[Chem. Pharm. Bull. 32(9)3594—3600(1984)]

Enzyme Immunoassay for Plasma 11-Deoxycortisol. Application to Metyrapone Test[1)]

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(Received January 9, 1984)

A sensitive and relatively specific enzyme immunoassay of 11-deoxycortisol in human plasma has been developed. Enzyme labeling of 11-deoxycortisol was accomplished by the active ester method. The use of 4-carboxymethylthio-11-deoxycortisol *N*-succinimidyl ester and β-galactosidase in an appropriate molar ratio provided an enzyme-labeled antigen suitable for enzyme immunoassay. The anti-11-deoxycortisol antiserum used was that elicited in the rabbit by immunization with the conjugate of the haptenic derivative with bovine serum albumin. In order to improve the assay specificity, cortisol was used as an agent for the blocking of less specific antibodies present in the antiserum. The specificity of the assay system was assessed by comparing the results of measurement of 11-deoxycortisol in human plasma with those obtained by a radioimmunoassay. The quantitation limit of 11-deoxycortisol was 800 fg per tube. The intra- and inter-assay coefficients of variation for 11-deoxycortisol in human plasma were 3.0—15.8% and 7.6—12.5%, respectively. The present enzyme immunoassay of 11-deoxycortisol was found to be useful in the metyrapone tests for evaluation of pituitary-adrenal function and for differential diagnosis of Cushing's syndrome. The assay can be done on methylene chloride extracts of plasma.

Keywords——enzyme immunoassay; 11-deoxycortisol; anti-11-deoxycortisol antiserum; enzyme labeling of 11-deoxycortisol; *N*-succinimidyl ester method; 11-deoxycortisol-β-galactosidase conjugate; plasma 11-deoxycortisol level; metyrapone test; Cushing's syndrome

Immunoassays of 11-deoxycortisol in human plasma are useful in the metyrapone test.[3)] The drug metyrapone inhibits the biosynthesis of cortisol, causing an increase in 11-deoxycortisol secretion in the presence of adrenocorticotropic hormone. The diagnostic test is done for evaluation of pituitary-adrenal function and in Cushing's syndrome. Radioimmunoassays of 11-deoxycortisol have been developed using various antisera elicited in animals by immunization with hapten molecules linked to a carrier protein,[4)] and applied to the metyrapone test by several groups.[4a,5)] It is desirable to develop a practical enzyme immunoassay of this steroid hormone. In general, however, development of a specific and sensitive enzyme immunoassay for a steroid is not always easy, since the specificity of antibodies is dependent upon the structure of the haptenic derivative used for the preparation of immunogen, and the combination between antiserum and enzyme-labeled antigen is an important factor determining the sensitivity.[6)] We have prepared antisera to 11-deoxycortisol, using carboxylated 11-deoxycortisol derivatives possessing bridges at the C-4 position as haptens.[7)] In previous papers of this series, the sensitivity[8)] and specificity[9)] of various enzyme immunoassay systems employing these antisera were reported. This paper deals with the development of an enzyme immunoassay for plasma 11-deoxycortisol and its application to the metyrapone test.

Materials and Methods

Materials——β-Galactosidase (EC 3.2.1.23) from *E. coli* (grade VI, 360 units per mg protein) was purchased from Sigma Chemical Co. (St. Louis, Mo.); 4-methylumbelliferyl β-D-galactopyranoside, from Nakarai Chemicals, Ltd. (Kyoto). Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs., Ltd. (Tokyo). The assay buffer used in the immunoassay procedure was 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl and 0.1% NaN_3. All other chemicals were of reagent grade. 4-Carboxymethylthio-11-deoxycortisol was prepared by the method previously established in these laboratories;[10] the haptenic compound was derivatized into an activated ester in the manner described below.

