ANTIGENIC PROPERTIES OF ESTRIOL 3-GLUCURONIDE-[C-6]-BOVINE SERUM ALBUMIN CONJUGATES HAVING OXIME BRIDGES

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ANTIGENIC PROPERTIES OF ESTRIOL 3-GLUCURONIDE-[C-6]-BOVINE SERUM ALBUMIN CONJUGATES HAVING OXIME BRIDGES[1)]

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The preparation and antigenic properties of estriol 3-glucuronide-bovine serum albumin (BSA) conjugate in which the hapten is linked to the carrier protein through an (*O*-3-carboxypropylcarbamoylmethyl)oxime bridge at the C-6 position on the steroid nucleus, have been described. 16,17-Di-*O*-acetyl-6-oxoestriol 6-(*O*-carboxymethyl)oxime 3-glucuronide acetate-methyl ester was condensed with γ-amino-*n*-butyric acid by the mixed anhydride method. Subsequent coupling with BSA followed by removal of the protecting groups with alkali gave the desired hapten-BSA conjugate. The antisera elicited in the rabbit with the conjugate were highly specific to estriol 3-glucuronide, discriminating from other 3-substituted compounds. The specificity of antisera improved by elongation of the 6-(*O*-carboxymethyl)oxime bridge has been discussed.

Keywords —estriol 3-glucuronide; radioimmunoassay; antiserum; specificity; cross-reaction; 6-(*O*-carboxymethyl)oxime; 6-(*O*-carboxypropylcarbamoylmethyl)oxime; bridge length

INTRODUCTION

The urinary level of estriol 3-glucuronide is recently recognized as an important index of the fertile period in women.[2,3)] The current methods for the determination of steroid glucuronides in biological fluids require prior chemical or enzymatic hydrolysis.[4)] These procedures have inevitable disadvantages: the lack of reliability in analytical results and the loss of information on the conjugated forms. Therefore, development of a direct radioimmunoassay method without deconjugation employing specific antisera is needed. In the previous paper of this series we reported the preparation of specific antisera to estrone 3-sulfate by immunization with the hapten-[C-6]-bovine serum albumin (BSA) conjugate.[5)] Among various types of linkage to BSA through the C-6 position on the steroid nucleus, an (*O*-carboxymethyl)oxime bridge is most favorable in the preparation of the hapten-BSA conjugate with respect to feasibility.[6)] As for estrogen 3-glucuronides, however, antisera elicited with the corresponding 6-oxime-BSA conjuagates exhibited significant cross-reactions with the closely related compounds, in particular the 3-substituted derivatives.[7)] This appears to be ascribable to the proximity between the glucuronyl moiety and BSA linked to the C-6 position. The present paper deals with the comparative studies on the antigenic properties of estriol-[C-6]-BSA conjugates having 6-oxime bridges of different lengths.

MATERIALS AND METHODS

Chemicals and Reagents — [6,7-^{3}H]-Estriol (56.4 Ci/mmol) was supplied by New England Nuclear (Boston, MA), and the radiochemical purity was checked by thin-layer chromatography (TLC) prior to use. [6,7-^{3}H]-Estriol 3-glucuronide and other conjugated steroids were

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prepared in these laboratories by known methods.[7] All free steroids were kindly donated from Teikoku Hormone Mfg. Co. (Tokyo, Japan). BSA fraction V and bovine serum gamma-globulin were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Kokusan Chemical Works, Ltd. (Tokyo, Japan), complete Freund's adjuvant and other general reagents were from Nakarai Chemical Ltd. (Kyoto, Japan).

Synthesis of Hapten-BSA Conjugate — 16,17-Di-*O*-acetyl-6-oxoestriol 3-glucuronide acetate-methyl ester 6-(*O*-3-carboxypropylcarbamoylmethyl)oxime **(2)**: Tri-*n*-butylamine (80 μl) and isobutyl chloroformate (25 μl) were added to a solution of 16,17-di-*O*-acetyl-6-oxoestriol 3-glucuronide acetate-methyl ester 6-(*O*-carboxymethyl)oxime **(1)** [7] (60 mg) in dry dimethylformamide (1.5 ml), and the whole was stirred for 30 min under ice-cooling. γ-Amino-*n*-butyric acid (50 mg) in water–dimethylformamide–1 N NaOH (2:4:0.05, v/v) (8 ml) was added to the above solution, and the whole was stirred at room temperature for 32 h. The resulting solution was evaporated *in vacuo*, diluted with water, acidified with 0.1 N HCl and then extracted with ethyl acetate. The organic layer was washed with water, drived over anhydrous Na_2SO_4, and evaporated down under reduced pressure. The residue was recrystallized from aq. methanol to give **2** (35 mg) as a colorless amorphous substance. The product showed a single spot on TLC using chloroform–methanol–water (80:20:2.5, v/v) as a developing solvent. The crude product was subjected to further elaboration without purification.

