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One-Step Synthesis of a Cortisol Derivative for Radioiodination and Application of the ^{125}I-Labeled Cortisol to Radioimmunoassay

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A one-step synthesis of a cortisol derivative for radioiodination is presented. The radioimmunoassay for cortisol using the bridge heterologous ^{125}I-labeled antigen was more sensitive than that using a bridge homologous antigen. Cortisol levels in saliva and serum were easily determined by direct radioimmunoassay with the proposed ^{125}I-labeled antigen.

Keywords——cortisol; cortisol derivative one-step synthesis; ^{125}I-labeled cortisol; direct radioimmunoassay; saliva; serum

In radioimmunoassay (RIA) for haptens such as steroids and pharmaceuticals, ^{3}H- or ^{125}I-labeled haptens have been widely used. Gamma-emitting ^{125}I is preferred for labeling of the hapten because it is easy to count. When the hapten does not possess a suitable chemical structure for direct radioiodination, it has to be bound with an appropriate functional group such as an imidazole or phenolic moiety before radioiodination. In this case, a bridge exists between the hapten and the functional group. The difference in the chemical structure of the radiolabeled hapten from the original hapten molecule affects the sensitivity and specificity of the RIA.[1] The importance of the combination of antiserum and enzyme-labeled steroid have also been pointed out in enzyme immunoassay.[2]

Using cortisol as a hapten, we prepared ^{125}I-labeled antigens having a bridge homologous or heterologous to that of the immunogen. For the preparation of the bridge heterologous antigen, a new type of coupling reagent, which can be applied to an one-step synthesis of derivatives for radioiodination, is proposed. A direct RIA procedure for cortisol in saliva and serum is presented as an application of the ^{125}I-labeled bridge heterologous antigen.

Experimental

All unlabeled steroids were obtained from Sigma Chem. Co. (U.S.A.), Na^{125}I was a gift from Daiichi Radioisotope Laboratory (Japan) and [1,2,6,7-^{3}H]cortisol (100 Ci/mmol) was purchased from Amersham (England). All other reagents were of reagent grade and were used without further purification. (^{1}H-NMR) spectra were determined on a JEOL 60H spectrometer with tetramethylsilane as an internal standard.

Preparation of Antiserum against Cortisol——Cortisol-3-*O*-carboxymethyl-oxime (F3-CMO)[3] was conjugated to bovine serum albumin (BSA) according to the method of Erlanger *et al.*[4] (the number of cortisol molecules linked to one molecule of BSA was determined to be 20). The conjugate (1 mg) was dissolved in 0.5 ml of saline and emulsified with an equal volume of complete Freund's adjuvant. A rabbit was given three booster injections subcutaneously at intervals of two weeks and thereafter injections once a month. Blood was collected 10 months after the initial injection and centrifuged at 2000 *g* for 10 min. The antiserum was stored at −20 °C. The titer was

approximately 20000.

Preparation of Coupling Reagent for Derivatization of Cortisol——3-(4-Imidazolyl)propionic Acid Hydrazide (IPH): Five grams of urocanic acid was dissolved in 72.4 ml of 0.5 N NaOH and catalytic reduction was carried out by using 0.5 g of a 10% palladium-on-charcoal catalyst for 2 h in the usual way. The reaction mixture was filtered and adjusted to pH 2 with concentrated HCl, and then concentrated under reduced pressure on a water bath. EtOH was added to the residue and the mixture was filtered. The filtrate was concentrated under reduced pressure and the residue, crude 3-(4-imidazolyl)propionic acid HCl, was esterified by heating to reflux with 50 ml of MeOH–HCl for 2 h. After removal of the excess HCl under reduced pressure, 7.2 g of 100% hydrazine hydrate was added and the whole was heated at 70°C for 2 h. The reaction mixture was concentrated to dryness in a rotary evaporator. EtOH was added to the residue and the mixture was filtered to remove hydrazine HCl. The filtrate was passed through an anion exchange resin column (Amberlite IR-45, OH form, 15 g, 1.5 × 20 cm) to remove HCl. The effluent was concentrated to dryness under reduced pressure and the residue was recrystallized from EtOH. Yield 0.8 g, mp 142 °C. ^{1}H-NMR (CD_3OD) δ: 2.31 (4H, m, $-CH_2CH_2-$), 6.49 (1H, s, imida 5H), 7.24 (1H, s, imida 2H). *Anal.* Calcd for $C_6H_{10}N_4O$: C, 46.74; H, 6.54; N, 36.34. Found: C, 46.77; H, 6.39; N, 35.78.

