DETERMINATION OF ESTROGENS BY RADIOIMMUNOASSAY WITH ANTIBODIES TO ESTROGEN-C6-CONJUGATES

I. SYNTHESIS OF ESTRONE-, ESTRADIOL-17ß-, AND ESTRIOL-6-ALBUMIN CONJUGATES

by E. Kuss and R. Goebel

Laboratorium für Biochemie der I. Univ.-Frauenklinik
München, Germany

Received: 12/2/71

ABSTRACT

The 6-carboxymethoximes of 6-oxo-estrone, -estradiol-17ß, and -estriol were prepared and conjugated to bovine serum albumin by the mixed-anhydride-technique. The products and the intermediates were characterised by usual methods.

INTRODUCTION

In recent years methods for the determination of estrogens by radioimmuno-assays have been described with increasing frequency (1 - 7). All the techniques available until now have been based upon the reaction of estrogens with antibodies corresponding to estrogen C17- (8,9) or to estrogen ring A-conjugates (10 -13). Like others (14,15) we supposed that in these conjugates the linkages which occupy functional groups of the hapten would diminish the specificity of the corresponding antibody. This publication describes the synthesis of antigens in which estrogens - estrone, estradiol-17ß, and estriol - were each

attached by their C atom 6 to the rest of the macromolecule, while work on the corresponding antibodies will be described in a forthcoming publication. Some of our results have appeared in a preliminary communication (16). LINDNER et al. and EXLEY also recently mentioned the application of estradiol-17ß 6-conjugate as an antigen (17).

MATERIAL

Chloracetic acid "zur Synthese", estrone [3-hydroxy-1,3,5(10)-estratriene-17-one] (E_1), estradiol-17ß [1,3,5(10)-estratriene-3,17ß-diol] (E_2) and estriol [1,3,5(10)-estratriene-3,16α,17ß-triol] (E_3) "für biochemische Zwecke" and other analytical grade reagents were all purchased from E. Merck, Darmstadt, Germany.

4-^{14}C-estradiol-17ß and 4-^{14}C-estriol (specific radioactivity 58 and 30 mc/mMol resp. from The Radiochemical Centre, Amersham, England, were diluted with unlabelled estradiol-17ß and estriol resp. to give 1 x 10^6 cpm/mMol; these materials were used for synthesis.

6-oxo-estrone [3-hydroxy-1,3,5(10)-estratriene-6,17-dione] (E_1-6-oxo) was obtained by oxidation of 6-oxo-estradiol with CrO_3 and purified by column chromatography.

6-oxo-estradiol [3,17ß-dihydroxy-1,3,5(10)-estratriene-6-one] (E_2-6-oxo) and 6-oxo-estriol [3,16α,17ß-trihydroxy-1,3,5(10)-estratriene-6-one] (E_3-6-oxo) obtained from Ikapharm,Ramat-Gan, Israel, were used as reference steroids. Our synthetic ^{14}C-6-oxo-estrogens had essentially the same properties (m.p.,uv, TC) as those described in the literature (19 - 21) and those supplied by Ikapharm.

Isobutyl-chlorcarbonate and acetoxime were purchased from Schuchardt, München, Germany, bovine serum albumin "trocken,reinst" from Behringwerke A.G., Marburg, Germany, and Sephadex G25 fine and Sephadex LH-20 from Pharmacia, Uppsala, Sweden.

2,5-Diphenyloxazol (PPO) and 1,4-bis-2(-4-methyl-5-phenyloxazolyl-benzene (Dimethyl-POPOP) were supplied by W. Zinsser, Frankfurt, Germany.

