione 3,17-bisethylene ketal (Ia) and the 7β epimer (Ib) derived from adrenosterone; 7α-methoxycarbonylmethyl-17α,20,20,21-bismethylenedioxy-5-pregnene-3,11,20-trione 3-ethylene ketal (IIa) and the 7β epimer (IIb) derived from cortisone; 7α-methoxycarbonylmethyl-21-hydroxy-5-pregnene-3,11,20-trione 3,20-bis-ethylene ketal (IIIa) and the 7β epimer (IIIb) derived from 11-dehydrocorticosterone; 3β-hydroxy-7α-methoxycarbonylmethyl-5-pregnen-20-one (IVa) and the 7β epimer (IVb) derived from pregnenolone; 17β-hydroxy-7α-methoxycarbonylmethyl-5-androsten-3-one 3-ethylene ketal (Va) and the 7β epimer (Vb) derived from testosterone.

RESULTS AND DISCUSSION

The TLC separation of the epimers was undertaken in various solvents (Table I) and it was found that hexane–ethyl acetate would be the most useful solvent for the analytical HPLC separation. However, the cortisone epimers (IIa and IIb) were eluted too rapidly. The separation was improved by decreasing the solvent strength and using 75:25 proportions. This mixture could not be applied to preparative or semi-preparative HPLC due to the low solubility of the compounds, the concentration of the injected sample being 100 times higher. Thus part of the ethyl acetate and

TABLE II

TLC R_F VALUES AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN HEXANE–ETHYL ACETATE–DICHLOROMETHANE

Capacity factor, $k' = (1 - R_F)/R_F$.

Sample	Hexane–ethyl acetate–dichloromethane (25:60:15)			Hexane–ethyl acetate–dichloromethane (30:50:30)		
	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β
I	0.39	0.33	1.30	0.39	0.34	1.24
II	0.41	0.37	1.19	0.45	0.41	1.18
III	0.12	0.11	1.10	0.10	0.09	1.12
IV	0.32	0.31	1.05	0.32	0.31	1.05
V	0.32	0.29	1.15	0.33	0.30	1.15

TABLE III

INFLUENCE OF THE ETHYL ACETATE AND DICHLOROMETHANE PROPORTIONS ON
THE SELECTIVITY FACTORS, k'_α/k'_β, IN ANALYTICAL HPLC t_0 = 1.6 min

Sample: IIa and IIb. Capacity factor, $k' = (t_R - t_0)/t_0$.

Hexane–ethyl acetate–dichloromethane	$t_{R\beta}$ (min)	$t_{R\alpha}$ (min)	k'_α/k'_β
50:45:00	3.4	4.0	1.33
50:30:30	3.2	3.8	1.37
75:25:00	7.2	8.2	1.18
75:20:10	7.2	8.4	1.21

hexane was replaced with dichloromethane. The same solvent strength was maintained, but the compounds exhibited a remarkably higher solubility. TLC of the epimers with this ternary mixture (Table II) showed that the addition of a third solvent has little influence on the selectivity factors, k'_α/k'_β. Similarly, Table III shows that, at the same solvent strength, a comparable variation of the ethyl acetate and dichloromethane proportions did not significantly alter the selectivity factors in analytical HPLC. Table IV shows the conditions used for separation of the various pairs of epimers with ternary mixtures in analytical HPLC.

An attempt to separate cortisone derivatives (IIa and IIb) by preparative HPLC using the solvent system described for analytical HPLC revealed that the capacity factors, $k' = (t_R - t_0)/t_0$, were greater than 15, resulting in a poor resolution. The direct transposition of the conditions for analytical HPLC to those suitable for preparative HPLC by means of a simple relationship was shown not to be feasible, thus necessitating several trials to define the most suitable solvent mixtures. However, this study permitted the identification of solvent systems which can be used directly to perform separations in semi-preparative HPLC (Fig. 3) at sample concentrations similar those ones used in preparative HPLC. Conditions for the separation of each pair of epimers in preparative HPLC are given in Table V. All compounds were separated in 90% yield. Analytical HPLC and NMR assays revealed that the impurities were less than 2%. Table V shows also a comparison of the results of the TLC separations either with the solvent mixture used for preparative HPLC or that already found to be optimal for TLC. Use of TLC to predict suitable HPLC conditions has already been suggested[6,7]. Decreasing the solvent strength of the optimum ternary mixture for TLC allows the extrapolation of the results to preparative HPLC. The TLC/HPLC solvent-strength ratio should be of the same order for each epimer pair with a R_F range between 0.1 and 0.25.

This study of the separation of 7-methoxycarbonylmethyl steroid epimers using TLC and analytical, semi-preparative and preparative HPLC allowed the establishment of a ternary mixture of solvents suitable for the resolution of the epimer pairs within a wide range of sample concentrations injected. Among these different liquid chromatography methods, the closest analogy was found between preparative HPLC and TLC, permitting the extrapolation of the conditions used for the latter to preparative HPLC.

TABLE IV

RETENTION TIMES, t_R, AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN ANALYTICAL HPLC t_0 = 1.6 min

Capacity factor, $k' = (t_R - t_0)/t_0$.

Sample	Hexane–ethyl acetate–dichloromethane	$t_{R\beta}$ (min)	k'_β	$t_{R\alpha}$ (min)	k'_α	k'_α/k'_β
I	70:20:10	9.8	5.12	14.8	8.25	1.60
II	75:25:00	7.2	3.50	8.2	4.12	1.18
III	25:50:25	7.0	3.37	8.8	4.50	1.33
IV	60:20:20	10.2	5.37	11.4	6.12	1.13
V	60:20:20	10.4	5.50	13.4	7.37	1.34

TABLE V

COMPARISON BETWEEN RETENTION TIMES, t_R (min), AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN PREPARATIVE HPLC (t_0 = 2.55 min) AND R_F VALUES AND SELECTIVITY FACTORS IN TLC

Capacity factors, k': in TLC, $(1 - R_F)/R_F$; in HPLC, $(t_R - t_0)/t_0$.

Sample	Preparative HPLC				TLC			TLC: optimum separation			
	Hexane–ethyl acetate–dichloromethane	$t_{R\beta}^*$	$t_{R\alpha}^*$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	Hexane–ethyl acetate–dichloromethane	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β
I	50:30:20	19	25	1.37	0.24	0.20	1.26	25:60:15	0.39	0.33	1.3
II	50:30:20	17.2	21	1.25	0.26	0.23	1.18	25:60:15	0.41	0.37	1.19
III	20:50:30	21.8	24.7	1.15	0.10	0.09	1.12	0:100:0	0.34	0.32	1.10
IV	40:40:20	17.2	18.7	1.10	0.20	0.18	1.14	10:60:30	0.37	0.34	1.10
V	40:40:20	18	21	1.20	0.20	0.18	1.14	10:60:30	0.37	0.34	1.14

* Measured after recycling once.

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