Synthesis of 4-Carboxymethylthio-11-deoxycortisol *N*-Succinimidyl Ester——*N*-Hydroxysuccinimide (37 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (62 mg) were added to a solution of 4-carboxymethylthio-11-deoxycortisol (100 mg) in 95% dioxane (1 ml), and the mixture was stirred at room temperature for 2 h. The resulting solution was diluted with AcOEt, washed with H_2O, and dried over anhydrous Na_2SO_4. The solution was passed quickly through an Al_2O_3 (2 g) layer on a sintered-glass funnel, and the filtrate was evaporated down. Recrystallization of the crude product from methylene chloride–hexane gave 4-carboxymethylthio-11-deoxycortisol *N*-succinimidyl ester (80 mg) as colorless leaflets. mp 204—205 °C. $[\alpha]_D^{25}$ +98° ($c=0.23$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$–CD_3OD (10 : 1)) δ : 0.69 (3H, s, 18-CH_3), 1.22 (3H, s, 19-CH_3), 2.28 (4H, s, succinimidyl), 3.60—3.92 (3H, 6α-H and 4-SCH_2CO), 4.27 and 4.65 (each 1H, d, $J=19$ Hz, 21-H). *Anal.* Calcd for $C_{27}H_{35}NO_8S\cdot 5/4H_2O$: C, 58.31; H, 6.80; N, 2.52. Found: C, 58.18; H, 6.62; N, 2.48.

Preparation of β-Galactosidase-Labeled 11-Deoxycortisol——Eight μl of a solution of 4-carboxymethylthio-11-deoxycortisol *N*-succinimidyl ester in methylene chloride (1.87×10^{-3} M) was transferred to a test tube with a micro syringe, and the solvent was evaporated off. A solution of β-galactosidase (1 mg, 200 μl) in PB was added to the residue at 0 °C, and the reaction mixture was immediately vortex-mixed, then allowed to stand overnight at 4 °C with occasional shaking. After dialysis against cold PB (1 l) for 2 d, the enzyme solution was diluted with assay buffer to make the total volume 2 ml, and stored at 4 °C. This was diluted 1 : 5000 with assay buffer containing 0.5% normal rabbit serum just before use.

Anti-11-deoxycortisol Antiserum——The antiserum used was that elicited in a rabbit by immunization with the conjugate of 4-carboxymethylthio-11-deoxycortisol with bovine serum albumin; this corresponds to CMT-2 in the previous papers.[7-9] For use in enzyme immunoassay, an antiserum solution containing cortisol (an agent for the blocking of less specific antibodies) was prepared as follows: an ethanol solution containing cortisol (600 ng) was transferred to a test tube, and the solvent was removed with the aid of a nitrogen gas stream. The anti-11-deoxycortisol antiserum (10 μl) and assay buffer (4 ml) were added to this residue, and the solution was vortex-mixed, then allowed to stand at 4 °C for at least 12 h. This was diluted 1 : 75 with assay buffer prior to use. The cross-reactivities (50% displacement) of related steroids in the present enzyme immunoassay were as follows: cortisol 0.48%; cortisone 1.9%; 11-deoxycorticosterone 4.4%; 17α-hydroxyprogesterone 9.4%; progesterone 0.5%; corticosterone 0.01%; 6β-hydroxy-11-deoxycortisol 30%; 6β-hydroxycortisol <0.01%.

Sample Preparation——Plasma specimens were provided by Dr. 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 4, 8, 24 and 48 h after the initial administration and plasma was immediately obtained. The standard extraction procedure is as follows: H_2O (1 ml) and methylene chloride (2 ml) were added to plasma (50 μl), and the whole was vortex-mixed for 30 s. A 200 μl aliquot of the organic layer was taken into a test tube by using an Eppendorf micropipette with disposable plastic tips. The organic solvent was evaporated off with a centrifugal evaporator and the residue was redissolved in assay buffer (100 μl). In the case of the metyrapone test, more diluted samples were also assayed. For precision studies, various dilutions of the samples obtained from a normal subject and a metyrapone-treated patient were used. In recovery tests, the extraction procedure was carried out on pooled normal plasma, to which were added known amounts of 11-deoxycortisol (0, 100, 200, 400, 600 and 800 pg per 100 μl of plasma).

