RADIOIMMUNOLOGICAL AND CHROMATOGRAPHIC PROPERTIES OF TYROSINE METHYL ESTER CONJUGATES WITH STEREOISOMERIC STEROID CARBOXY DERIVATIVES

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Pure 3*Z* (*syn*) and 3*E* (*anti*) stereoisomers of testosterone 3-[*O*-(2-carboxyethyl)]oxime were synthesized, separated by HPLC or TLC, and used for preparation of tyrosine methyl ester (TME) conjugates by using mixed anhydride or carbodiimide–*N*-hydroxysuccinimide methods. While the latter method provided more than 96% of product with retained configuration, the mixed anhydride method yielded a mixture containing 26–40% of the opposite stereoisomer. The stereoisomers were used as model compounds, to which the other steroid TMEs and the corresponding radioiodinated products could be aligned according to their chromatographic properties. The TME conjugates of 3-(*O*-carboxymethyl)oximes of seven 4-en-3-oxo steroids were further prepared by carbodiimide–*N*-hydroxysuccinimide method. With exception of cortisol, the stereoisomeric (*Z* and *E*) radioiodinated TME conjugates could be separated by TLC. In addition, the conjugates with TME and consequently radioiodinated tracers were synthesized from hemisuccinates of cortisol and its 11α-isomer, via 11β- and 11α-hydroxy group. The radioiodinated conjugates were tested as radioligands with rabbit polyclonal antisera raised by using position-homologous conjugates of the respective steroid carboxy derivatives with bovine serum albumin as immunogens. With the exception of 11-deoxycorticosterone, the stereoisomeric *Z* and *E* radioiodinated TMEs did not differ in their binding properties. In the case of isomeric cortisol tracers conjugated through position 11 the antisera recognized only the sterically homologous radioligands, but the specificity of the system was poor.

Key words: Steroid carboxymethyl derivatives; Tyrosine methyl esters; Radioiodination; Radioimmunoassay.

This study was evoked by the need for radioligands of sufficient specific radioactivity to be used in the immunoassay of several less common 4-en-3-oxo steroids present in human serum. Two methods are generally available for the preparation of radioiodinated steroid conjugates for radioimmunoassay: *1*. The compound is first radioiodinated and subsequently coupled with a reactive steroid intermediate, and *2*. The conjugate is prepared as a stock and radioiodinated when needed[1,2]. Histamine, tyramine, and tyrosine methyl ester represent the most common candidates for conjuga-

tion with steroids[1–3]. In general, the methods for preparing protein conjugates are applicable to the synthesis of steroid conjugates as well[4]. Of these, the mixed anhydride and carbodiimide–*N*-hydroxysuccinimide methods are the most common.

Carboxyalkyl oximes, hemisuccinates, carboxyalkyl ethers or thioethers are the most frequently used derivatives for conjugate preparation[3]. Since the first exist as *Z* and *E* isomers with respect to the C=N double bond formed[5–8], the following problems may be encountered: (i) Further derivatization of carboxymethyl oximes (CMO) may influence the stereochemistry of the products, and (ii) the stereoisomeric radiolabelled conjugates may differ in their binding properties when used as tracers in immunoassays.

An effective separation of the stereoisomeric products is therefore needed at first. Because already the starting 3-(*O*-carboxymethyl)oximes used for tyrosine methyl ester (TME) conjugate synthesis usually represent a mixture of *Z* and *E* stereoisomers[7,8], well characterized pure *Z* and *E* isomers of 3-[*O*-(2-carboxyethyl)]oximes of testosterone were first prepared and used as model compounds to which the conjugates of the other steroid 3-(*O*-carboxymethyl)oximes could be aligned according to their chromatographic properties. The radioiodinated conjugates were tested as radioligands with rabbit antisera raised by using position-homologous immunogens.

In addition, tyrosine methyl ester conjugates and consequently radioiodinated tracers were synthesized from stereoisomeric 11β- and 11α-hemisuccinates of cortisol and its 11α-isomer in order to find out how the stereoisomeric tracers would recognize the antisera raised with sterically homologous or heterologous haptens.

