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A Direct Enzyme Immunoassay of 6β-Hydroxycortisol in Human Urine[1]

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A sensitive enzyme immunoassay for urine 6β-hydroxycortisol has been developed. Enzyme labeling of 6β-hydroxycortisol was accomplished by the N-succinimidyl ester method. The use of N-succinimidyl ester of 6β-hydroxycortisol 21-hemisuccinate and β-galactosidase in an appropriate molar ratio provided a conjugate suitable for enzyme immunoassay. Antiserum was prepared by immunization with the 6β-hydroxycortisol 21-hemisuccinate-bovine serum albumin conjugate in the rabbit. Sufficient sensitivity and improved specificity of the assay system could be obtained by the selective blocking of less specific antibodies. The quantitation limit of 6β-hydroxycortisol was approximately 10 pg, which is comparable to that of radioimmunoassay. The intra- and inter-assay coefficients of variation for 6β-hydroxycortisol in human urine were 5.9—8.1% and 2.8—12.3%, respectively.

Keywords——enzyme immunoassay; 6β-hydroxycortisol; N-succinimidyl ester method; 6β-hydroxycortisol 21-hemisuccinate-BSA; 6β-hydroxycortisol-β-galactosidase conjugate; sensitivity; cross-reaction; urine level

6β-Hydroxycortisol is a main unconjugated metabolite of cortisol in human urine[3] and constitutes approximately 1—2% of the total daily cortisol secretion.[4] The excreted amount is increased in the newborn,[5] in pregnancy,[6] and in cancer.[7] This polar substance is formed by the mixed function oxidase in the liver and is excreted by the kidney. The measurement of urine 6β-hydroxycortisol may be useful for monitoring the effects of hormones and drugs acting as inducers of microsomal enzymes.[8] Common methods for the determination of urine 6β-hydroxycortisol involve the Porter–Silber reaction with extensive purification steps, such as paper chromatography and thin-layer chromatography. These methods, however, are somewhat tedious, time-consuming and insensitive. Recently, determination methods by radioimmunoassay[9] and high-performance liquid chromatography[10] have been reported. Enzyme immunoassay is an attractive method, in particular when extraction and/or purification steps can be eliminated and therefore, no correction for the procedural loss is needed. Such an assay requires high sensitivity and specificity comparable to those of radioimmunoassay. This paper deals with the development of an enzyme immunoassay of 6β-hydroxycortisol and its application to human urine specimens.

Materials and Methods

Reagents——All organic solvents and reagents were of analytical-reagent grade. β-Galactosidase from *E. coli* (grade VI, 290 Units per mg protein) and bovine serum albumin (BSA) (crystallized) were obtained from Sigma Chemical Co. (St. Louis, MO), *o*-nitrophenyl β-D-galactopyranoside from Nakarai Chemical, Ltd. (Kyoto), and complete Freund's adjuvant from Iatron Laboratories (Tokyo). Goat anti-rabbit IgG antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs. Ltd. (Tokyo). The antigen and antiserum were prepared according to the method of Kishida and Fukushima,[9a] with a slight modification.

Preparation of 6β-Hydroxycortisol 21-Hemisuccinate——A solution of 6β-hydroxycortisol (100 mg) and succinic anhydride (80 mg) in pyridine (1 ml) was stirred at room temperature for 5 hr. After addition of water, the resulting mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated down. The residue was purified by preparative TLC using chloroform–methanol–acetic acid (70: 10: 0.1) as a developing solvent and repurified using another solvent system, benzene–acetone–methanol–acetic acid (24: 24: 2: 0.1). Recrystallization of the eluate from aqueous methanol gave 6β-hydroxycortisol 21-hemisuccinate (60 mg) as colorless leaflets. mp 177—179° (lit. mp 178.5—180°).[9a]

Conjugation of Hapten with BSA——Tri-*n*-butylamine (20 μl) and isobutyl chlorocarbonate (10 μl) were added to a solution of 6β-hydroxycortisol 21-hemisuccinate (30 mg) in dry dioxane (0.7 ml) at 11° and the mixture was stirred for 30 min. The resulting solution was added to a solution of BSA (90 mg) in water (2.3 ml)–dioxane (1.5 ml)–1 N NaOH (90 μl) at 0° and stirred for 3 hr. The resulting mixture was dialyzed against cold running water overnight, and the turbid protein solution was brought to pH 4.5 with 1 N HCl. The suspension was centrifuged at 3000 rev./min for 20 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the manner described above. Lyophilization of the solution afforded the 6β-hydroxycortisol-BSA conjugate (*ca.* 90 mg) as fluffy powder. The number of steroid molecules linked to a BSA molecule was elucidated to be 18 by UV spectrometric analysis.

