Radioimmunoassay of 2-Hydroxyestrone Using Antisera Raised against Antigenic Complexes Obtained by Convenient Methods

Mitsuteru NUMAZAWA,*,[a] Katsuhiko KIMURA,[a] Masao NAGAOKA[a] and Akira KAMBEGAWA*,[b]

Tohoku College of Pharmacy,[a] 4–1 Komatsushima-4-chome, Sendai 981, Japan and Department of Obstetrics and Gynecology, School of Medicine, Teikyo University,[b] 11–1 Kaga-2-chome, Itabashi, Tokyo 173, Japan. *Received October 31, 1988*

Antigenic complexes of 2-hydroxyestrone (2-OHE_1) were obtained by Mannich reaction of 2-OHE_1 and bovine serum albumin (BSA) and by coupling of 2-OHE_1 1-glutathione thioether to BSA using glutaraldehyde. Antiserum raised against the antigen obtained by the Mannich reaction had high affinity ($K_d = 3.8 \times 10^9\ M^{-1}$) and relatively high specificity; cross reactivities for estrone, 4-hydroxyestrone and 2-methoxyestrone were 2.1%, 10% and 1.5%, respectively. The other antiserum also had high affinity ($4.5 \times 10^9\ M^{-1}$) but its cross reactivities for the above three steroids were more than 100%. Concentrations of 2-hydroxyestrone in human plasma were determined by radioimmunoassay with the more specific antiserum and Sephadex LH-20 chromatography to be less than a minimum detectable amount (< 10 pg/ml) (men), 20.9 pg/ml (women, proliferation) and 26.0 pg/ml (women, periovulation).

Keywords 2-hydroxyestrone; radioimmunoassay; hapten antigen; Mannich reaction; glutaraldehyde; anti-2-hydroxyestrone-antiserum; cross reactivity; human plasma

Catechol estrogens, a group of major estrogen metabolites formed by aromatic hydroxylation of primary estrogens at the C-2 position, affect neuroendocrine mediated events at pharmacological doses.[1] Concentrations of 2-hydroxyestrone (2-OHE_1) in human plasma were first determined by Yoshizawa and Fishman[2] using radioimmunoassay (RIA). Antiserum used in the RIA was raised against 2-OHE_1 17-*O*-carboxymethoxime coupled to bovine serum albumin (BSA). However, it seems difficult to obtain antiserum specific enough for the non-chromatographic determination of 2-OHE_1 with this antigen.[3] Recently, more specific anti-2-hydroxyestrogen antisera were raised against 2-hydroxyestradiol-6-hemisuccinate–BSA[4] and 2-OHE_1-15-carboxyethylthio–BSA[5] conjugates. However, synthesis of these haptens involves many steps and requires careful treatment to prevent oxidative cleavage of the extremely labile catechol steroids.

Previously, Nambara *et al.*[6] reported the preparation of antisera with excellent specificity to the estrogen ring D glucuronides by immunization with antigens in which steroid haptens are coupled to BSA through ring A using the Mannich reaction and glutaraldehyde. We report here a convenient preparation of antigens for 2-OHE_1 which involves Mannich reaction of 2-OHE_1 and BSA and coupling of 2-OHE_1 1-glutathione thioether to BSA with glutaraldehyde, and RIA of 2-OHE_1 in human plasma using the antiserum induced by the antigen.

Materials and Methods

Materials [6,7-^{3}H]2-OHE_1 (40 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA) and stored in the standard ascorbic acid solution[3a] containing ascorbic acid (15 g) and acetic acid (4 ml) in MeOH (400 ml). Its radiochemical purity was verified by thin-layer chromatography prior to use; the purity was more than 95%. BSA (Fraction V) and bovine serum gamma-globulin were supplied by Sigma Chemical Co. (St. Louis, MO), Sephadex LH-20 and G-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden). 2-OHE_1 and its 1-glutathione thioether were synthesized according to the methods[7] reported previously.

