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The Specificity in Enzyme Immunoassay for Plasma and Urine Cortisol[1)]

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The specificity in heterogeneous enzyme immunoassays for cortisol has been investigated. Enzyme labeling of cortisol was accomplished by the N-succinimidyl ester method at an appropriate molar ratio of steroid to enzyme. Three cortisol derivatives possessing different bridges at C-4 were covalently linked to β-galactosidase. The anti-cortisol antisera used were those elicited in rabbits by immunization with the conjugates of these haptenic derivatives with bovine serum albumin. With the aim of obtaining high sensitivity, enzyme immunoassays were carried out in the bridge heterologous combinations of antiserum and enzyme-labeled cortisol. The specificity of these assay systems was assessed by measuring the amount of cortisol in biological fluids, and by comparison of the results with those of radioimmunoassays. The cross-reactivities were also tested with fifteen kinds of closely related steroids. The assay using the antiserum raised against the 4-(2-carboxyethylthio)cortisol-protein conjugate was found to be relatively specific and applicable to the determination of cortisol in human plasma and urine.

Keywords——enzyme immunoassay; cortisol; enzyme-labeling of cortisol; N-succinimidyl ester method; cortisol-β-galactosidase conjugate; bridge heterology; specificity; plasma level; urine level; cross-reaction

Enzyme immunoassays of cortisol have recently been developed using antisera raised against the conjugates of 21-hemisuccinoylcortisol[2)] and cortisol 3-(O-carboxymethyl)oxime[3)] with bovine serum albumin (BSA). The specificity of antibodies is significantly influenced by the position on the steroid molecule used for conjugation to the carrier and also by the stereochemistry of the steroid hapten. The position C-4 in the cortisol molecule appears to be an attractive site for attachment of the carrier because the trigonal carbon provides characteristic stereochemistry, and hydroxylation at this position has not yet been reported in the metabolism of cortisol. It is also advantageous that several types of haptenic derivatives linked to this position are readily available, since the combination of antibody and enzyme-labeled antigen is an important factor determining the sensitivity of enzyme immunoassay, and hence a "bridge" heterologous system is often required. In a previous paper of this series, we reported the sensitivities obtained in various enzyme immunoassays using antisera raised against cortisol-4-BSA conjugates.[4)] This paper deals with the specificity in the enzyme immunoassays for human plasma and urine cortisol.

Materials and Methods

Materials——β-Galactosidase (EC 3.2.1.23) from *E. coli* (grade VI, 360 units per mg protein) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and *o*-nitrophenyl β-D-galactopyranoside from Nakarai Chemical, Ltd. (Kyoto). [1,2,6,7-^{3}H]-Cortisol (90 Ci/mmol) was supplied by CEA (Gif-Sur-Yvette, France). 4-(Carboxymethylthio)cortisol (CMT), 4-(2-carboxyethylthio)cortisol (CET), 4-(2-hemisuccinoyloxyethylthio)-cortisol (HST), and 4-hemisuccinoyloxycortisol (HS) were prepared by the methods previously established in these laboratories.[5)] The abbreviations are also used for antiserum and enzyme-labeled antigen, which were prepared by use of the corresponding carboxylated derivatives. Anti-cortisol antisera used were those reported in the previous paper.[6)] The antisera were diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (Buffer A). Goat anti-rabbit IgG antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs., Ltd. (Tokyo).

Preparation of Cortisol-β-Galactosidase Conjugates——This was carried out in the manner described previously.[4)] In short, the carboxylated cortisol (CMT, CET and HS) was derivatized into the N-succinimidyl

ester by treatment with N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Reaction of the activated ester with β-galactosidase at a molar ratio of 4 or 6 gave the enzyme-labeled cortisol. After dialysis against cold PB, the conjugates were stored at 4°C in the solution at a concentration of 500 μg per ml, adjusted with PB containing 0.1% gelatin and 0.1% NaN_3 (buffer B). For the immunoassay procedure, this was diluted with the buffer solution containing 0.5% normal rabbit serum.