Preparation of Cortisol Derivatives——Cortisol-3-[3-(4-imidazolyl)propionic Acid] Hydrazone (F3-IPH): Cortisol (157 mg) and IPH (100 mg) were dissolved in 2 ml of MeOH and refluxed for 6 h. Purification by thin layer chromatography (TLC) (Kiesel-gel 60) using $CHCl_3$–MeOH–H_2O (24 : 7 : 1) as a developing solvent gave F3-IPH (37 mg, recrystallized from MeOH). mp 190°C. ^{1}H-NMR (CD_3OD) δ: 0.51 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 5.48 and 5.78 (1H, both s, 4-H, *anti*:*syn*=2 : 1), 6.47 (1H, s, imida 5H), 7.23 (1H, s, imida 2H). *Anal.* Calcd for $C_{27}H_{38}N_4O_5 \cdot 2H_2O$: C, 60.65; H, 7.92; N, 10.48. Found: C, 60.05; H, 7.72; N, 10.40. Cortisol-3-(O-carboxymethyl)oxime-histamine (F3-CMO-his) was prepared from F3-CMO, ethyl chloroformate and histamine according to the method of Allen and Redshaw.[1c]

Radioiodination of Antigens——Antigens were labeled with ^{125}I by the chloramine T procedure of Hunter *et al.*[5] Specific activity (according to the method of Morris[6]): F3-IPH-^{125}I, 700 Ci/mmol; F3-CMO-his-^{125}I, 100 Ci/mmol.

Standard Curves——Standard (cortisol) and antiserum were diluted in 0.1 M phosphate buffer (pH 7.4) containing 0.5% BSA. Radiolabeled antigens were diluted in 0.1 M phosphate buffer (pH 7.4). Radiolabeled antigen (*ca.* 10000 cpm, 0.1 ml) and diluted antiserum (0.1 ml) were added to a series of standard solutions (0.1 ml), and each mixture was incubated overnight at 4°C. After addition of 0.5 ml of dextran-coated charcoal, prepared by mixing dextran T 70 (50 mg) and Norit A (500 mg) in 100 ml of 0.1% gelatin at 4°C, the suspension was vortex-mixed and allowed to stand for 30 min at 4 °C, and then centrifuged at the same temperature for 15 min at 2000 *g*. The radioactivity in the precipitate or supernatant was measured in the usual way.

Direct RIA of Cortisol——The flow sheet of the direct assay is shown in Chart 1. Paired samples of saliva and blood were collected from normal, apparently healthy volunteers. In the study of analytical recovery of cortisol from serum or saliva to which had been added a known amount of cortisol, a 2.5 μl aliquot of normal human pooled serum (Nescol X, Chemo-Sero-Therapeutic Research Inst., Japan) or 0.1 ml of saliva was used. Free cortisol values in sera were determined by the equilibrium dialysis method[7] and the salivary cortisol level was determined by our IPH-RIA method presented here.

Results and Discussion

The structure of the immunogen, antigens, and related compounds are shown in Chart 2. Histamine or tyrosine methyl ester derivatives of steroids have been used as substrates for radioiodination of 3-carboxymethyloxime steroids,[1c,8] but their preparation involves several steps. Hydrazide derivatives of phenolic compounds have also been prepared and treated with steroid.[1d] Histamine forms mono-iodinated derivatives, whereas tyrosine methyl ester (TME) can form, in addition to the mono-iodo derivatives, di-iodinated TME derivatives which have a lower immunoactivity and are less stable.[9] Consequently, we used a histamine tracer. An antigen (F3-IPH) having a heterologous bridge was prepared by coupling cortisol with hydrazide derivatives of imidazole (IPH) and then radioiodinated. The F3-IPH-^{125}I derived from a new coupling reagent (IPH) had high specific activity. The hydrazide group can easily react with the conjugated ketone of cortisol and can subsequently be easily radioiodinated. An antigen (F3-CMO-his) having a bridge homologous structure to the immunogen was prepared by reaction of F3-CMO with histamine, and was derived to F3-CMO-his-^{125}I.

The standard curves for cortisol are shown in Fig. 1. The curve obtained using the radiolabeled bridge heterologous antigen (F3-IPH-^{125}I) was shifted to a region of lower concentration of cortisol and was more sensitive than the rediolabeled bridge homologous

sample or standard solution
+
BB-BSA
} total 0.2 ml
↓ mix
F3-IPH-^{125}I in BB, 0.1 ml
↓ mix
antiserum in BB-BSA, 0.1 ml
↓ mix and incubate overnight at 4°C
3% γ-globulin in BB, 0.1 ml
↓ mix
15% PEG in BB, 1 ml
↓ mix
centrifugation for 20 min at 4°C (2000 *g*)
↓
aspiration of supernatant
↓
counting (precipitation, bound)

BB: 0.5 M borate buffer (pH 8.6)
BB-BSA: 0.5% BSA in BB
PEG: polyethylene glycol 6000