6-Oxoestriol 3-glucuronide 6-(*O*-3-carboxypropylcarbamoylmethyl)oxime-BSA conjugate **(4)**: Tri-*n*-butylamine (70 μl) and isobutyl chloroformate (20 μl) were added to a solution of **2** (30 mg) in dry dimethylformamide (2.0 ml), and the whole was stirred for 30 min under ice-cooling. A solution of BSA (100 mg) in water–dimethylformamide–1 N NaOH (5:4:0.2, v/v) (9.2 ml) was added to the above solution, and the whole was stirred at 4°C for 20 h. The resulting solution was dialyzed against cold running water for 48 h. Lyophilization of the solution gave 16,17-di-*O*-acetyl-6-oxoestriol 3-glucuronide acetate-methyl ester 6-(*O*-3-carboxypropylcarbamoylmethyl)oxime-BSA conjugate **(3)** (125 mg). A solution of **3** (125 mg) in water (30 ml) was adjusted with 5 N NaOH to pH 12 and stirred at room temperature for 12 h.[8] The resulting solution was dialyzed against cold running water for 48 h. Lyophilization of the solution gave **4** (94 mg) as a fluffy powder. The molar steroid: protein ratio of the conjugate was determined to be 13 by UV absorption spectrophotometric analysis.

Immunization of Rabbits — Two male albino rabbits were used for immunization. The antigen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). This emulsion was injected into rabbits subcutaneously at multiple sites over the scapulae and in the thigh. This procedure was repeated at intervals of 2 weeks for a further 2 months and then once a month. The rabbits were bled 10 d after the booster injection. The sera were separated by centrifugaton at 3000 rev/min for 10 min and stored at −20°C. The antisera were thawed and diluted with 0.05 M phosphate buffer (pH 7.4) containing 0.04% BSA.

Assay Procedure — A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing 0, 50, 100, 200, 500, 1000, and 2000 pg of non-labeled estriol 3-glucuronide and ^{3}H-labeled estriol 3-glucuronide (76 pg, 20000 dpm). The diluted antiserum (1:5000, 1:7000) (0.2 ml) was added, and the mixture was incubated at 4°C for 12 h. After treatment with dextran-coated charcoal (0.5% Norit and 0.1% dextran in assay buffer) (0.2 ml) for 10 min at 0°C, the bound and free steroids were separated by centrifugation (3000 rev/min). A 0.2 ml aliquot of each supernatant was taken into a counting vial and added with a tT 21 scintillation cocktail (4 ml).[9] The radioac-

TABLE I. *Percent Cross-Reactivities of Anti-Estriol 3-Glucuronide Antisera Raised against the 6-(O-Carboxymethyl)oxime-BSA Conjugate with Selected Steroids*

Steroid	% cross-reactivity (50%)	
	No. 1	No. 2 a)
Estriol 3-glucuronide	100	100
Estrone 3-glucuronide	<0.01	<0.01
Estradiol 3-glucuronide	1.01	2.27
Estradiol 17-glucuronide	<0.01	<0.01
Estriol 16-glucuronide	0.08	0.06
Estriol 17-glucuronide	<0.01	<0.01
2-Hydroxyestradiol 2-glucuronide	<0.01	<0.01
Estrone sulfate	<0.01	<0.01
Estradiol 3-sulfate	0.70	0.88
Estradiol 17-sulfate	<0.01	<0.01
Estriol 3-sulfate	50.00	27.78
Estrone	<0.01	<0.01
Estradiol	1.20	0.91
Estriol	34.00	17.64
Estrone 3-methyl ether	<0.01	<0.01
Estradiol 3-methyl ether	0.98	0.68
Estriol 3-methyl ether	26.15	9.37

a) The data presented in the previous report.[7]