Immunoassay Procedures——Enzyme immunoassay was carried out in duplicate in glass test tubes (10 ml) as follows: the diluted antiserum (100 μl) and β-galactosidase-labeled 11-deoxycortisol (10 ng, 100 μl) in assay buffer were added to a series of standard solutions (0, 1, 2, 4, 10, 20, 40, 100 and 200 pg of 11-deoxycortisol) or plasma samples in the buffer (100 μl), and the mixture was incubated at 4 °C for 4 h. Goat anti-rabbit antiserum (100 μl) diluted 1 : 20 with assay buffer containing 0.3% ethylenediaminetetraacetic 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 assay buffer (1.5 ml), the resulting solution was centrifuged at 3000 rev./min for 15 min, and the supernatant was discarded by aspiration. The immune precipitate was washed twice with the buffer (1 ml) and used for the measurement of the enzymic activity. Radioimmunoassay was carried out using [^{3}H]-11-deoxycortisol, as reported in the previous paper.[7]

Measurement of β-Galactosidase Activity——The immune precipitate was diluted with assay buffer (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and preincubated at 37 °C for 3 min. 4-Methylumbelliferyl β-D-galactopyranoside (0.007%, 1 ml) in assay buffer was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml). Fluorescence due to 4-

methylumbelliferone formed was measured at 450 nm with excitation at 360 nm with a Hitachi MPF-2A fluorescence spectrophotometer.

Results and Discussion

Development of a steroid enzyme immunoassay having high specificity and sensitivity comparable to those of radioimmunoassay is not always easy. In fact, the assay system employed here was selected out of thirty-two systems previously tested[8,9] and a particular method was necessary in order to improve the specificity of assay. The anti-11-deoxycortisol antiserum used was that raised against the 4-carboxymethylthio-11-deoxycortisol-bovine serum albumin conjugate.[7] β-Galactosidase-labeled 11-deoxycortisol, a labeled antigen, was prepared by the active ester method previously developed in our laboratories.[11] The *N*-succinimidyl ester obtained from 4-carboxymethylthio-11-deoxycortisol by condensation with *N*-hydroxysuccinimide in the presence of a water-soluble carbodiimide was covalently linked to β-galactosidase (Fig. 1). The steroid/enzyme molar ratio of 8 was used for the coupling reaction in phosphate buffer (pH 7.3). The conjugate was dialyzed against the buffer to remove *N*-hydroxysuccinimide and the unreacted steroid. The loss of enzymic activity was less than 10% under the coupling conditions employed. Although the number of steroid molecules incorporated per enzyme was not determined, the labeled antigen was found to have a satisfactory binding affinity to the antiserum.

All the assays of plasma specimens were done on methylene chloride extracts. The bound and free labeled antigens were separated by a double antibody technique and the enzymic activity of immune precipitate was determined fluorophotometrically with 4-methylumbelliferyl β-D-galactopyranoside as a substrate. The dose-response curves were obtained by incubating 0—200 pg of unlabeled 11-deoxycortisol and a fixed amount (10 ng) of the labeled antigen with an appropriately dilute antiserum.

We reported previously that when an enzyme immunoassay is less specific than a radioimmunoassay using the same antiserum, the addition of an appropriate compound to the system is sometimes effective to block the less specific antibodies present in the antiserum.[12] This was also the case in this work and cortisol was used as a blocking agent.[9] The effect of the addition of cortisol on the determination of plasma 11-deoxycortisol levels is shown in Table I. It is evident that the addition of 200 pg of cortisol per tube is effective in the case of the samples from normal subjects, resulting in a significant decrease in the 11-deoxycortisol value, whereas such an effect is not observed in the post-metyrapone values. The latter finding reasonably 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. In view of the cross-reactivity of cortisol (0.48%) and its high levels in normal subjects and in patients with Cushing's syndrome, overestimations of 11-deoxycortisol values in these plasma samples may still be inevitable in this assay system. In the metyrapone test,

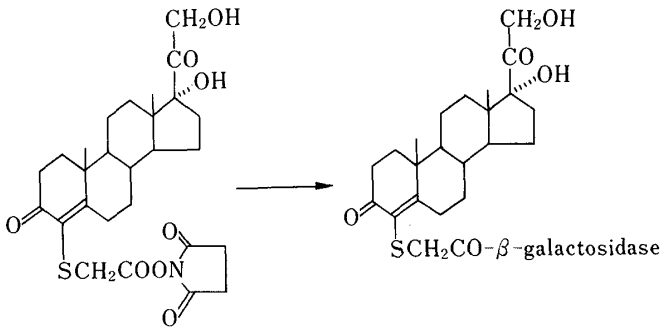

Fig. 1. Preparation of Enzyme-Labeled Antigen

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

Cortisol added (pg/tube)	Plasma sample[b]				
	Normal		Metyrapone		
0	13	6.2	198	222	200
200	5.7	2.6	210	190	150

a) Enzyme immunoassays were carried out with a 1 : 30000 dilution of the anti-11-deoxycortisol antiserum. Dose-response curves were constructed separately in the absence and presence of cortisol.
b) Two samples from normal subjects and three from metyrapone-treated patients were tested.

