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THIN-LAYER CHROMATOGRAPHY OF STEROID DERIVATIVES FOR RADIOIMMUNOASSAY

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SUMMARY

Separation by thin-layer chromatography of the intermediates and final products in the synthesis of steroid–protein and steroid–tyrosine methyl ester conjugates is described for the following steroids: aldosterone, cholesterol, cortisol, progesterone and testosterone.

INTRODUCTION

The search for new analytical methods for the determination of picogram amounts of steroids in biological samples has interested many investigators for a number of years. A major advance in this field occurred with the advent of radioimmunoassay, which can be applied to steroids, provided that truly specific antibodies are available. As steroids are non-antigenic *per se*, they must be chemically coupled to a large protein molecule or to a poly(amino acid)copolymer (polylysine) to induce a specific response in animals. To obtain antibodies able to discriminate among closely similar steroidal structures, the chemical coupling must be performed in such a way as to minimize the steric masking of the specific functional groups; this can be done by the correct choice of the position in the basic cyclopentenophenanthrene nucleus through which the steroid molecule is conjugated to the protein. Conjugation is usually carried out by converting the steroid into a derivative containing a COOH group, which in turn is linked, via an amido bond, to the ε-amino groups of the protein. The COOH derivative can be coupled to tyrosine methyl ester (TME) and the resulting product can easily be labelled with radioiodine in the *ortho* position of the aromatic nucleus. As the antibodies have been raised against a steroid–protein conjugate, they usually bind the steroid–TME derivative with similar affinity. Thus, the ^{125}I-labelled TME–steroid derivatives can be used succesfully as tracers in radioimmunoassay instead of the ^{3}H-labelled steroids, with a substantial gain in cost and operative simplicity.

In this paper are collected the R_F values, in suitable solvent systems, of the intermediates and final compounds that have been prepared in the course of a research programme for the setting up of the radioimmunoassay of the following steroidal compounds: aldosterone, progresterone, testosterone, cortisol and cholesterol. If synthesis is carried out using labelled steroids as tracers, thin-layer radiochromato-

graphy is the simplest method to use to quantitate the reaction yields, to check the purity of intermediates and of the final products and to prepare pure compounds for immunization and for labelling.

EXPERIMENTAL

Reference compounds

Testosterone (17β-hydroxy-4-androsten-3-one), aldosterone (18,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al), progesterone (4-pregnene-3,20-dione), 11α-hydroxyprogesterone (11α-hydroxy-4-pregnene-3,20-dione), cortisol (11β, 17α,21-trihydroxy-4-pregnene-3,20-dione) and cholesterol (5-cholesten-3β-ol) were a gift from Warner-Vister Steroid Research Institute, Casatenovo Como, Italy, which is gratefully acknowledged. L-Tyrosine methyl ester hydrochloride (TME-HCl) was obtained from Schwarz Mann, U.S.A. [1,2-^{3}H]testosterone, [16-^{3}H]progesterone, [1,2-^{3}H]cortisol and [^{3}H]cholesterol (uniformly labelled) were obtained from CEA-CEN-Sorin and [1,2-^{3}H]aldosterone from The Radiochemical Centre, Amersham, Great Britain.

Cortisol 21-hemisuccinate, aldosterone 21-hemisuccinate, progesterone 11α-hemisuccinate, testosterone hemisuccinate and cholesterol hemisuccinate were prepared according to the procedure described by ERLANGER *et al.*[1], slightly modified according to the properties of each steroid[2]. The 3-hydrazone and 3,20-dihydrazone derivatives of progesterone were prepared using *p*-hydrazinobenzoic acid (*p*HBA). Testosterone 3-(*o*-carboxymethyl)oxime was prepared according to ERLANGER *et al.*[1]. Cortisol 21-hemisuccinyl-TME, aldosterone 21-hemisuccinyl-TME, progesterone 11-hemisuccinyl-TME, testosterone hemisuccinyl-TME, testosterone 3-(*o*-carboxymethyl)oxime-TME and cholesterol hemisuccinyl-TME were prepared by reacting TME with the corresponding carboxyl derivative of each steroid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide or of N,N'-dicyclohexylcarbodiimide using tetrahydrofuran as the solvent[2]. This reaction also leads to the formation of a steroid-substituted urea[2], which must be separated from the TME derivative: cortisol 21-hemisuccinyl-N,N'-dicyclohexylurea, aldosterone 21-hemisuccinyl-1-ethyl-3-(3-dimethylaminopropyl)urea, progesterone 11-hemisuccinyl-N,N'-dicyclohexylurea, testosterone hemisuccinyl-1-ethyl-3-(3-dimethylaminopropyl) urea, testosterone 3-(*o*-carboxymethyl)oxime-1-ethyl-3-(3-dimethylaminopropyl)urea and cholesterol hemisuccinyl-N,N'-dicyclohexylurea.