Immunization of Rabbit——The antigen (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into a domestic female albino rabbit subcutaneously at multiple sites along the back. This procedure was repeated once a week for 3 weeks and then once every fortnight. Blood was collected 6 months after the initial injection from the rabbit and centrifuged at 3000 rev./min for 10 min. The antiserum thus prepared was stored at 4° in 0.1% NaN_3. This solution was diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (buffer B), when required for immunoassay.

Preparation of 6β-Hydroxycortisol-β-galactosidase Conjugate——1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (3 mg) and N-hydroxysuccinimide (2 mg) were added to a solution of 6β-hydroxycortisol 21-hemisuccinate (2 mg) in 95% dioxane (0.1 ml), and the mixture was allowed to stand at room temperature for 1.5 hr. The reaction mixture was diluted with ethyl acetate, washed with water, and dried over anhydrous Na_2SO_4. The solution was passed quickly through Al_2O_3 (100 mg) packed in a capillary pipet, and the filtrate was evaporated down under an N_2 gas stream. The residue was dissolved in methylene chloride (1 ml), and the concentration of 6β-hydroxycortisol 21-hemisuccinate N-succinimidyl ester was determined by UV spectrometric analysis. A definite volume of the solution, calculated from the molar ratios of steroid to enzyme, was transferred to a test tube with a microsyringe, and the solvent was evaporated off. A solution of β-galactosidase (2 mg) in PB (0.4 ml) was added to the residue at 0° and the reaction mixture was immediately vortex-mixed, then allowed to stand overnight at 4° with occasional shaking. The resulting solution was dialyzed against cold PB (1 liter) for 2 days, diluted with PB containing 0.1% gelatin and 0.1% NaN_3 (buffer B) to bring the concentration to 500 μg/ml, and stored at 4°. The steroid-enzyme conjugates prepared by this method were stable for several months as regards enzymic activity and immunoreactivity under these storage conditions. For the immunoassay procedure, this solution was diluted with the buffer solution containing 0.5% normal rabbit serum.

Enzyme Immunoassay Procedure——Diluted antiserum (0.1 ml) containing 500 pg of cortisol and the 6β-hydroxycortisol-enzyme conjugate (0.1 μg, 0.1 ml) in the buffer were added to a series of standard solutions of 6β-hydroxycortisol (0—1 ng) and to diluted urine in buffer B (0.1 ml), and the mixture was incubated at 4° for 4 hr. Goat anti-rabbit IgG antiserum (0.1 ml) diluted to 1: 30 with buffer A containing 0.3% ethylenediaminetetraacetic acid (EDTA) was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4° for 16 hr. After addition of buffer B (1 ml), the resulting mixture was centrifuged at 3000 rev./min 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.

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° 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 with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer.

Results and Discussion

Enzyme-labeled 6β-hydroxycortisol was prepared by the activated ester method previously developed in our laboratories.[11,12] The N-succinimidyl ester obtained from 6β-hydroxycortisol 21-hemisuccinate[9a] by condensation with N-hydroxysuccinimide in the presence of a water-soluble carbodiimide was covalently linked to β-galactosidase (Fig. 1). Various molar ratios (2, 4, 6 and 10) of the 6β-hydroxycortisol derivative to enzyme were used for the coupling reaction in phosphate buffer (pH 7.3). The number of steroid molecules incorporated per enzyme was not determined. The loss of enzymic activity was less than 20% under the coupling conditions employed.

The anti-6β-hydroxycortisol antiserum used in the enzyme immunoassay was that raised against the 6β-hydroxycortisol 21-hemisuccinate-BSA conjugate (prepared by the method of Kishida and Fukushima[9a]), which is a homologous combination. The bound and free enzyme-

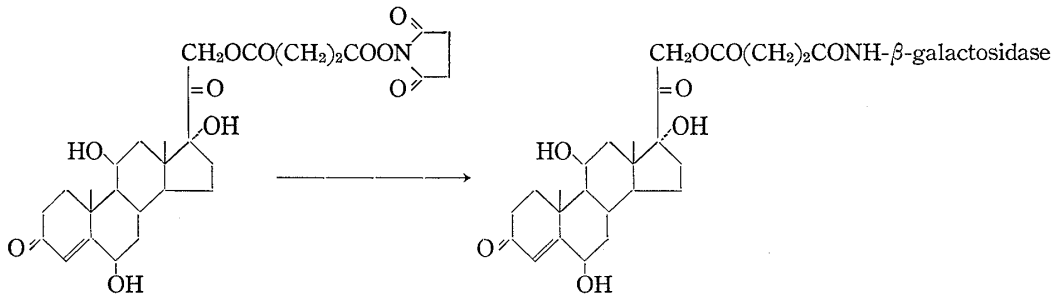

Fig. 1. Preparation of 6β-Hydroxycortisol-β-galactosidase Conjugate

steroid conjugates were separated by a double antibody technique, and the enzymic activity of immune precipitate was determined colorimetrically with *o*-nitrophenyl β-D-galactopyranoside as a substrate.

