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The Specificity of Enzyme Immunoassays for Plasma 11-Deoxycortisol[1)]

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In order to develop an 11-deoxycortisol enzyme immunoassay applicable to metyrapone tests, the specificity obtainable with various assay systems has been investigated. Four 11-deoxycortisol derivatives possessing different bridges at C-4 were covalently linked to β-galactosidase to give enzyme-labeled antigens. The anti-11-deoxycortisol antisera used were those elicited in rabbits by immunization with the conjugates of these haptenic derivatives with bovine serum albumin. Enzyme immunoassays were carried out with the homologous and bridge heterologous combinations between antiserum and enzyme-labeled steroid. The specificity of these assay systems was assessed by measuring the amount of 11-deoxycortisol in human plasma specimens, and by comparison of the results with those of radioimmunoassay. The assay employing the antiserum and enzyme-labeled antigen which were prepared by the use of 4-(carboxymethylthio)-11-deoxycortisol was found to be relatively specific. The cross-reactivities in this assay were tested with six kinds of closely related steroids. The selective blocking of less specific antibodies present in the antiserum was also examined. It was found that the addition of cortisol to the antiserum resulted in improvement of the specificity. The enzyme immunoassay thus developed was reasonably specific and sensitive.

Keywords——enzyme immunoassay; 11-deoxycortisol; anti-11-deoxycortisol antiserum; enzyme-labeling of 11-deoxycortisol; 11-deoxycortisol-β-galactosidase conjugate; specificity; plasma 11-deoxycortisol level; metyrapone test; cross-reaction

Immunoassays of 11-deoxycortisol in human plasma are useful in the metyrapone test,[3)] assessment of pituitary-adrenal reserve. Radioimmunoassays of the steroid hormone have been developed using various antisera elicited in animals by immunization with hapten molecules linked to a carrier protein.[4)] The specificity of antibodies is significantly influenced by the position on the steroid molecule used for conjugation to the carrier. We have recently reported on the specificity of antisera raised against hapten–bovine serum albumin (BSA) conjugates linked through the C-4 position of 11-deoxycortisol in the radioimmunoassay procedure.[5)] It is desirable to develop an enzyme immunoassay system having high sensitivity and specificity comparable to those of the radioimmunoassay. In a previous paper of this series, we investigated the sensitivities obtainable with various enzyme immunoassay systems using these antisera.[6)] This paper deals with the specificity of the enzyme immunoassays for human plasma 11-deoxycortisol.

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.); *o*-nitrophenyl β-D-galactopyranoside and 4-methylumbelliferyl β-D-galactopyranoside were from Nakarai Chemicals, Ltd. (Kyoto). Goat anti-rabbit IgG antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo). [1,2-^{3}H]-11-Deoxycortisol (50 Ci/mmol) was supplied by Amersham (England). The haptenic derivatives used were 4-(carboxymethylthio)-11-deoxycortisol (CMT), 4-(2-carboxyethylthio)-11-deoxycortisol (CET), 4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol (HST), and 4-hemisuccinoyloxy-11-deoxycortisol (HS).[5,7)] The abbreviations are also used for antiserum and enzyme-

labeled antigen, which were prepared by using the corresponding carboxylated derivatives. Anti-11-deoxycortisol antisera used were those reported in the previous paper.[5] 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).

Preparation of 11-Deoxycortisol-β-galactosidase Conjugates——This was carried out in the manner described previously.[6] In short, the carboxylated 11-deoxycortisol (CMT, CET, HST and HS) was derivatized into the *N*-succinimidyl ester by treatment with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The activated esters were reacted with β-galactosidase at a molar ratio (steroid to enzyme) of 8 to give enzyme-labeled antigens. 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). The labeled antigens were stable for several months as regards enzymic activity and immunoreactivity under these storage conditions. 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 11-deoxycortisol-enzyme conjugate (100 ng in spectrophotometry or 10 ng in fluorophotometry) in the buffer (0.1 ml) diluted 1:20 with buffer A containing 0.3% ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid 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 rev./min for 15 min, and the supernatant was removed. The immune precipitate was washed twice with buffer B (1 ml) and used for measurement of the enzymic activity. In experiments using cortisol as an agent for the blocking of less specific antibodies, the steroid was added to the anti-11-deoxycortisol antiserum diluted 1:400, which was then allowed to stand at 4 °C for at least 12 h. Radioimmunoassay was carried out using [^{3}H]-11-deoxycortisol, as reported in the previous paper.[5]
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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 60—90 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml). The absorbance was then measured at 420 nm. In the fluorophotometric method, 4-methylumbelliferyl β-D-galactopyranoside (0.007%) was used as a substrate, and fluorescence was measured at 450 nm with excitation at 360 nm.