EXPERIMENTAL

Chemicals

11α,17α,21-Trihydroxy-4-pregnene-3,20-dione (epicortisol) and 17β-hydroxy-4-estren-3-one (19-nortestosterone) were obtained from Steraloids Inc. (Wilton, NH, U.S.A.). All other steroids including the 3-*O*-CMO derivatives of cortisol and testosterone were purchased from Sigma (U.S.A.). The 3-*O*-CMO derivatives of 17α,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol), 11β,17α-dihydroxy-4-pregnene-3,20-dione (21-deoxycortisol), 21-hydroxy-4-pregnene-3,20-dione (11-deoxycorticosterone), 17α-hydroxy-4-androsten-3-one (epitestosterone) and 19-nortestosterone were prepared according to the method of Janoski et al.[9] in modification of Perry et al.[10]. The synthesis of 11β- and 11α-hemisuccinates of cortisol and epicortisol has been described elsewhere[11]. Preparation of 3*Z* and 3*E* isomers of 3-[*O*-(2-carboxyethyl)]oxime of testosterone is described in ref.[12].

L-Tyrosine methyl ester, tributylamine, *N*-hydroxysuccinimide, *N,N′*-dicyclohexylcarbodiimide, isobutyl chloroformate, aminooxyacetic acid hemihydrochloride and chloramine T were purchased from Sigma (U.S.A.). All other chemicals were of analytical grade, except for the solvents which were of HPLC grade.

[1,2-^{3}H]Cortisol, [1,2,6,7(N)-^{3}H]testosterone, and [1,2-^{3}H]11-deoxycortisol had specific activities 1.49, 3.15, and 1.48 TBq/mmol, respectively, and were purchased from NEN (U.S.A.). Carrier-free Na[^{125}I] was obtained from the Institute of Radiochemistry, Hungarian Academy of Sciences, Hungary.

Methods

TLC. Steroids were chromatographed on DC-Alufol silica sheets (Art 5883, Merck, Germany) in the following systems: S1, benzene–ethanol–acetic acid (75 : 24 : 1); S2, dichloromethane–2-propanol (96 : 4); S3, dichloromethane–2-propanol (94 : 6); S4, dichloromethane–2-propanol (9 : 1); S5, benzene–ethanol (9 : 1); S6, dichloromethane–methanol–acetic acid (96 : 4 : 0.5). 3-*O*-Oximino-4-ene steroids were visualized under UV light (254 nm), non-absorbing compounds were detected by a sulfuric acid–ethanol (9 : 1 v/v) mixture and TME-conjugates were visualized with Folin-phenol reagent. Radioactivity of ^{125}I was scanned by an automatic TLC linear analyzer Tracemaster 40 (Berthold, Wildbad, Germany). The areas containing steroids which were further processed or used as tracers, were cut out and eluted with ethanol.

HPLC. The HPLC system (ECOM, Czech Republic) consisted of a LCP 3001 micropump, LCO 100 column oven, and an LCD 2082 UV detector. The reverse-phase column (SGX C18, 5 μm, 240 × 4 mm) was from TESSEK (Czech Republic). Chromatography was carried out at 60 °C under isocratic conditions using methanol–water (7 : 3 v/v) as the mobile phase, the flow rate was 0.7 ml/min.

UV spectra in ethanol were measured on Unicam 8600 (U.K.) UV-VIS spectrophotometer.

Synthesis of L-Tyrosine Methyl Esters by Mixed Anhydride Method

Tributylamine (10 μl) and isobutyl chloroformate diluted with dioxane (1 : 9 v/v, 10 μl) were added to the carboxy derivative of the steroid (1 mg), which was dissolved in sodium dried dioxane (360 μl). The mixture was stirred at 10–12 °C for 1 h. L-Tyrosine methyl ester (2 mg), dissolved in a mixture of dioxane (100 μl) and 0.05 M sodium phosphate buffer (pH 8.0, 10 μl) was added to the above-mentioned solution, alkalinized with 1 M aqueous NaOH (10 μl), and stirred for an additional 2 h at controlled temperature (10–12 °C). The reaction mixture was then partitioned between ethyl acetate (2 ml) and 0.1 M HCl (1 ml), the organic phase was evaporated under nitrogen and the TME conjugates were separated by TLC or HPLC (see below). The yields of the conjugates were assessed by UV spectroscopy (253 nm) in ethanol and ranged from 65 to 84%.