Preparation of Antigens 1) Mannich Reaction Method: BSA (80 mg) dissolved in 50 ml of 0.12 M borate buffer (pH 9.8), 2-OHE_1 (50 mg) dissolved in 7 ml of dimethylformamide, and 7.5% formalin (0.08 ml) were mixed, and the reaction mixture was stirred under an N_2 atmosphere at room temperature for 4 d. The resulting solution was dialyzed, purified by gel filtration (Sephadex G-25) and then lyophilized essentially according to the procedure[6] previously reported to give a hapten–BSA conjugate (**1**) (70 mg).

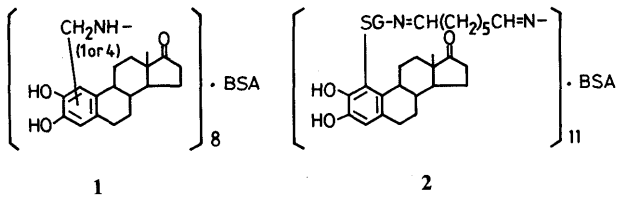
2) Glutaraldehyde Method: A 1% aqueous glutaraldehyde solution (0.7 ml) was added dropwise to a solution of 2-OHE_1 (30 mg) and BSA (66 mg) in 6 ml of 0.1 M phosphate buffer (pH 6.8), and the mixture was allowed to stand at room temperature overnight. Hapten–BSA conjugate (**2**) (23 mg) was isolated in the same way as above.

Immunization and Blood Collection Three male albino rabbits (2—2.5 kg) were used for immunization with each of the two antigens. An antigen (2 mg) was dissolved shortly before injection in 0.5 ml of sterile isotonic saline and emulsified in 1 ml of complete Freund's adjuvant (Nakarai Chemicals Ltd., Kyoto). This emulsion was injected subcutaneously in multiple sites over the scapulae and in the thighs once a month for 3 months. Blood was obtained from the ear vein 10 d after the booster injection. Serum samples were stored at −20 °C. The antisera were thawed and diluted with 0.05 M borate buffer (pH 8.0) containing 0.06% BSA and 0.05% bovine serum gamma-globulin. The serum of the rabbit that gave the highest titer was selected for characterization in each group.

Characterization of Antisera A standard curve was obtained in each case by setting up duplicate centrifuge tubes (7 ml) containing 10—1000 pg of non-labeled 2-OHE_1 and [6,7-^{3}H]2-OHE_1 (21 pg, 5500 dpm), which were initially added to the tube in the standard ascorbic acid solution, followed by evaporation. The diluted antiserum (0.5 ml) was added to all tubes and incubated at 0 °C for 4 h. After this time, 0.5 ml of the dextran–charcoal suspension was added and the mixture was incubated for a further 10 min at 0 °C. After centrifugation (3000 rpm, 10 min), 0.5 ml of the supernatant was used for radioactivity counting.

Specificity was investigated according to Abraham[8] where the degree of cross reaction is expressed numerically on the basis of the mass of each steroid required to produce 50% displacement of labeled 2-OHE_1. Association constants were obtained from Scatchard plot.[9]

Determination of 2-OHE_1 in Plasma The standard ascorbic acid solution[3a] (0.1 ml) containing [6,7-^{3}H]2-OHE_1 (1000 dpm) was added to plasma and then extracted with ether (4 ml × 2). The organic layer was evaporated to dryness under N_2 gas at room temperature. The residue obtained was redissolved in 0.2 ml of the elution solvent and applied to a Sephadex LH-20 column (5 cm × 0.6 cm i.d.). The column was eluted with benzene–MeOH (9 : 1, v/v) containing 0.05% ascorbic acid. The 2-OHE_1 fraction (3.5—6 ml) was collected and evaporated to dryness under N_2 gas. Estrone and 2-methoxyestrone, for which the cross-reactivities of the antiserum are relatively high, could be completely separated from 2-OHE_1 by the chromatography. However, 60% of 4-hydroxyestrone applied to the column was eluted in the 2-OHE_1 fraction. A 0.05 M borate buffer (0.2 ml), as a diluent of the antiserum, was added to each tube, and vortexed, and then a 0.1 ml aliquot was removed for a recovery test. The labeled steroid (5000 dpm, 0.1 ml) and antiserum (0.3 ml) were added to the remainder, and the resulting mixture was incubated at 0 °C for 4 h. Radioactivity of the bound form was determined essentially as above.