METHODS

Carboxymethoxylamine-hydrochloride

Acetone-carboxymethoxime was prepared by condensation of acetoxime with chloracetic acid in 20% sodium hydroxide as described by BOREK and CLARKE (18). To hydrolyse the oxime we preferred 5 hrs boiling of 20 g in 200 ml acetic acid followed by repeated evaporations and treatment of the residue with 1N hydrochloric acid instead of saponification with 6N HCL as described by these authors. After the fourth evaporation the residue was dissolved in 100 ml ethanol, 350 ml ether was added and the hydrochloride crystallised overnight at 0°C (13 g, 75%).

6-Oxoestriol 6-carboxymethoxime (E_3-6-cmo)

8,5 g estriol-triacetate (1 x 10^6 cpm/mMol) was oxidized with CrO_3 as described by MARRIAN and SNEDDON (19). 4 g of crude 6-oxo-estriol-triacetate was obtained which was hydrolysed during 24 hrs in 60 ml 20% (w/v) KOH in methanol at room temperature in an atmosphere of N_2 without prior separation of the ketonic fraction. Water was added to a total of 1 l. Most of the hydroxide was neutralized with concentrated HCL and then neutralization to phenolphtalein was completed with solid CO_2 . The organic material was extracted with ethyl acetate (4 x 200 ml), the combined extracts were washed with water (2 x 75 ml) and evaporated. 1,2 g of crude 6-oxo-estriol was obtained (46%).

560 mg of the crude 6-oxo-estriol was refluxed 1 hr with 13,5 ml ethanol, 1,5 ml water, 750 mg sodium acetate and 490 mg carboxymethoxylamine hydrochloride. The mixture was cooled, 90 ml ether was added and the organic layer was extracted with 5% solution of Na_2CO_3 saturated with sodium bicarbonate (2 x 15 ml). The combined extracts were acidified with 5 ml concentrated HCL. The solid obtained after filtration, 525 mg (75%), was dissolved in 2,5 ml 50% acetic acid and was chromatographed on a column of Sephadex G25 (1,0 x 200 cm) with 0,5N acetic acid. The fraction 160 - 360 ml was collected and evaporated. The 6-oxoestriol 6-carboxymethoxime was crystallised from ethanol/water 1:1 and from ethanol/cyclohexane 1:1; m.p. 198 - 105°C, decomposition with gas evolution.

6-Oxoestradiol 6-carboxymethoxime (E_2-6-cmo)

Starting from estradiol-diacetate (1 x 10^6 cpm/mMol) the above procedure was applied to synthesize the estradiol-6-carboxy-methoxime. The final product was chromatographed on a column of Sephadex G25 with 0,5N acetic acid, the fraction 320 - 400 ml was collected and evaporated. The 6-oxoestradiol 6-carboxymethoxime was crystallized from acetic acid/water 1:1 and from ethanol/cyclohexane 1:1; m.p. 194 - 197 °C, decomposition with gas evolution.

6-Oxoestrone 6-carboxymethoxime (E_1-6-cmo)

36 mg 6-oxoestradiol 6-carboxymethoxime (1 x 10^6 cpm/mMol) was dissolved in 6 ml acetone which had been distilled from $KMnO_4$. 0,3 ml of JONES chromic acid oxidant (10,3 g CrO_3, 30 ml water, 8,7 ml H_2SO_4 conc.) (20,21,22) was added. After being allowed to stand 10 min at room temperature the mixture was diluted with 100 ml water and extracted with ether (4 x 30 ml). The ether was evaporated and the residue, 34 mg (95%), was purified by chromatography on a column of Sephadex LH-20 (1,8 x 40 cm) with benzene/0,01N acetic acid in methanol (90:10). The fraction 150 - 180 ml was collected and evaporated. 6-oxoestrone 6-carboxymethoxime was crystallized from ethyl-acetate/cyclohexane and from ethanol/water; m.p. 235 - 240 °C, decomposition with gas evolution.

Thin layer and paper chromatography

To check the course of the reactions, the starting material and the products were chromatographed on precoated plastic sheets and on paper impregnated with formamide; compounds were visualised by the reagent of FOLIN-CIOCALTEU.