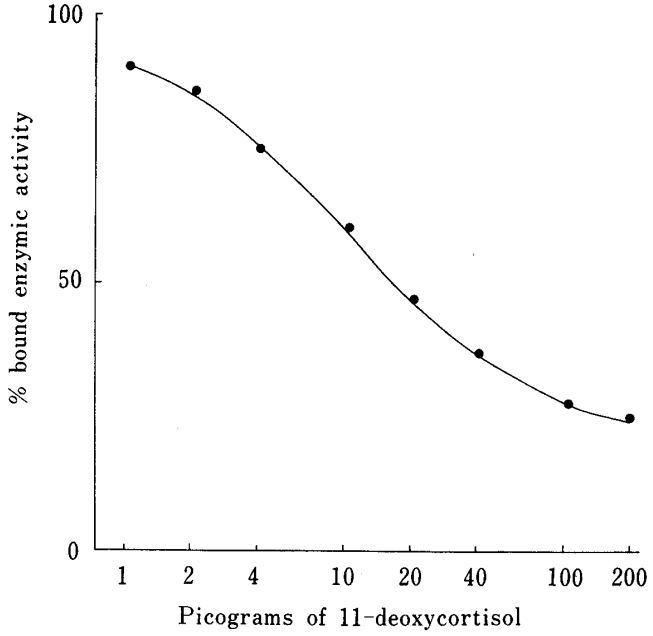

Fig. 2. Dose-Response Curve for 11-Deoxycortisol Enzyme Immunoassay

however, the acceptable baseline values are less than 10 μg/l, and hence, this assay system should be useful.

A typical dose-response curve is shown in Fig. 2; the amount of 11-deoxycortisol needed to displace 50% of the bound label was 17 pg. The minimal detectable amount of 11-deoxycortisol, that is twice the standard deviation of the zero determination ($n=10$), was 800 fg per tube, and the water blank was below the limit of detection. Thus, the sensitivity of the assay is higher than that of the radioimmunoassay.[7]

Assessment of the assay was then carried out by checking the recovery rate and the intra- and inter-assay coefficients of variation, and by comparing the results of measurement of plasma 11-deoxycortisol with those obtained by the radioimmunoassay. Recovery experiments were performed on plasma specimens containing known amounts of 11-deoxycortisol and the results are listed in Table II. The recovery rates ranged from 94 to 110%, with a mean value of 102.8 ± 6.6 (SD)%. Intra-assay coefficients of variation were 6.0—15.8% and 3.0—11.6% ($n=6$) for the samples obtained from a normal subject and a metyrapone-treated patient, respectively. Excellent inter-assay coefficients of variation were also obtained (Table III). Thus, the present enzyme immunoassay proved to be satisfactory in both accuracy and precision.

TABLE II. Recovery of 11-Deoxycortisol Added to Pooled Human Plasma

11-Deoxycortisol (pg/tube)			Recovery (%)
Added	Found	Expected	
0	10		
5	16	15	107
10	21	20	105
20	33	30	110
30	39	40	98
40	47	50	94
			Mean 102.8±6.6

TABLE III. Precision of the Enzyme Immunoassay

Plasma sample[a)]	Plasma volume (μl)	Intra-assay ($n=6$)		Inter-assay ($n=6$)	
		11-Deoxycortisol (μg/l)	CV (%)	11-Deoxycortisol (μg/l)	CV (%)
A	2.5	2.85	15.8	3.00	8.0
	5.0	2.34	9.4	2.50	7.6
	10	2.33	6.0	2.32	12.5
B	0.05	173	11.6	176	8.5
	0.10	169	3.0	175	8.6
	0.20	180	6.0	171	8.8

a) Samples A and B were obtained from a normal subject and a metyrapone-treated patient, respectively.
CV = coefficients of variation.