1 2

H_2N–GSH: glutathione
BSA: bovine serum albumin

Chart 1

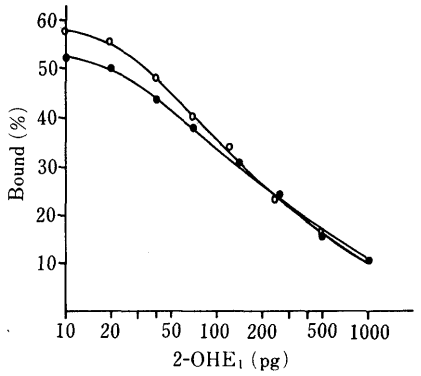


Fig. 1. Dose–Response Curves for Radioimmunoassay of 2-OHE_1 Using Anti-2-OHE_1-antisera

—○—, the Mannich-antiserum (1:3000 dilution). —●—, the glutaraldehyde-antiserum (1:10000 dilution).

TABLE I. Per Cent Cross-Reaction of Anti-2-OHE_1-antisera with Selected Steroids

Steroid	% cross-reaction	
	Antiserum A	Antiserum B
2-Hydroxyestrone	100	100
2-Hydroxyestradiol	1.8	5.3
2-Hydroxyestriol	0.5	1.4
2-Methoxyestrone	1.5	181
2-Methoxyestradiol	<0.1	<0.1
2-Methoxyestriol	<0.1	<0.1
4-Hydroxyestrone	10	131
4-Hydroxyestradiol	<0.1	<0.1
Estrone	2.1	189
Estradiol	0.7	0.4
Estriol	<0.1	<0.1
Androstenedione	<0.1	<0.1
Testosterone	<0.1	<0.1
Progesterone	<0.1	<0.1
Cortisone	<0.1	<0.1

A, Mannich-antiserum; B, glutaraldehyde-antiserum.

Results

Preparation of antigen two novel antigens for production of anti-2-OHE_1 antisera were initially prepared according to the procedure[6] previously reported. Mannich reaction under basic condition occurred at the *ortho* positions to the phenolic group in ring A[10] to give a hapten–BSA conjugate (**1**). 2-OHE_1 1-glutathione thioether was coupled to BSA using glutaraldehyde as a cross-linking agent to yield a hapten–BSA conjugate (**2**). The number of steroid residues incorporated per molecule of BSA as determined by ultraviolet (UV) absorption measurement at 280 nm was 8 for antigen **1** and 11 for antigen **2**.

TABLE II. Plasma Concentration of 2-OHE_1

Subject	Age (year)	No.	2-OHE_1 (pg/ml)
Men	20—40	10	N.D.
Women	20—40		
Proliferation		10	20.9±4.9
Periovulation		10	26.0±3.7

Plasma was collected from normal healthy men and women. N.D.: not detectable (less than 10 pg/ml).

Characterization of Antisera A final dilution of 1 : 10000 (glutaraldehyde method) or 1 : 3000 (Mannich reaction method) of antisera giving 50—60% binding of [6,7-^{3}H]2-OHE_1 (21 pg, 5500 dpm) was used for the study. The standard curves obtained with the antisera are presented in Fig. 1. The specificity of the antisera was investigated by determining the cross-reactivity of various steroids. As can be seen in Table I, the compounds showing significant cross-reactivity were estrone (2.1 and 189%), 2-methoxyestrone (1.5 and 181%), 4-hydroxyestrone (10 and 131%), and 2-hydroxyestradiol (1.8 and 5.3%).

The association constants were found to be $3.8\times10^9\,M^{-1}$ for the Mannich-antiserum and $4.5\times10^9\,M^{-1}$ for the glutaraldehyde-antiserum.