Immunoassay Procedures——Enzyme immunoassay was carried out as follows: diluted antiserum (0.1 ml) and the cortisol-enzyme conjugate (0.1 μg, 0.1 ml) in the buffer were added to a series of standard solutions and to the sample in buffer B (0.1 ml), and the mixture was incubated at 4°C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1: 20 with buffer A containing 0.3% EDTA was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4°C for 16 h. After addition of buffer B (1.5 ml), the resulting solution was centrifuged at 3000 rpm for 15 min, and the supernatant was removed. The immune precipitate was washed once with buffer B (1 ml) and used for measurement of the enzymic activity. Radioimmunoassay was carried out using [^{3}H]-cortisol, as reported in the previous paper.[6]

Measurement of β-Galactosidase Activity——The immune precipitate was diluted with buffer A (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and preincubated at 37°C for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in PB was added to the resulting solution, and the mixture was incubated for 90 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml). The absorbance was then measured at 420 nm with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer.

Sample Preparation——A solution of [^{3}H]-cortisol (*ca.* 1800 dpm) in ethanol (0.1 ml) was transferred to a test tube, and the solvent was removed with the aid of an N_2 gas stream. Urine (0.1 ml) was added to this residue, and the solution was vortex-mixed, then allowed to stand at 4°C for 1 h. After addition of water (0.5 ml), the urine sample was extracted with methylene chloride (1 ml), and the aqueous layer was discarded. The organic solvent was evaporated off under an N_2 gas stream, and the residue was redissolved in the assay buffer. An aliquot of this solution was used for radioactivity counting. Extraction of plasma cortisol was carried out in a similar manner. The denatured sample was prepared by heating the plasma diluted with buffer B at 60°C for 30 min.

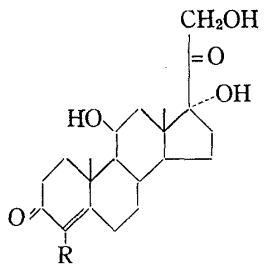
Cross-Reaction Study——The specificity of the assay systems was tested by cross-reaction study with fifteen kinds of steroids related to cortisol. The relative amounts required to reduce the initial binding of enzyme-labeled cortisol by half, where the mass of unlabeled cortisol was arbitrarily taken as 100%, were calculated from standard curves.

Results and Discussion

The purpose of this work was to examine whether high specificity to cortisol could be obtained in heterogeneous enzyme immunoassays employing anti-cortisol antisera and β-galactosidase-labeled antigens which were prepared by the use of carboxylated cortisol derivatives possessing bridges at the C-4 position. The haptens used were the thioether derivatives, 4-(carboxymethylthio)cortisol (CMT), 4-(2-carboxyethylthio)cortisol (CET), 4-(2-hemisuccinoyloxyethylthio)cortisol (HST), and another type of hapten, 4-hemisuccinoyloxycortisol (HS).[5] We have previously reported the sensitivities obtainable with the homologous and bridge heterologous assay systems.[4] In the present study, enzyme immunoassay was carried out with seven systems, which include two preparations of antisera (1 and 2) derived from the same hapten-BSA conjugate (Table I). Selection of these out of thirty-two systems reported[4] was based upon the sensitivity in the enzyme immunoassay and the specificity in the radioimmunoassay.[6] With these systems (I—VII), sensitivities comparable to that of radioimmunoassay have been obtained.

The bound and free enzyme-cortisol conjugates were separated by a double antibody method, and the enzymic activity of immune precipitate was determined colorimetrically with *o*-nitrophenyl β-D-galactopyranoside as a substrate. Useful dose-response curves could be constructed in the range of 20 pg to 1 ng per tube. A typical standard curve obtained with the assay II is shown in Fig. 1.