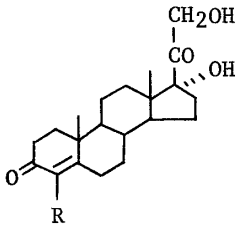
Sample Preparation——Plasma specimens were provided by Drs. Sasaki and Shimizu, Tohoku University School of Medicine. In metyrapone tests, patients received 0.5 g of metyrapone every 4 h for a total of six doses. Blood was withdrawn 24 and 48 h after the initial administration and plasma was immediately obtained. A solution of [^{3}H]-11-deoxycortisol (*ca.* 1800 dpm) in ethanol (0.1 ml) was transferred to a test tube, and the solvent was removed with the aid of a nitrogen gas stream. Plasma (0.1 ml) was added to this residue, and the soluition was vortex-mixed, and allowed to stand overnight at 4 °C. After addition of water (1 ml), the plasma sample was extracted with methylene chloride (3 ml), and the aqueous layer was discarded. The organic solvent was evaporated off under a nitrogen gas stream, and the residue was redissolved in the assay buffer. An aliquot of this solution was used for radioactivity counting to estimate the recovery rate; recovery rates of the tracer were 90—100%.

Cross-Reaction Study——Six kinds of steroids related to 11-deoxycortisol were used. The relative amounts (weight-to-weight) required to reduce the initial binding of enzyme-labeled 11-deoxycortisol by half, where the mass of unlabeled 11-deoxycortisol was arbitrarily taken as 100%, were calculated from standard curves.

Results and Discussion

In general, development of a specific and sensitive enzyme immunoassay for a steroid hormone is not always easy owing to various factors. Anti-steroid antibody elicited with an immunogen haptenized *via* a "chemical bridge" usually recognizes the structure of the bridge between the enzyme and steroid in the labeled antigen, and hence, the binding affinity of the labeled antigen to antiserum is higher than that of the antigen to be measured, resulting in insensitive assay systems. Thus, the combination of antiserum and enzyme-labeled steroid is an important factor determining the sensitivity and specificity.[6,8] The purpose of this work was to examine whether high specificity for 11-deoxycortisol could be obtained in heterogeneous enzyme immunoassay employing anti-11-deoxycortisol antisera and β-galactosidase-labeled steroids which were prepared by the use of carboxylated 11-deoxycortisol derivatives possessing different bridges at the C-4 position. The haptenic derivatives used were CMT, CET, HST and HS. We have previously reported the sensitivities obtainable with the homologous and bridge heterologous combinations, which include two preparations of antisera (1 and 2) derived from the same hapten–BSA conjugate.[6] The antisera CMT-2 and

CET-2 have been shown to be more specific than the others in the radioimmunoassay procedure.[5] In the present study, however, enzyme immunoassay was carried out with sixteen systems (I—XVI) covering all the antisera (Table I). Selection of these out of thirty-two systems reported[6] was based upon the sensitivity; the homologous systems except with the antisera CET-1 and HST-1 showed relatively high sensitivities, and hence, were also employed.



CMT: $R=SCH_2COOH$
CET: $R=S(CH_2)_2COOH$
HST: $R=S(CH_2)_2OCO(CH_2)_2COOH$
HS: $R=OCO(CH_2)_2COOH$

Chart 1

TABLE I. Assay Systems Used in Enzyme Immunoassay of 11-Deoxycortisol

Antiserum	β-Galactosidase-labeled 11-deoxycortisol			
	CMT	CET	HST	HS
CMT-1	I	—	—	—
2	II	—	—	III
CET-1	IV	—	—	V
2	VI	VII	—	VIII
HST-1	IX	—	—	X
2	XI	—	XII	—
HS-1	XIII	—	—	XIV
2	XV	—	—	XVI