Chart 1. Flow Sheet of IPH-RIA

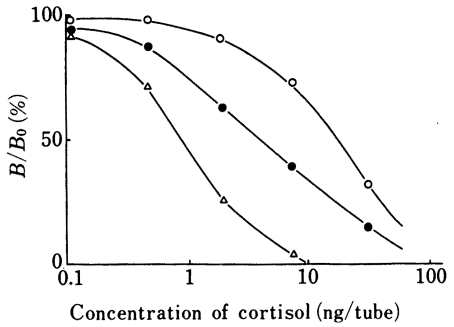

Fig. 1. Semi-logarithmic Standard Curves Obtained with Various Tracers for Cortisol RIA
○, ^{3}H-cortisol; ●, F3-CMO-his-^{125}I; △, F3-IPH-^{125}I.

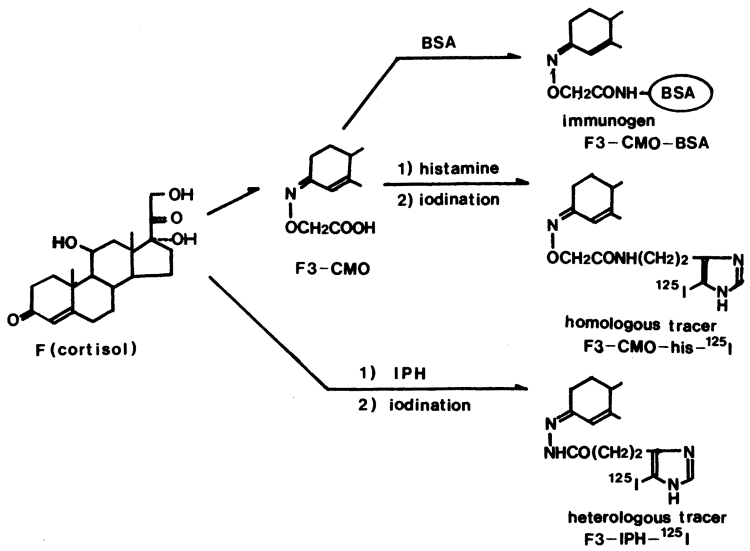

Chart 2

antigen (F3-CMO-his-^{125}I). ^{3}H-Cortisol, which has no bridge site, produced the least variation of B/B_0 (where B is the bound activity and B_0 is the activity bound in the absence of unlabeled cortisol) with the change in cortisol concentration.

The amount of cortisol in the samples (serum and saliva) was determined by using the bridge heterologous radiolabeled antigen (F3-IPH-^{125}I). The detection limit of the IPH-RIA for cortisol (according to the method of Ekins and Newman[10]) was 49 ng/dl. Tables I and II

TABLE I. Recovery of Cortisol Added to Normal Human Pooled Serum

Present	Cortisol (ng/tube) Added	Found	Recovery (%)
0.15	0.75	0.85	94.4
0.15	0.43	0.58	100.0
0.15	0.20	0.33	94.3
0.15	0.13	0.28	100.0
0.15	0.06	0.21	100.0
		Mean:	97.7

TABLE II. Recovery of Cortisol Added to Saliva

Present	Cortisol (ng/tube) Added	Found	Recovery (%)
0.31	1.73	1.99	97.5
0.31	0.79	1.14	103.6
0.31	0.40	0.71	100.0
0.31	0.15	0.47	102.1
0.31	0.09	0.41	102.5
		Mean:	101.1

show the recovery of cortisol added to serum and to saliva, respectively. The recovery was satisfactory. IPH-RIA can be used to determine cortisol in the presence of corticosteroid-binding globulin in serum without pretreatment of the sample. The concentration of cortisol in saliva is known to reflect the free cortisol level in serum, and determination of the cortisol level in the saliva, especially when it is difficult to draw blood from a vein, such as in children, seems to be very useful.[7b] The cortisol level in saliva is much lower than that in serum and saliva sometimes can not be collected in large amounts. Thus, it is important to determine the amount of cortisol in the smallest possible quantity of saliva. Free cortisol levels in serum determined by equilibrium dialysis were 7—14% of the concentration of total cortisol in serum. Salivary cortisol showed a good correlation with the values of serum free cortisol ($Y=1.731\ X+0.079\ \mu g/dl$, n, 10; r, 0.92; Y, serum free cortisol; X, salivary cortisol).

In conclusion, a new type of coupling reagent (IPH) could be applied to a one-step synthesis of a derivative for radioiodination. The IPH-RIA, which uses a bridge heterologous antigen, can be used to determine the cortisol level in serum and saliva with high sensitivity through a simple procedure using a small quantity of sample (serum 2.5 μl, saliva 100 μl).

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