It is well recognized that, when various assays are compared using biological samples, the assay result yielding the lowest estimation can usually be assumed to be proximate to the true value. Therefore, the specificity of assay was assessed by measuring cortisol in human plasma and urine specimens. The assays were done on plasma samples extracted with methylene chloride and samples heated to denature corticosteroid-binding globulin. In the former, the observed values were corrected on the basis of the recovery rates of [^{3}H]-cortisol



CMT: R=SCH_2COOH
CET: R=SCH_2CH_2COOH
HST: R=$SCH_2CH_2OCO(CH_2)_2COOH$
HS : R=$OCO(CH_2)_2COOH$

Chart 1

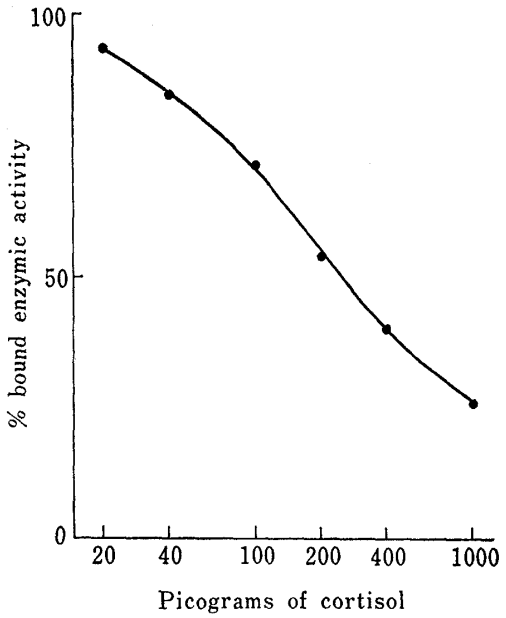


Fig. 1. Dose-Response Curve for Cortisol Enzyme Immunoassay using the Antiserum CET-1 and Enzymelabeled CMT

added to each sample. The results of the estimation of cortisol levels are listed in Table II. A specific assay should give the same value for the samples prepared by different pretreatment methods. As can be seen in Table II, the observed values varied with both assay systems and pretreatment methods in most cases. The assay II gave the lowest value for all the samples, and similar results with the two pretreatment methods. Thus, this assay is considered to possess the highest specificity among the systems tested, and "direct" determination was found to be possible. Inter-assay coefficients of variation ($n=4$) were 3.9 and 10.1%, respectively, for the plasma samples A and B. In the case of the assay III, specificity comparable to that of the assay II was obtained. This showed that alteration of the bridge in enzyme labeling did not significantly influence the specificity, as expected from previous findings.[7] Other systems were found to overestimate the amount of cortisol, especially with the unextracted plasma, and were therefore less specific than the assays II and III. The assays using the antisera HST-1 and HS-1, though less sensitive, were also examined. However, they did not exceed the assay II in specificity. When compared with the antisera CMT, HS, HST and those raised against 3- and 21-BSA conjugates, CET-2 showed excellent specificity in the radioimmunoassay,[6] but the enzyme immunoassays (IV, V) using this antiserum were less specific.

Table III shows the results of plasma and urine cortisol levels obtained by the enzyme immunoassays and radioimmunoassays. In the case of the assays II and III using CET-1, specificity comparable to those of the radioimmunoassays could be obtained. On the other hand, the assay IV using CET-2 exhibited a lack of specificity, which was not improved even when the enzyme immunoassay was carried out fluorometrically in the homologous combination using the enzyme-labeled CET. Such a discrepancy in specificity between enzyme immunoassay and radioimmunoassay is often observed, and may be ascribed to the difference in structure between labeled-antigen and the antigen to be measured.