The specificity of assay was assessed by measuring 11-deoxycortisol in plasma specimens obtained from normal subjects and from patients after metyrapone administration. The assay was done on methylene chloride extracts of plasma. The dose-response curves were obtained by incubating 5—500 pg of unlabeled 11-deoxycortisol and a fixed amount (100 ng) of the label with appropriately diluted antisera, and the enzymic activity of the immune precipitate formed by a double antibody method was determined spectrophotometrically with *o*-nitrophenyl β-D-galactopyranoside as a substrate. Previously, we have developed a reasonably specific radioimmunoassay of 11-deoxycortisol.[5] The specificity was ascertained by several criteria: comparisons of various antisera, pretreatments of plasma specimens, and cross-reactions and agreement of the results with the physiological state of the subject. In this work, therefore, the specificity of enzyme immunoassay systems was examined mainly by comparison of the results with those of the radioimmunoassay. Preliminary experiments showed that the systems using the antisera CMT-2, CET-2, HST-1, HST-2 and HS-1 were worthy of further examination; the other five systems were less specific (data not shown). With the former assay systems, the estimation of 11-deoxycortisol levels in various plasma samples was carried out. The results, together with those obtained by the radioimmunoassay, are listed in Table II. It is well recognized that, when various antisera are compared using biological samples, the result yielding the lowest estimation can usually be assumed to be proximate to the true value.[9] It is evident that the assays II, VI and VII are more specific than the other systems, and give results similar to those of the radioimmunoassay with respect to the samples

Table II. Plasma 11-Deoxycortisol Levels Obtained by Enzyme Immunoassay and Radioimmunoassay (μg/l)[a]

Sample[b]	Enzyme immunoassay									RIA[c]
	II	III	VI	VII	VIII	IX	XI	XII	XIII	
1	129	172	128	140	208	162	212	228	231	160
2	175	182	199	193	278	236	211	236	241	200
3	111	128	118	130	152	158	116	152	152	113
4	121	144	144	139	185	134	150	185	169	144
5	97	108	108	93	150	88	107	176	152	94
6	6.4	11	6.8	8.8	17	—	—	—	—	1.1
7	10	14	12	15	20	—	—	—	—	3.4
8	8.2	13	9.1	12	19	—	—	—	—	1.6

a) Values are averages of triplicate determinations: coefficients of variation were less than 10%.
b) Samples **1**—**5** were obtained from metyrapone-treated subjects; samples **6**—**8**, from normal subjects.
c) Radioimmunoassay using the antiserum CMT-2.

Table III. Per Cent Cross-Reaction with Selected Steroids

Steroid	EIA[a]				RIA[b]
	1	**2**	**3**	**4**	
11-Deoxycortisol	100	100	100	100	100
Cortisol	1.6	0.56	1.5	0.48	0.58
Cortisone	2.8	1.7	5.5	1.9	0.86
Corticosterone	0.07	0.02	0.08	0.01	<0.001
11-Deoxycorticosterone	9.2	6.3	14	4.4	6.4
17α-Hydroxyprogesterone	13	7.7	13	9.4	4.1
Progesterone	0.88	0.71	0.88	0.50	0.33

a) Enzyme immunoassay using system II. The enzymic activity was determined spectrophotometrically (**1**, **2**) or fluorophotometrically (**3**, **4**). In the EIAs **2** and **4**, the antiserum CMT-2 contained 500 and 200 pg of cortisol per tube, respectively.
b) Radioimmunoassay using the antiserum CMT-2.

collected after metyrapone administration. The use of the antisera CMT-1, CET-1, HST-1, HST-2, HS-1 and HS-2, which are less specific than the antisera CMT-2 and CET-2 in the radioimmunoassay procedure,[5] was shown to give unsatisfactory results. These results indicate that antiserum exhibiting a lack of specificity in radioimmunoassay using tritium as a tracer is not suitable for use in enzyme immunoassay. It should be noted that employment of a bridge heterologous system resulted in the loss of specificity in some cases —system VIII as compared with the homologous assay VII, for example.

Systems II, VI and VII may be useful in the metyrapone test. However, the values obtained for normal subjects by these enzyme immunoassays were considerably high when compared with those determined by the radioimmunoassay; baseline values for the diagnostic test seem to be less than 10 μg/l. Such a discrepancy in specificity between enzyme immunoassay and radioimmunoassay is often observed, despite the use of the same antiserum. This is ascribed to the structural difference of the labeled antigens used, and to the participation of less specific antibodies present in the antiserum. Commonly, the most abundant steroid in normal human plasma is cortisol, whose level may be several hundred times higher than that of 11-deoxycortisol. Therefore, in assay II, which is considered to possess the highest specificity among the systems listed in Table II, the cross-reactivities of cortisol and related

TABLE IV. Effect of Addition of Cortisol on the Assay Value of 11-Deoxycortisol (μg/l)[a]

Cortisol added (pg)	Antiserum initial dilution	Plasma sample[b]					
		Normal			Metyrapone		
0	1:15000	7.7	8.3	14	169	176	256
500	1:10000	1.6	3.1	5.8	161	156	250

a) Enzyme immunoassays corresponding to EIA-1 and EIA-2 in Table III were carried out.
b) Three samples obtained from normal subjects and three from metyrapone-treated subjects were tested.