System 1: silica gel (Polygram-Sil G from Macherey-Nagel & Co., Düren, Germany) cyclohexane/chloroforme/acetic acid 2:1:1

System 2: cellulose powder (Polygram-Cel 300 from Macherey-Nagel & Co., Düren, Germany) acetate buffer solution pH5 (14,7 ml 2N acetic acid + 20 ml 1M NH_4OH; 1 vol diluted with 9 vol water).

System 3: paper (Nr. 2043b Mgl from Schleicher & Schüll, Dassel, Germany) impregnated with formamide (in methanol, 20%) chlorobenzene/ethyl acetate 3:1 (saturated with formamide).

Rf-values approx.:

	system 1	system 2	system 3
E_1	0,4	0,02	0,8
E_2	0,3	0,02	0,7
E_3	0,1	0,2	0,1
E_1-6-oxo	0,2	0,07	0,6
E_2-6-oxo	0,15	0,15	0,4
E_3-6-oxo	0,02	0,3	0,01
E_1-6-cmo	0,15	0,4	-
E_2-6-cmo	0,09	0,45	-
E_3-6-cmo	0,00	0,57	-

Uv-spectra

The uv-spectra of the three 6-oxoestrogen 6-carboxymethoximes exhibit maxima at 261 nm and 310 nm, $\varepsilon \approx 11{,}5 \times 10^3$ and $4{,}3 \times 10^3$ resp. (in ethanol, spectrophotometer Beckman DB-G).

Mass spectra

Methyl esters for mass spectrographic analysis were prepared by treatment of the 6-oxoestrogen 6-carboxymethoximes with diazomethane. Mass spectra were determined on a mass spectrometer CH-7 from Varian MAT GmbH, Bremen, Germany.

E_1-6-cmo-methylester m/e 371 (M^+), E_2-6-cmo-methylester m/e 373 (M^+), E_3-6-cmo-methylester m/e 389 (M^+).

Nmr-spectra

Nmr-spectra were obtained for the O-carboxymethyloximes in D_5-pyridine; internal standard : tetramethylsilane.

chemical shifts in ppm

	$C_{(18)}\underline{H}_3$	$C_{(7)}\underline{H}_{1eq.}$	$C_{(17)}\underline{H}_1$-OH	=N-O-$C\underline{H}_2$-COOH
E_1-6-cmo	0,74	3,44	---	5,16
E_2-6-cmo	0,90	3,40	3,88*	5,12
E_3-6-cmo	0,98	3,44	4,06**	5,10

* triplet, J = 8,5
** doublet, J = 6,0

Tab. 1

Estrogen-6-albumin conjugates

Each of the three estrogen-6-carboxymethoximes was coupled to bovine serum albumin by means of the mixed anhydride technique following the procedure which ERLANGER et al. (23) used to prepare estrone-17-albumin conjugate.

Radioactivity

An aliquot of the samples (0,100 - 0,050 ml was dropped on to a filter paper (Ø 2,4 cm, SS 595, from Schleicher & Schüll, Dassel, Germany) which was dried (120°C) and counted in a Tricarb 3002 spectrometer, Packard Instrument Company Inc. Downers Grove Ill., USA. Scintillation liquid:4 g PPO, 0,3 g Dimethyl-POPOP in 1 l toluene.

Protein determination

Aliquots of the samples were mixed with biuret reagent; the protein concentration (g/100 ml) was calculated using the extinction coefficient 2,77 (546 nm).

Determination of estrogen residues per molecule albumin conjugate

The radioactivity, the protein content of the samples and the extinction of the solutions at 313 nm were measured. The estrogen/protein ratio was calculated by assuming

a) the molecular weight of bovine serum albumin is 70×10^3;
b) there is no quenching in radioactivity counting of the steroid protein conjugates;
c) the extinction of the conjugates at 313 nm is totally due to the steroid chromophores; their extinction coefficients are not grossly influenced by the protein.