Solvents and procedure

The following solvent systems were used: S_1, chloroform–methanol–water (9:1:0.1); S_2, chloroform–acetone (9:1); S_3, benzene–acetone–methanol (5:5:2).

Pre-scored glass plates, 20 × 20 cm, pre-coated with layers of Silica Gel F_{254} (Merck) were activated for 1 h at 110° and allowed to cool in a moisture-free chamber. The development was carried out in flat-bottomed jars, 23 × 23 × 8 cm, saturated with the appropriate solvent system and lined with filter-paper for good equilibration.

By means of Drummond disposable pipettes, 5 μl (5–10 μg) of each reference compound were applied to each plate, 2 cm from the bottom edge. Development took about 1 h in each solvent system. The zones were located in short-wave (254 nm) UV light.

TABLE I

R_F VALUES OF STEROID DERIVATIVES ON SILICA GEL THIN LAYERS

Compound	*R_F values for solvent system*[a]		
	S_1	S_2	S_3
Testosterone	0.60	0.25	0.59
Testosterone hemisuccinate	0.41	0.05	0.41
Testosterone hemisuccinyl-TME	0.57	0.18	0.65
Testosterone hemisuccinyl-1-ethyl-3-(3-dimethylaminopropyl)urea	0.19	St.p.	0.10
Testosterone 3-(*o*-carboxymethyl)oxime	0.10	St.p.	0.12
Testosterone 3-(*o*-carboxymethyl)oxime-TME	0.49	0.09	0.58
Testosterone 3-(*o*-carboxymethyl)oxime-1-ethyl-3-(3-dimethyl-aminopropyl)urea	0.03	0.04	0.05
Progesterone	0.70	0.50	0.64
Progesterone 3-*p*HBA	0.47	0.21	0.56
Progesterone 3,20-*p*HBA	0.26	0.16	0.43
11α-Hydroxyprogesterone	0.55	0.12	0.58
Progesterone 11-hemisuccinate	0.37	0.022	0.44
Progesterone 11-hemisuccinyl-TME	0.54	0.10	0.60
Progesterone 11-hemisuccinyl-N,N′-dicyclohexylurea	0.70	0.38	0.62
Aldosterone	0.41	0.034	0.51
Aldosterone 21-hemisuccinate	0.17	St.p.	0.36
Aldosterone 21-hemisuccinyl-TME	0.44	0.013	0.60
Aldosterone 21-hemisuccinyl-1-ethyl-3-(3-diethylaminopropyl)urea	St.p.	St.p.	St.p.
Cortisol	0.26	0.025	0.55
Cortisol 21-hemisuccinate	0.12	St.p.	0.39
Cortisol 21-hemisuccinyl-TME	0.36	0.03	0.62
Cortisol 21-hemisuccinyl-N,N′-dicyclohexylurea	0.50	0.10	0.67
Cholesterol	0.53	0.41	0.68
Cholesterol hemisuccinate	0.37	0.21	0.41
Cholesterol hemisuccinyl-TME	0.50	0.33	0.68
Cholesterol hemisuccinyl-N,N′-dicyclohexylurea	0.62	0.61	0.72
L-Tyrosine methyl ester (TME)	0.23	St.p.	0.42
N,N′-Dicyclohexylcarbodiimide	0.60	0.52	0.56
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide	St.p.	St.p.	St.p.
p-Hydrazinebenzoic acid (*p*HBA)	0.35	0.06	0.53

[a] Compositions of the solvent systems: S_1 = chloroform–methanol–water (9:1:0.1); S_2 = chloroform–acetone (9:1); S_3 = benzene–acetone–methanol (5:5:2).