Determination of 2-OHE_1 in Human Plasma The Mannich-antiserum was used for the plasma study. The blank value obtained by the assay procedure using 3 ml of charcoal-treated plasma was not significantly different from zero. The minimum detectable amount averaged 10 pg. Recovery of 2-OHE_1 which was subjected to the entire analytical protocol (extraction and Sephadex LH-20 chromatography) was about 50% on average. The accuracy and precision of the assay were determined by addition of definite amounts of 2-OHE_1 (50 pg) to charcoal-treated human plasma (3 ml). After equilibration for 30 min at 37 °C the amounts of 2-OHE_1 were determined by RIA after Sephadex LH-20 chromatography to be 53±4.2 pg. The coefficient of variation was 8.0% ($n=6$). The inter assay precision study on pooled plasma of women (35 pg/ml) resulted in a coefficient of variation of 11.5%. The concentrations of 2-OHE_1 in plasma are given in Table II.

Discussion

Anti-2-OHE_1 antisera were obtained by immunization using new hapten antigens in which the haptens are linked to BSA through the C-1 and/or C-4 position(s) in a steroid molecule. The affinity of the antisera is as high as those[4,5] reported previously. The specificity of the Mannich-antiserum is relatively high and comparable to those[4,5] reported previously, while the glutaraldehyde-antiserum does not discriminate estrogen A-ring derivatives at all. The stereo-complementary of the antigenic determinations is thought to provide the major contribution towards specificity, and hapten–BSA conjugate containing decomposed hapten usually reduces the specificity of antisera raised against it. These considerations, together with the structures of the hapten antigens **1** and **2** and the instability of 2-OHE_1 under aerobic conditions can account for the present results. Although we have no evidence concerning the decomposition of the haptens, the high cross-reactivity of the glutaraldehyde-antiserum might be principally due to

decomposed haptens. On the other hand, the concentrations of 2-OHE_1 in human plasma could be determined accurately and precisely by using the Mannich antiserum. 4-Hydroxyestrone could not be completely separated from 2-OHE_1 by Sephadex LH-20 chromatography. However, since the 4-hydroxyestrone concentration in plasma is less than 10% of the 2-OHE_1 concentration, the present results on the 2-OHE_1 concentration should have been little affected by the contaminating 4-hydroxy isomer in the assay system.

In conclusion, a novel hapten antigen which can be easily obtained by the Mannich method produced anti-2-OHE_1 antiserum with high affinity and relatively high specificity. The plasma 2-OHE_1 could be determined by RIA using the antiserum, when chromatographic purification was also employed.

Acknowledgment The authors wish to thank Dr. S. Honma of Teikoku Hormone Manufacturing Co. for a generous gift of various steroids used in the cross-reaction experiments.

References

1) P. Ball and R. Knuppen, *Acta Endocrinol.*, **93**, 1 (1980); N. J. MacLuskey, F. Naftolin, F. L. C. Krey and S. Franks, *J. Steroid Biochem.*, **15**, 111 (1981).
2) I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, **32**, 3 (1971).
3) *a*) P. Ball, G. Emons, O. Haupt, H.-O. Hoppen and R. Knuppen, *Steroids*, **31**, 249 (1978); *b*) S. C. Chattoraj, A. S. Fanous, D. Cecchini and E. W. Lowe, *ibid.*, **31**, 375 (1978).
4) Y. Fujii, M. Teranishi, Y. Ikeda, M. Yamazaki, S. Kishida and S. Miyabo, *Steroids*, **46**, 857 (1985).
5) T. Ohkubo, F. Tsuchiko, T. Wakasawa and T. Nambara, *Chem. Pharm. Bull.*, **36**, 3519 (1988).
6) T. Nambara, M. Numazawa, T. Tanaka and T. Ohkubo, *J. Steroid Biochem.*, **9**, 785 (1978); *idem, J. Pharmacobio-Dyn.*, **1**, 55 (1978).
7) G. Stuberauch and R. Knuppen, *Steroids*, **28**, 733 (1971); M. Numazawa and K. Kimura, *ibid.*, **41**, 675 (1983); E. Kuss, *Hoppe-Seyler's Z. Physiol. Chem.*, **352**, 817 (1971).
8) G. E. Abraham, *J. Clin. Endocrinol.*, **29**, 866 (1969).
9) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
10) B. B. Thompson, *J. Pharm. Sci.*, **57**, 715 (1968).