The cross-reactivities with fifteen kinds of closely related steroids were then tested and the results obtained with the assay II are listed in Table IV. The per cent cross-reaction was calculated at 50% displacement of the enzyme-labeled cortisol. We found 56% cross-reaction with 11-deoxycortisol, 37% with 21-deoxycortisol, 12% with corticosterone, 24%

Table I. Assay Systems used in Enzyme Immunoassay of Cortisol

System	Antiserum	Enzyme-labeled steroid	Antiserum dilution
I	CMT-1	HS	1:8000
II	CET-1	CMT	1:1000
III	CET-1	HS	1:1000
IV	CET-2	CMT	1:1000
V	CET-2	HS	1:3000
VI	HS-2	CMT	1:600
VII	HS-2	CET	1:600

Table II. Plasma Cortisol Levels obtained by Enzyme Immunoassay (ng/ml)

Sample	Assay system						
	I	II	III	IV	V	VI	VII
Plasma A							
Extracted	130	108	114	131	122	115	114
Heated	142	109	112	133	148	156	152
Plasma B							
Extracted	63	44	48	59	54	51	53
Heated	52	46	46	59	60	70	64

with 5α-dihydrocortisol, and smaller values with cortisone, 17α-hydroxyprogesterone and 11-deoxycorticosterone. In order to identify interfering substances in the assay IV, cross-reactions in the various assays including radioimmunoassay were compared. However, a basis for explaining the lack of specificity could not be obtained.

In view of the metabolism of cortisol, the determination of cortisol in urine requires higher specificity than that in plasma. The levels of urine cortisol were measured in eight normal subjects by the assay II. The values varied from 65 to 108 μg/24 h, with a mean value of 85.6±14.2 (S.D.) μg/24 h, which correlated well with those estimated by the radioimmunoassays using the antisera CET-1 (92.4±13.3 μg/24 h) and CET-2 (94.2±13.1 μg/24 h). Intra-assay and inter-assay coefficients of variation were satisfactory (<10%). Since extraction of cortisol from urines with methylene chloride yields 80—90% recovery, no correction for the procedural loss by the use of radio tracer is needed for practical purposes.

Table III. Plasma and Urine Cortisol Levels obtained by Enzyme Immunoassay and Radioimmunoassay[a)]

Sample	Radioimmunoassay		Enzyme immunoassay		
	CET-1	CET-2	II	III	IV
Plasma C					
Extracted	132	123	118	123	—
Heated	120	116	111	116	—
Plasma D					
Extracted	44	44	46	46	—
Heated	44	42	46	47	—
Urine					
A	69	69	65	64	150
B	84	75	76	82	194
C	96	85	88	90	177

a) ng/ml and μg/24 h, respectively, for plasma and urine cortisol levels.

TABLE IV. Cross-Reactivities of Selected Steroids in the Enzyme Immunoassay using the System II

Steroid	% cross-reaction (50%)
Cortisol	100
11-Deoxycortisol	56
21-Deoxycortisol	37
Corticosterone	12
Cortisone	3.7
17α-Hydroxyprogesterone	8.9
11-Deoxycorticosterone	7.1
Progesterone	2.9
6β-Hydroxycortisol	2.8
Tetrahydrocortisol	0.1
Tetrahydrocortisone	0.04
Tetrahydro-11-deoxycortisol	0.6
5α-Dihydrocortisol	24
5β-Dihydrocortisol	6.2
20α-Dihydrocortisol	0.8
20β-Dihydrocortisol	0.5

Thus, a sensitive and relatively specific enzyme immunoassay of cortisol could be developed, and was found to be applicable to the measurement of cortisol in human urine as well as plasma. The quantitation limit of cortisol was approximately 20 pg. In separate experiments using a fluorogenic substrate, the sensitivity was increased to 10 pg. Recently, enzyme immunoassays of cortisol have been reported by several groups.[2,3] The sensitivity colorimetrically obtained was not very high, and fluorescence[3] and chemiluminescence[2b] techniques were used to improve the sensitivity.

To the best of our knowledge, this is the first reported enzyme immunoassay applicable to the determination of cortisol in human urine. Development of a practical enzyme immunoassay is not always easy owing to various factors influencing the sensitivity and specificity. It should be mentioned that the high sensitivity obtained in the present study was ascribed to the use of the heterologous combination between antiserum and enzyme-labeled steroid, and of an appropriate molar ratio of haptenic derivative to enzyme in the enzyme labeling. Employment of a bridge heterologous system rather than site heterology seems to be preferable as regards assay specificity.

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