Radioactive compounds were located by autoradiography or by direct scanning using an Actigraph Model 3 instrument (Nuclear Chicago Corp., U.S.A.).

RESULTS AND DISCUSSION

The R_F values measured for various compounds are listed in Table I. For each steroid, different positions of conjugation to the protein were chosen, in an attempt to obtain the highest specificity in the immunological response.

Progesterone

Using *p*-hydrazinobenzoic acid, a mixture of the 3-monohydrazone (85 %) and of the 3,20-dihydrazone (15 %) is obtained. The 3-monohydrazone can be purified

by preparative thin-layer chromatography (TLC) using the solvent system S_1 (see Table I), and it can be used both for conjugation and for the preparation of the TME derivative. An alternative route starts from 11α-hydroxyprogesterone, which is converted into the corresponding hemisuccinate. The potential advantage of following the latter route is that of leaving intact all the functional groups of progesterone. Actually, the antibodies formed against the corresponding conjugate were found to be less cross-reacted with other steroids than those formed against the conjugate prepared from the 3-monohydrazone[3].

Testosterone

The simplest way to conjugate testosterone to a protein is through the corresponding hemisuccinate; conjugation through the 3-(*o*-carboxymethyl)oxime, which leaves the 17-β-hydroxyl group intact, would be expected to favour the formation of more specific antibodies. However, this did not occur in this instance, as antibodies of quite similar specificity were obtained with conjugation on C_3 and C_{17} (ref. 3).

Aldosterone

With the correct choice of the experimental conditions, succinylation leads to the formation of the aldosterone 21-hemisuccinate. This can be purified by preparative TLC, using solvent system S_3, from the small amounts of products of unknown composition that are formed during the reaction; these products originate from aldosterone, as proved by using [1,2-^{3}H]aldosterone, and show R_F values in the range 0.5–0.8. The alternative solution for aldosterone is that of conjugating the steroid to the protein at position C_3, via the 3-monohydrazone or the 3-(*o*-carboxymethyl)oxime. However, in both instances, we failed to obtain the expected derivative in acceptable yield, as most of the original aldosterone was converted into the 3,20-dihydrazone or the 3,20-dioxime accompanied by large amounts of other products of unknown composition, giving several distinct spots when using solvent system S_3 (ref. 3).

Cortisol and cholesterol

For both of these steroids, conjugation was carried out via the hemisuccinate, in positions C_{21} and C_3, respectively. Solvent systems S_1 and S_3, respectively, were used to purify cholesterol hemisuccinate and cortisol 21-hemisuccinate.

TME derivatives

Coupling of the steroid derivatives to the tyrosine methyl ester was carried out using N,N'-dicyclohexylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and tetrahydrofuran as solvent[2–4]. Both the steroid-substituted urea, which originates from the reaction, and the unreacted tyrosine methyl ester can be easily separated from the TME–steroid derivative by preparative thin-layer chromatography (TLC). The conversion into the monoiodinated derivative that occurs when labelling the steroid–TME conjugates does not appreciably affect their chromatographic behaviour, at least for the solvent systems we used. The inorganic iodine that originates from autoradiolysis during storage is well separated in each solvent system (position in solvents S_1 and S_2: starting point; in solvent S_3: $R_F = 0.35$), thus permitting a fast purity check by TLC of the labelled TME–steroid derivatives.

All the steroid conjugates, with both rabbit serum albumin or γ-globulins and polylysine, remain at the origin with each solvent system, thus permitting the use of thin-layer radiochromatography to quantitate the reaction yield and to check the purity and the stability of the conjugates during their purification by gel filtration.

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