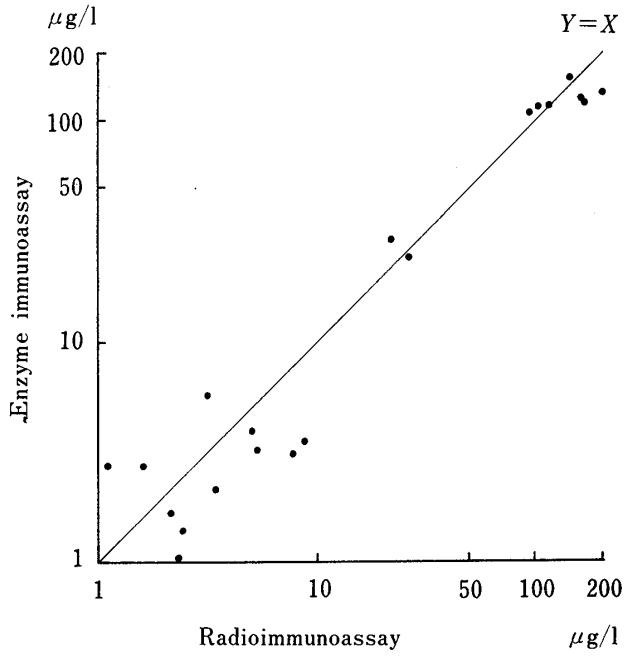

Fig. 3. Correlation between Plasma 11-Deoxycortisol Levels as Determined by Enzyme Immunoassay and Radioimmunoassay

A comparison of the 11-deoxycortisol levels determined by the enzyme immunoassay and radioimmunoassay[7] is shown in Fig. 3. The regression equation was $Y=0.81X+3.6$ ($r=0.96$, $n=20$), showing that the specificity of the enzyme immunoassay is comparable to that of the radioimmunoassay. The mean 11-deoxycortisol level in plasma of normal subjects, as

TABLE IV. Plasma 11-Deoxycortisol Levels before and after Metyrapone Administration to Patients with Cushing's Syndrome

Patient		Time (h)[a]				
		0	4	8	24	48
Hyperplasia	1	1.6	58	58	88	62
	2	1.9	23	58	—	100
	3	8.4	84	96	97	120
Adenoma	1	3.2	6.2	8.6	5.7	6.0
	2	2.8	1.4	1.3	4.9	—
	3	9.8	16	16	18	14

a) After the initial administration of metyrapone. Time 0 means before administration.

determined by the enzyme immunoassay, was $3.0 \pm 1.2\ \mu g/l$ ($n=9$). In the case of the samples from patients with intact pituitary-adrenal reserve, the pre-metyrapone value was $1.4 \pm 0.46\ \mu g/l$ ($n=6$), and the samples collected after 48 h of metyrapone administration showed much higher 11-deoxycortisol values, ranging from 73 to 240 $\mu g/l$, with a mean value of $139 \pm 56\ \mu g/l$ ($n=6$).

The enzyme immunoassay procedure was applied to the metyrapone tests done on patients with Cushing's syndrome and the results are listed in Table IV. The pre-metyrapone plasma 11-deoxycortisol values were less than 10 $\mu g/l$ with a mean value of $4.6 \pm 3.5\ \mu g/l$ ($n=6$). In patients with Cushing's syndrome due to adrenocortical hyperplasia, plasma 11-deoxycortisol levels increased significantly after metyrapone administration, showing the mean value of 94 $\mu g/l$ after 48 h. On the other hand, the patients with Cushing's syndrome due to adrenal adenoma showed a low response.

Thus, the present enzyme immunoassay of 11-deoxycortisol was found to be useful in the metyrapone tests for evaluation of pituitary-adrenal function and for differential diagnosis of Cushing's syndrome. It should be noted that in a direct assay (without extraction) somewhat higher 11-deoxycortisol values, but not over 10 $\mu g/l$, were obtained for patients with Cushing's syndrome and normal subjects, whereas significant overestimations were observed in the case of patients after metyrapone administration. Although in this work the measurement of the enzymic activity was carried out fluorophotometrically, if desired, the spectrophotometric detection can be used with slight modifications.[9] Recently, we have prepared a monoclonal anti-11-deoxycortisol antibody.[13] Development of immunoassays using this antibody is under way in these laboratories.

Acknowledgement The authors thank Dr. Y. Shimizu, School of Medicine, Tohoku University, for providing plasma specimens and for helpful advice.

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