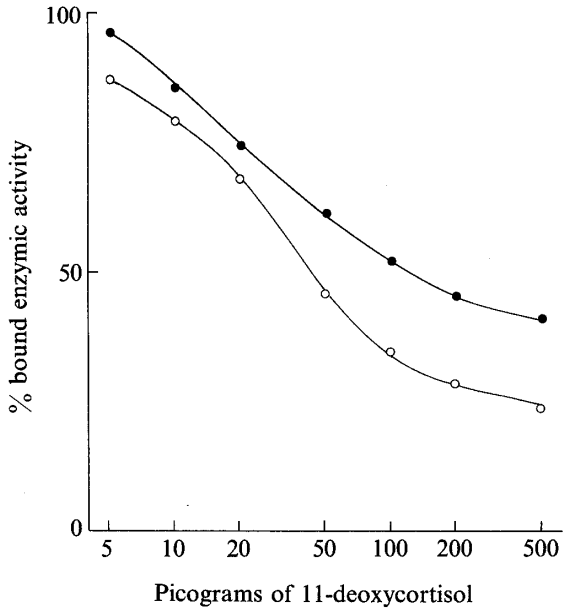


Fig. 1. Dose–Response Curves for 11-Deoxycortisol Enzyme Immunoassay with (—●—, EIA-2) and without (—○—, EIA-1) Addition of Cortisol

Assay conditions are given in Table IV.

steroids were tested (Table III, EIA-1). The percent cross-reaction was calculated at 50% displacement of the enzyme-labeled 11-deoxycortisol. We found 1.6% cross-reaction with cortisol. It is also obvious that all the steroids tested exhibit higher cross-reactivities in the enzyme immunoassay than in the radioimmunoassay,[5] in accord with the results obtained by the measurement of the plasma samples.

With the aim of improving the specificity, we examined the effect of addition of cortisol to the antiserum in assay II. This was based upon the previous finding that the selective blocking of less specific antibodies is sometimes effective.[10] When 500 pg of cortisol was added to each test tube, the desired effect was observed. A comparison of 11-deoxycortisol values obtained with and without addition of cortisol is presented in Table IV. In the case of the normal plasma, significantly decreased values were obtained when the antiserum containing cortisol was used, whereas such an effect was not observed in the post-metyrapone values. The latter finding relates to the fact that in the metyrapone test a subject with intact pituitary-adrenal reserve shows a markedly enhanced 11-deoxycortisol level with a decrease in the cortisol level. The cross-reaction study was then carried out and the results are listed in Table III, EIA-2. It appeared that the cross-reactivities of not only cortisol but also the other steroids were lowered in comparison with the EIA-1. Thus, the method proved to be satisfactory with respect to specificity. However, the addition of cortisol resulted in a loss of sensitivity, as can be seen in the dose-response curves (Fig. 1).

Employing this method, we explored more appropriate assay conditions. Finally,

satisfactory results were obtained by the use of a smaller amount of enzyme label (10 ng) at a higher dilution of the antiserum CMT-2 (1 : 30000) in the presence of 200 pg of cortisol per tube. In this case, the enzymic activity was determined by a fluorophotometric method using 4-methylumbelliferyl β-D-galactopyranoside as a substrate. The sensitivity of the assay was several times higher than that of the EIA-2 in Fig. 1. The cross-reactivities of the related steroids in this assay (EIA-4) were also tested. The results, together with those obtained without addition of cortisol (EIA-3), are listed in Table III. The specificity obtained in the EIA-4 seems to be comparable to that of the radioimmunoassay.

Thus, a sensitive and relatively specific enzyme immunoassay system for the determination of 11-deoxycortisol in human plasma was developed. It should be mentioned that the present high sensitivity is ascribable to the use of an appropriate molar ratio of steroid to enzyme in enzyme labeling[8b,11] as well as to the use of a small amount of the labeled antigen, the enzymic activity of which was fluorophotometrically determined. Further, selective blocking of less specific antibodies was effective for the purpose of improving the specificity. Application of this assay system to the metyrapone test[3] on patients with Cushing's syndrome are being conducted, and the details will be reported elsewhere.

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