Synthesis of L-Tyrosine Methyl Esters by Carbodiimide Method

N,*N*-Dimethylformamide (DMF) solutions of *N*,*N*′-dicyclohexylcarbodiimide (7.25 mg/100 μl) and *N*-hydroxysuccinimide (4.6 mg/100 μl), 10 μl each, were added to the carboxy derivative of the steroid (1 mg) dissolved in DMF (40 μl). The mixture was left overnight at room temperature. L-Tyrosine methyl ester solution in DMF (2.5 mg/100 μl, 40 μl) was added, and the mixture was left to react for an additional 24 h. Aqueous 0.1 M hydrochloric acid (200 μl) was subsequently added, and the solution was extracted with ether (2 × 1 ml). The ether was evaporated and the product was purified by chromatography as described above. The yields of the conjugates ranged from 70 to 89%.

Radioiodination of Tyrosine Methyl Ester Conjugates

A slightly modified version of the Cook and Beastall procedure[3] was applied. In brief, the TME conjugate (approximately 5 μg) in ethanolic solution was transferred into a conical glass tube, and the ethanol was evaporated. Na[^{125}I] (18.5 MBq in 0.5 M sodium phosphate, 50 μl, pH 7.4) was added, and the reaction was started by addition of freshly prepared chloramine T solution (6 mg/ml, 10 μl). After 2 min, the iodination was stopped by adding sodium metabisulfite (30 mg/ml, 50 μl). The solution was extracted three times with ethyl acetate (1 ml). The extracts were combined, and the solvent was evaporated under nitrogen.

The radioligands were purified by chromatography (see below). The yields of iodination varied from 21 to 74%.

Antisera

The carboxy derivatives of the steroids were coupled to bovine serum albumin (BSA) and used for immunization of rabbits by a standard procedure[3]. The characteristics of the antisera used in this study are given elsewhere in the literature[11,13–17].

RESULTS AND DISCUSSION

Following separation, the *Z* and *E* isomers of testosterone 3-[*O*-(2-carboxyethyl)]oxime were used for preparation of TME conjugates by both mixed anhydride and carbodiimide–*N*-hydroxysuccinimide methods. The conjugates were radioiodinated and all the products were chromatographed by TLC and HPLC. The chromatographic data are shown in Table I. Although the starting acids (3-CEOs) could not be separated by the methods used, the TME conjugates as well as their iodo derivatives were separated well by both TLC and HPLC. In the latter case, the same mobile phase and reverse-phase column were used as recently reported for the 3-CMOs of four common hormonal steroids and their methyl esters[8]. The less polar *E* isomers moved faster on TLC than the *Z* isomers, whilst the latter moved faster on reverse phase HPLC. As demonstrated in Table II, HPLC of the conjugates prepared by the two above-mentioned methods revealed that the carbodiimide–*N*-hydroxysuccinimide method provided more than 96% of a product with retained configuration, while the mixed anhydride method yielded a

TABLE I
Chromatographic properties of *Z* and *E* isomers of testosterone 3-[*O*-(2-carboxyethyl)]oximes and their derivatives. The data represent means of three determinations

Testosterone 3-CEO derivatives	TLC (R_F)		HPLC (R_T, min)
	chloroform–IPA[a]		methanol–water
	94 : 6	96 : 4	7 : 3
Z-acid	0.11	0.05	4.58
E-acid	0.12	0.05	4.62
Z-TME	0.30	0.19	10.0
E-TME	0.37	0.25	10.8
Z-TME-^{125}I	0.42	0.26	11.2
E-TME-^{125}I	0.53	0.33	12.3

[a] IPA, 2-propanol.

mixture of both stereoisomers (Table II). Neither iodination with [Na^{125}I] nor 3 month's storage in ethanol at –20 °C changed their configuration.

Both radioactive iodo derivatives were checked as radioligands in a testosterone radioimmunoassay using common rabbit antiserum[13] to testosterone-3-*O*-(carboxymethyl)oxime : BSA. The radioligands (*E* and *Z*) did not differ from each other in their binding properties (i.e. in the "titer" and 50% intercept of the calibration curve in a log–logit plot), and they could replace the routinely used [^{125}I]iodohistaminyl testosterone 3-*O*-(carboxymethyl)oxime.