TABLE II. *Percent Cross-Reactivities of Anti-Estriol 3-Glucuronide Antisera Raised against the 6-(O-3-carboxypropylcarbamoylmethyl)oxime-BSA Conjugate with Selected Steroids*

Steroid	% cross-reactivity (50%)	
	No. 1	No. 2
Estriol 3-glucuronide	100	100
Estrone 3-glucuronide	0.24	0.06
Estradiol 3-glucuronide	9.47	5.77
Estradiol 17-glucuronide	<0.01	<0.01
Estriol 16-glucuronide	<0.01	<0.01
Estriol 17-glucuronide	<0.01	<0.01
2-Hydroxyestradiol 2-glucuronide	<0.01	<0.01
Estrone sulfate	<0.01	<0.01
Estradiol 3-sulfate	<0.01	<0.01
Estradiol 17-sulfate	<0.01	<0.01
Estriol 3-sulfate	0.36	2.83
Estrone	<0.01	<0.01
Estradiol	<0.01	<0.01
Estriol	0.54	5.10
Estrone 3-methyl ether	<0.01	<0.01
Estradiol 3-methyl ether	0.02	0.14
Estriol 3-methyl ether	0.60	2.00

tivity was counted in a Beckman LS 7000 liquid scintillation spectrometer.

Cross-Reaction Study——The specificities of antisera raised against the estriol 3-glucuronide-BSA conjugates were assessed by cross-reaction studies with 17 kinds of selected steroids (Table I, II). The relative amounts required to reduce the initial binding of ^{3}H-labeled steroid by half, where the mass of non-labeled estriol 3-glucuronide was arbitrarily chosen as 100%, were calculated by the standard curve.[10]

RESULTS AND DISCUSSION

An initial effort was directed to the preparation of the estriol 3-glucuronide-BSA conjugate having a longer bridge linked to the C-6 position on the steroid nucleus. For this purpose 16,17-di-*O*-acetyl-6-oxoestriol 3-glucuronide acetate-methyl ester 6-(*O*-carboxymethyl)-oxime (**1**)[7] was taken as a pertinent starting material. Elongation of the bridge was undertaken to relieve the steric hindrance between the glucuronyl moiety and carrier protein. Condensation of **1** with γ-amino-*n*-butyric acid was effected by the mixed anhydride method to afford the 6-(*O*-3-carboxypropylcarbamoylmethyl)-oxime (**2**). Coupling with BSA by the mixed anhydride method followed by removal of the protecting groups with alkali under mild conditions provided the desired 6-oxoestriol 3-glucuronide 6-(*O*-3-carboxypropylcarbamoylmethyl)oxime-BSA conjugate (**4**). As judged from the UV absorption spectrophotometry a satisfactory number of steroid molecules were incorporated into each BSA.

The hapten-BSA conjugate thus prepared was used for immunization of animals. Several months after an initial injection of antigen, immunized rabbits yielded antibodies exhibiting remarkably increased binding activity to estriol 3-glucuronide. The preliminary test indicated that there was no substantial difference in the affinity among antisera elicited in the two rabbits with this antigen.

Evaluation of the titer was performed by incubating various dilutions of antiserum with a constant amount of ^{3}H-labeled estriol 3-glucuronide. The dilution of antiserum which was able to bind 50% of the labeled antigen, was defined as a titer. The dose-response curves were