Confirmation of identity of estrogen residues in the albumin conjugates; column chromatography of 6-oxo-estrogens

3 mg E_1- or E_2-6-albumin conjugate was boiled 5 hr in 5 ml 1N HCL. After extraction with ether (5 x 3 ml) the yield of free steroids was determined by measuring radioactivity and extinction at 326 nm (ethanolic solution).

Yields: from 6-oxo-estrone-conjugate, 90%,
from 6-oxo-estradiol-conjugate, 70%.
The free steroids were dissolved in 1 ml benzene/methanol 85/15 and chromatographed on Sephadex LH-20 (1,2 x 30 cm) with the same solvent. 6-oxo-estrone: fraction 18 - 27 ml, 6-oxo-estradiol: fraction 31 - 42 ml. When the estrone-albumin conjugate had been hydrolysed, the fraction 31 - 42 ml was collected, evaporated and submitted to TL chromatography. The same was done with the fraction 18 - 27 ml after hydrolysis of the estradiol-albumin conjugate.

RESULTS AND DISCUSSION

The identity of the prepared 6-oxoestrogen 6-carboxymethoximes seems to be well established by the mass- and especially by the nmr spectra (Tab.1).

Coupling of the O-carboxymethyl-oximes to protein was effected by the mixed anhydride technique with use of isobutyl chlorcarbonate. The resulting covalent linkages, probably amide bonds with the lysine residues of albumin molecules, were proven by the stability of the conjugates to organic solvents as described by ERLANGER et al. (24).

The number of estrogen residues per molecule of albumin was determined by measuring radioactivity and by uv-spectra. The results are summarised in Table 2.

NUMBER OF ESTROGEN RESIDUES PER MOLECULE OF CONJUGATE*)

	Method 1	Method 2
estrone conjugate	24-28	29-31
estradiol-17ß conjugate	21-31	24-37
estriol conjugate	23-29	28-39

*) Range obtained by several coupling reactions. Albumin was determined by the biuret method, estrogens were determined in Method 1 by measuring E_{313} ($\varepsilon \approx 4,3 \times 10^3$) and in Method 2 by measuring radioactivity (1×10^6 cpm/mMol).

Tab. 2

The absorption maxima of the 6-oxoestrogen 6-carboxymethoximes at 310 nm were shifted to 313 nm in the conjugates. ERLANGER et al. (24) noted a hypochromic shift but only when the chromophoric group was not covalently linked to the point of attachment to albumin as in estrogen 6-albumin conjugates.

Since a redox reaction at C17 hydroxy-/oxo-group, which would decrease the specificity of the corresponding antibody, seemed to be possible during storage of the conjugates, the identity of estrogen residues was checked. After hydrolysis of the conjugates the estrogens were separated by column chromatography and the amount of expected estrogen was determined by spectrophotometry of the appropriate fraction. The amount of suspected contaminant was measured by TL assay after concentration of the relevant fractions as described in the experimental section. The sensitivity of this method allows the conclusion that less than 0,1% of estrone- or estradiol-17ß residues had undergone redox reaction.

Thus, our results allow us to conclude that the estrone-, estradiol-17ß- and estriol-6-albumin conjugates, prepared as described, can be used as antigens which induce specific antibodies to the corresponding haptens.

ACKNOWLEDGEMENTS

The authors are indebted to Miss Helga Enderle for skilled technical assistance, to Dr.W. Schäfer, and Dr.J. Sonnenbichler, Max-Planck-Institut für Biochemie, München, for mass and nmr spectra.The study was supported by the Sonderforschungsbereich 51, Deutsche Forschungsgemeinschaft.