The binding abilities of 0.1 μg each of the enzyme-steroid conjugates obtained at various molar ratios were investigated with a 1: 500 dilution of anti-6β-hydroxycortisol antiserum. The immunoreactivity increased with increasing molar ratio and the binding ability ranged from 13 to 30%. The antibody dilution curves showed that the appropriate dilution range was 1: 2000—1: 4000. The dose-response curves of 6β-hydroxycortisol with each conjugate were then constructed at 1: 2000 and 1: 4000 dilutions of the antiserum. The results on the inhibition of bound enzymic activity by the addition of 200 pg of 6β-hydroxycortisol per test tube are listed in Table I. It is evident from the data that sufficient inhibition was observed at molar ratios of 2, 4 and 6. However, satisfactory sensitivity and optical density of B_0 could not be obtained at a molar ratio of 10 even when the dilution of antiserum was varied. Thus, the previous finding that the molar ratio is an important factor influencing the sensitivity[12,13] was confirmed.

Table I. Inhibition of Bound Enzymic Activity by 200 pg of 6β-Hydroxycortisol[a]

Molar ratio (steroid/enzyme)	Antiserum dilution	Inhibition (%)	
		without cortisol	with cortisol
2	1 : 2000	46	63
4	1 : 2000	40	60
	1 : 4000	54	—
6	1 : 2000	38	44
10	1 : 2000	20	36
	1 : 4000	17	—

a) Assays were carried out with and without addition of cortisol (500 pg) to antiserum. —: not carried out.

Table II. Effect of Addition of Cortisol on the Assay Value of 6β-Hydroxycortisol in Pooled Urine (μg/24 hr)

Urine dilution	Cortisol added (pg)		
	0	200	500
1 : 50	1650	1000	235
1 : 100	480	270	240
1 : 200	350	260	230
1 : 300	345	232	216
1 : 400	—	240	260
1 : 500	255	260	250

Since the range of measurement of the dose-response curve with the conjugate prepared at a molar ratio of 4 was 20—4000 pg at a 1: 2000 dilution of the antiserum, and the cross-reactions of related steroids were not significant, the direct measurement of 6β-hydroxycortisol in human urine was then attempted. Contrary to expectation, no linearity was observed between the value of 6β-hydroxycortisol and the dilution of urine. Higher values were obtained with low dilutions of urine and an acceptable value with a high dilution of urine, that is 1: 500. Similar results were also obtained in the experiments with a 1: 4000 dilution of the antiserum. This phenomenon seemed to be ascribable to the presence of less specific antibodies and to the heterogeneity of specificity of antibodies in the antiserum. In such a case, purification of antiserum by affinity chromatography[14] or the selective blocking of less specific antibodies[15] is sometimes effective. Separate experiments using the conjugate of cortisol 21-hemisuccinate with β-galactosidase revealed that the antiserum contained antibody populations highly responsive to cortisol rather than 6β-hydroxycortisol. This undesirable property of the antiserum may cause the overestimation at low dilutions of urine, because the level of free cortisol may be up to one-half of that of 6β-hydroxycortisol in normal human urine. In the present work, the latter technique, namely addition of cortisol to the antiserum, was examined with a 1: 2000 dilution of the antiserum. When 500 pg of cortisol was added to each test tube, parallelism was found between the value of 6β-hydroxycortisol and the urine dilution in the range from 1: 50 to 1: 500 (Table II). The observed values corresponded to that obtained with a 1: 500 dilution of urine without addition of cortisol, thus making it possible to determine urinary 6β-hydroxycortisol even in low dilutions of urine. It seemed likely that interfering compounds other than cortisol in urine, if present, were also blocked by this technique. Furthermore, the sensitivity of assay could be markedly increased; *i.e.* addition of 500 pg of cortisol raised the inhibition of bound enzymic activity by 200 pg of 6β-hydroxycortisol from the original value of 40% to 60% (Table I). The same effect was observed in the assay using the conjugates prepared at molar ratios of 2, 6 and 10. Typical dose-response