Tyrosine methyl esters were further prepared by carbodiimide–*N*-hydroxysuccinimide method from 3-*O*-(carboxymethyl)oximes of seven biologically active steroids. These included cortisol, three less common corticoids of the glucocorticoid pathway, testosterone, its 17α-hydroxyisomer, and 19-nortestosterone. Following chromatographic purification on TLC the conjugates were radioiodinated with Na[^{125}I]. Chromatographic mobilities of the starting acids (CMOs), the corresponding TME conjugates, and their radioactive iodo derivatives in various TLC systems are summarized in Table III. With the exception of cortisol, in all instances two isomeric TMEs and two radioiodinated products were isolated. The 4-en-3-oxo steroid TMEs could be aligned to *Z* and *E* series according to their chromatographic mobilities, which were analogous to those of the testosterone 3-CEO isomers and their derivatives.

The radioiodinated TME conjugates were tested as radioligands in assay systems using bridge- and position-homologous polyclonal antisera and the corresponding non-radioactive standards.

Table IV shows the working dilutions at which 50% of the constant amount of radioactive ligand (15 000 cpm/tube) remains specifically bound (the "titers") as well as the 50% intercepts derived from the calibration curves in log–logit plots. No difference was found when either *Z* or *E* isomers were used with the only exception of 11-deoxycorticosterone where about a five times higher "titer" was achieved with the *Z*

TABLE II

Ratios of stereoisomeric tyrosine methyl ester conjugates of testosterone 3-CEO prepared by the mixed anhydride (MIX) and carbodiimide–*N*-hydroxysuccinimide (CD–NHS) methods. The data represent means of three independent determinations

Starting isomer	Method	*Z*/*E*, %
Z	MIX	74 : 26
Z	CD–NHS	96 : 4
E	MIX	40 : 60
E	CD–NHS	4 : 96

TABLE III

Chromatographic properties of 3-*O*-(carboxymethyl)oximes of the seven 4-en-3-oxo steroids studied, of their derivatives, and of the corresponding [^{125}I]radioiodinated ligands on silica gel TLC. The data represent means of three measurements

Derivative	Supposed isomer	Chromatographic system					
		S1	S2	S3	S4	S5	S6
				11-Deoxycorticosterone			
Acid	*E*, *Z*	0.62	0.04	0.07[a]	0.05	0.08	0.28
TME I	*Z*	0.74	0.25	0.48[a]	0.29	0.32	0.56
TME II	*E*	0.74	0.25	0.48[a]	0.32	0.32	0.56
^{125}I-TME I	*Z*	0.77	0.32	0.70[a]	–	0.37	0.60
^{125}I-TME II	*E*	0.77	0.36	0.71[a]	–	0.38	0.60
				11-Deoxycortisol			
Acid	*E*, *Z*	0.51	0.04[a]	0.02	0.03	0.06	0.18[a]
TME I	*Z*	0.72	0.17[a]	0.09	0.10	0.25	0.48[a]
TME II	*E*	0.72	0.17[a]	0.09	0.13	0.25	0.48[a]
^{125}I-TME I	*Z*	0.74	0.13	0.12	0.16	–	0.57[a]
^{125}I-TME II	*E*	0.74	0.18	0.14	0.19	–	0.57[a]
				21-Deoxycortisol			
Acid	*E*, *Z*	0.48	0.01[a]	0.01	–	0.04	0.10
TME I	*Z*	0.71	0.14[a]	0.11	–	0.27	0.35
TME II	*E*	0.71	0.22[a]	0.20	–	0.27	0.36
^{125}I-TME I	*Z*	0.75	0.41[a]	0.25	–	0.40	0.42
^{125}I-TME II	*E*	0.76	0.46[a]	0.36	–	0.42	0.44
				Cortisol			
Acid	*E*, *Z*	0.67	0.00	0.00	–	0.02[a]	–
TME	*E*, *Z*	0.50	0.12[a]	0.10	–	0.21[a]	–
^{125}I-TME	*E*, *Z*	0.71	0.15[a]	0.16	–	0.23[a]	–
				Testosterone			
Acid	*E*, *Z*	0.45	0.01	0.01	–	0.09	–
TME I	*Z*	0.57	0.24	0.34	–	0.30	–
TME II	*E*	0.63	0.29	0.40	–	0.32	–
^{125}I-TME I	*Z*	–	0.32	0.44	–		