REFERENCES

1. Steroid assay by protein binding, Transactions of the second Symposium held in Geneva on March 23-25, 1970, Editor E. Diczfalusy, ACTA ENDOCR.(Kbh.), SUPPL. 147 (1970).
2. Abraham, G.E., J. CLIN. ENDOCR. 29, 866 (1969).
3. Aoki, T., and Hreshchyshyn, M.M., AMER.J.OBESTET. GYNEC. 111, 382 (1971).
4. Mikhail, G., Wu, C.-H., Ferin, M., and Vande Wiele, R.L., STEROIDS 15, 333 (1970).
5. Wu, C.-H., and Lundy, L.E., STEROIDS 18 , 91 (1971).
6. Gurpide, E., Giebenhain, M.E., Tsen, L., and Kelly, W.G., AMER.J.OBSTET.GYNEC. 109, 897 (1971).
7. Yoshizawa, I., and Fishman, J., J. CLIN.ENDOCR. 32, 3 (1971).
8. Lieberman, S., Erlanger, B.F., Beiser, S.M., and Agate, F.J., RECENT PROGR. HORMONE RES. 15, 165 (1959).
9. Ferin, M., Zimmering, P.E., Lieberman, S., and Vande Wiele, R.L., ENDOCRINOLOGY 83, 565 (1968).
10. Goodfriend, L., and Sehon, A., IMMUNOLOGIC METHODS IN STEROID DETERMINATION, Editors F.G. Péron, and B.V. Caldwell, Appleton-Century-Crofts, Meredith corporation, New York, 1970, p. 15.
11. Gross, S.J., IMMUNOLOGIC METHODS IN STEROID DETERMINATION, Editors F.G. Péron, and B.V. Caldwell, Appleton-Century-Crofts, Meredith corporation, New York, 1970, p.41

12. Jungblut, P.W., Fischer, I., Gaues, J., and Tykal, P., 16. SYMPOSION DTSCH.GES.ENDOKR., Springer-Verlag, Berlin, Heidelberg, New York, 1970, p. 142.

13. Midgley, A.R.,Jr., Niswender, G.D., and Ram, J.S., STEROIDS 13, 731 (1969).

14. Lindner, H.R., Perel, E., and Friedlander, A., RES.ON STEROIDS IV, Pergamon Press, Oxford, 1970, p. 197.

15. Axelrod, L., IMMUNOLOGIC METHODS IN STEROID DETERMINATION, Editors F.G. Péron, and B.V. Caldwell, Appleton-Century-Crofts, Meredith corporation, New York, 1970, p. 169.

16. Kuss, E., and Goebel, R., FORTSCHRITTE DER KLINISCHEN CHEMIE; ENZYME UND HORMONE, Verlag der Wiener Medizinischen Akademie, in press (Proc. joint meeting of the Deutschen, Schweizerischen und Österreichischen Gesellschaft für Klinische Chemie, Wien, 22.-24. April, 1971).

17. Exley, D., J.ENDOCRINOL., in press; (Proc. joint meeting of the British Biochemical Society and Society for Endocrinology, Cardiff, September 12-16, 1971).
Lindner, H.R., Perel, E., Friedlander, A., and Zeitlin, A., J.ENDOCRINOL., in press; (Proc. joint meeting of the British Biochemical Society and Society for Endocrinology, Cardiff, September 12-16, 1971).

18. Borek,E., and Clarke, H.T., J.AMER.CHEM.SOC. 58, 2020 (1936).

19. Marrian, G.F., and Sneddon, A., BIOCHEM.J. 74, 430 (1960).

20. Longwell, B., and Wintersteiner, O., J.BIOL.CHEM. 133, 219 (1940).

21. Knuppen, R., and Breuer, H., ANN. 639, 194 (1961).

22. Djerassi, C., Engle, R.R., and Bowers, A., J.ORG. CHEM. 21, 1547 (1956).

23. Erlanger, B.F., Borek, F., Beiser, S.M., and Lieberman, S., J.BIOL.CHEM. 234, 1090 (1959).

24. Erlanger, B.F., Borek, F., Beiser, S.M., and Lieberman, S., J.BIOL.CHEM. 228, 713 (1957).