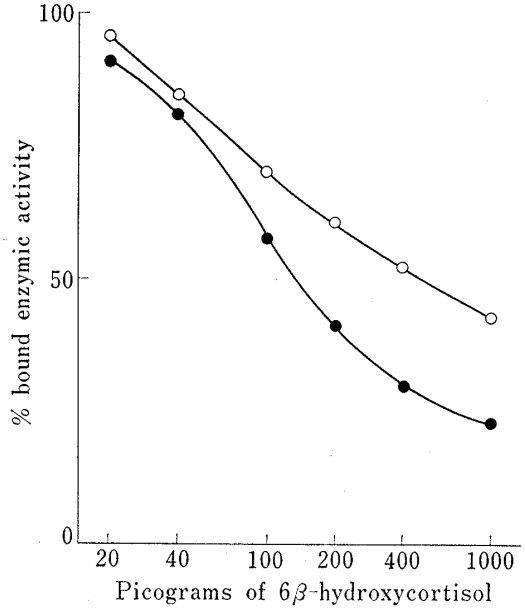

Fig. 2. Dose–Response Curves for Enzyme Immunoassay of 6β-Hydroxycortisol with (—●—) and without (—○—) Addition of Cortisol

The antiserum diluted to 1: 2000 and containing 500 pg of added cortisol was used.

Table III. Cross–Reactivities of Selected Steroids in the Enzyme Immunoassay

Steroid	% cross-reaction (50%)
6β-Hydroxycortisol	100
Cortisol	0.9
6α-Hydroxycortisol	0.1
Cortisone	0.1
6β-Hydroxycortisone	1.4
6α-Hydroxycortisone	0.1
11-Deoxycortisol	0.3
21-Deoxycortisol	0.6
Corticosterone	0.06
Progesterone	0.05
17α-Hydroxyprogesterone	0.4
3α,17,21-Trihydroxy-5β-pregnan-20-one	<0.02
3α,17,21-Trihydroxy-5β-pregnane-11,20-dione	<0.02
3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one	<0.02

Table IV. Recovery of 6β-Hydroxycortisol added to Pooled Human Urine

6β-Hydroxycortisol (pg)			Recovery (%)
Added	Found	Expected	
0	51		
20	72	71	101
40	90	91	99
60	110	111	99
100	165	151	109
140	200	191	105
200	255	251	102
			Mean 102.5±3.9

Table V. Precision of the Enzyme Immunoassay

Urine dilution	Intra-assay ($n=10$)		Inter-assay ($n=6$)	
	6β-Hydroxycortisol (ng/ml)	CV (%)	6β-Hydroxycortisol (ng/ml)	CV (%)
1 : 50	135	5.9	154	12.3
1 : 100	128	7.4	138	5.8
1 : 200	131	7.6	133	3.0
1 : 300	141	8.1	137	3.7
1 : 500	149	6.8	143	2.8

curves are shown in Fig. 2. At the 95% confidence limit, 10 pg was significantly different from zero pg.[16] The sensitivity of the assay is comparable to that of radioimmunoassay.[9] The useful range of measurement was 20—300 pg, which was adequate for determining 6β-hydroxycortisol in 0.5 μl of urine of normal subjects. The specificity of the assay was assessed by testing the ability of related compounds to compete for binding sites on the antibody. The per cent cross-reaction was calculated at 50% displacement of the enzyme-labeled 6β-hydroxycortisol. The results with 13 kinds of closely related steroids are listed in Table III. We found 0.9% cross-reaction with cortisol, 1.4% with 6β-hydroxycortisone, and smaller values with 11-deoxycortisol, 21-deoxycortisol and 17α-hydroxyprogesterone. Assessment of the assay was carried out by checking the recovery rate and the intra-assay and inter-assay coefficients of variation at several dilutions of pooled urine. A known amount of 6β-hydroxycortisol added to pooled urine was recovered at a rate of 102.5±3.9% (SD) (Table IV). Intra-assay and inter-assay coefficients of variation were 5.9—8.1% ($n=10$) and 2.8—12.3% ($n=6$), respectively (Table V). Thus, the present method proved to be satisfactory in both precision and accuracy.

The urine 6β-hydroxycortisol level of normal subjects was then measured. In the enzyme immunoassay, 1 : 200 and 1 : 400 dilutions were used for all urine samples. The levels obtained with both dilutions were nearly identical, when the values were in the range of 20—300 pg on the standard curve. The urine 6β-hydroxycortisol levels of 27 normal male subjects varied from 96 to 428 μg/24 hr, with a mean value of 237±84 μg/24 hr (SD). The values obtained in this study were consistent with those hitherto reported.[9,10] Although a comparison with an independent method was not carried out, the newly developed enzyme immunoassay can be expected to be valid and useful for studies of the metabolic significance of 6β-hydroxycortisol in diseases and the effect of foreign substances on the microsomal drug-metabolizing enzymes.

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 present

high sensitivity was ascribable to the use of an appropriate molar ratio of steroid to enzyme in enzyme labeling, and in part to the selective blocking of less specific antibodies, which was primarily done to improve the specificity.

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