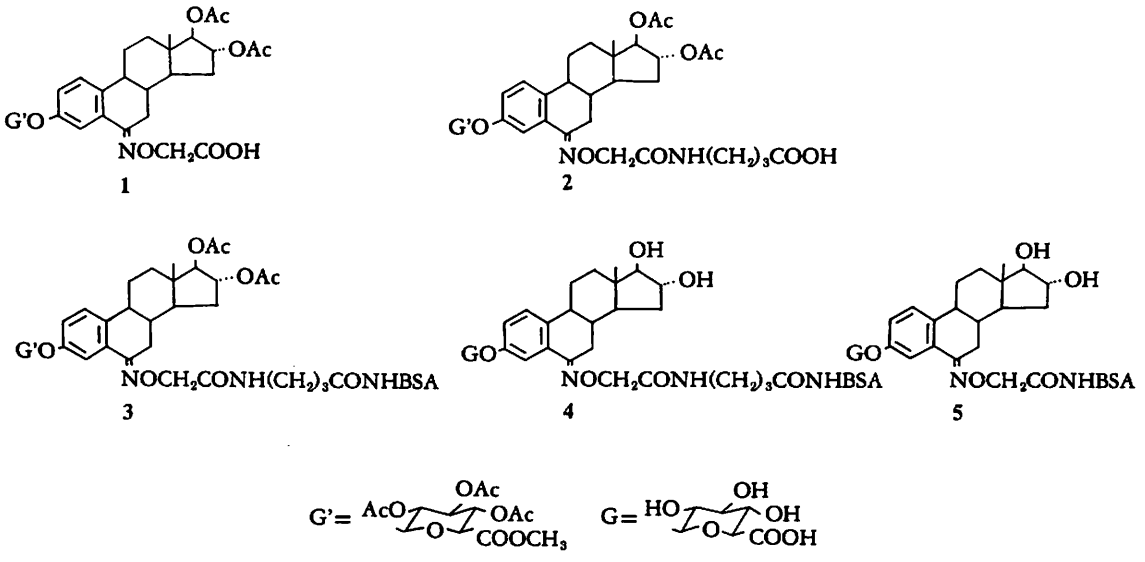

CHART 1

constructed with 1:5000 and 1:7000 dilution of anti-estriol 3-glucuronide antisera. The ratio of the bound to free (B/F) observed with each antiserum was plotted against the concentration of antigen according to the method of Scathard.[11] The association constants of anti-estriol 3-glucuronide antisera were thus estimated to be 2.5×10^9 and $2.7 \times 10^9 M^{-1}$, respectively.

The specificities were assessed by the displacement effect on ^{3}H-estriol 3-glucuronide with closely related steroids. The anti-estriol 3-glucuronide antisera were also prepared by immunization with the 6-(*O*-carboxymethyl)-oxime-BSA (**5**) conjugate in the manner previously reported.[7] The antisera exhibited cross-reactions to a considerable extent with estriol 3-sulfate (27.8, 50.0%), 3-methyl ether (9.4, 26.2%), and free estriol (17.6, 34.0%), respectively (Table I). Steroids other than estrogens showed negligibly low cross-reactivities. The results on cross-reactivities of antisera raised against the 6-oxoestriol 3-glucuronide 6-(*O*-3-carboxypropylcarbamoylmethyl)oxime-BSA conjugate with selected steroids are listed in Table II. These antisera were highly specific to estriol 3-glucuronide, discriminating from estriol 3-sulfate (0.4, 2.8%), 3-methyl ether (0.6, 2.0%), and free estriol (0.5, 5.1%). Other related steroids exhibited no cross-reactions. It is evident from the data that the specificity of anti-estriol 3-glucuronide antisera was remarkably improved by the use of immunogen having a longer bridge coupled to BSA.

Previously, we reported the preparation of specific antisera to estradiol 17-glucuronide and estriol 16-glucuronide.[6] The antisera elicited with immunogens in which the steroid haptens were coupled to a carrier protein through the C-6 position remote from both ring D and A, were capable of discriminating estrogen conjugates from the closely related steroids. In addition, the specific antiserum to estrone 3-sulfate was obtained by immunization with the 6α-hydroxyestrone 3-sulfate 6-hemisuccinate-BSA conjugate.[5] Contrary to the expectation, however, antisera raised against 6-oxoestriol 3-glucuronide 6-(*O*-carboxymethyl)oxime- and 6-oxoestrone 3-glucuronide 6-(*O*-carboxymethyl)oxime-BSA conjugates showed the cross-reactions to a considerable extent with important estrogens.[7] This appears to be ascribable to the proximity between the glucuronyl moiety at C-3 nd BSA linked to the C-6 position. It seems very likely that elongation of the bridge would serve to relieve the steric hindrance between these two, yielding antisera of higher specificity to estriol 3-glucuronide. Such a device for the hapten design may be widely applicable to the preparation of specific antisera to other steroid hormones having a bulky substituent at C-3.

The availability of specific antisera serves to develop a radioimmunoassay method without prior extraction and chromatographic separation, and hence is favorable for the routine assay.[12–14] The application of direct radioimmunoassay for estriol 3-glucuronide to clinical specimens will be reported elsewhere in the near future.

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