TABLE III
(*Continued*)

Derivative	Supposed isomer	Chromatographic system					
		S1	S2	S3	S4	S5	S6
			Epitestosterone				
Acid	*E, Z*	0.44	0.03	0.01	–	0.04	0.08
TME I	*Z*	0.60	0.31	0.40	–	0.28	0.18
TME II	*E*	0.60	0.35	0.45	–	0.32	0.23
^{125}I-TME I	*Z*	–	0.39	0.48	–	0.43	0.29
^{125}I-TME II	*E*	–	0.45	0.58	–	0.49	0.30
			Nortestosterone				
Acid	*E, Z*	0.59	0.07[a]	0.05	–	0.14[a]	–
TME I	*Z*	0.75	0.33[a]	0.26	–	0.37[a]	–
TME II	*E*	0.75	0.39[a]	0.31	–	0.38[a]	–
^{125}I-TME I	*Z*	–	0.42[a]	0.39	–	0.44[a]	–
^{125}I-TME II	*E*	–	0.52[a]	0.48	–	0.46[a]	–

[a] Repeated development in the same system.

isomer. This is not surprising because mixtures of isomers were used as haptens for preparation of the immunogens. In the three cases where tritiated ligands were available (cortisol, testosterone and 11-deoxycortisol), the titers were higher by about one order of magnitude with ^{125}I-labelled ligands than with tritiated ones. Thus sufficient assay sensitivity was achieved for detection of physiological or even supraphysiological concentrations of the above-mentioned less common steroids present in human serum[14,16]. In the case of epitestosterone, the tracer has been successfully applied to detection of this steroid in tissue samples from human prostate[18].

Tyrosine methyl esters and consequently ^{125}I-iodo derivatives were prepared also from isomeric 11β- and 11α-hemisuccinates of 11,17α,21-trihydroxy-4-pregnene-3,20-dione (cortisol and epicortisol). In the latter case, the intention was to prepare alternative tracers to the 3-CMO derivative to be used for RIA of 11-deoxycortisol in combination with the antisera raised against position- and bridge-homologous immunogens. It was also of interest to test whether the antisera raised against the 11α-hapten would recognize the "mirror-suited" 11β-derivatized tracer and vice versa. Chromatographic mobilities of these compounds in various TLC systems are shown in Table V.

Both radioiodinated TMEs of the isomeric 11-hemisuccinates of cortisol and epicortisol bound to the corresponding antisera with "titers" of 1 : 8 000 (11α) and 1 : 1 800 (11β), respectively, and could be displaced by non-radioactive 11-deoxycortisol. The specificity of the systems, however, was very poor; the radioligands were displaced by 11-deoxycortisol as well as by cortisol (cross-reaction nearly 100%) rendering the sys-

TABLE IV
Radioimmunological characteristics of [^{125}I]iodotyrosine methyl ester conjugates of seven 4-en-3-oxo steroids

Steroid	Titer[a]	50% Intercept[b], pmol/tube
11-Deoxycorticosterone (*Z*)	40 000	0.47
11-Deoxycorticosterone (*E*)	5 000	0.52
11-Deoxycortisol	2 500	1.34
21-Deoxycortisol	4 000	1.89
Cortisol	8 000	0.24
Testosterone	60 000	0.021
Epitestosterone	32 000	0.25
19-Nortestosterone	40 000	0.22

[a] Working dilution of the antiserum at which 50% of the radioactivity present in the system was specifically bound. [b] Derived from the calibration curve in a log–logit plot.

TABLE V
Chromatographic properties on silica gel TLC of isomeric 11,17α,21-trihydroxy-4-pregnene-3,20-dione (cortisol and epicortisol) hemisuccinates derivatized at position 11

Derivative	Chromatographic system		
	S1	S2	S3
11α-HS[a] (acid)	0.53	0.01	0.08
11α-HS-TME	0.57	0.08	0.16
11α-HS-^{125}I-TME	0.75	0.10	0.19
11β-HS (acid)	0.73	0.02	0.10
11β-HS-TME	0.74	0.18	0.22
11β-HS-^{125}I-TME	0.77	0.39	0.41

[a] HS, hemisuccinate.

tems useless for RIA. On the other hand, no binding of the ligands to the antisera raised against the opposite immunogens was observed.

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