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SHORT COMMUNICATION

RADIOIMMUNOASSAY OF CORTOL AND CORTOLONE IN HUMAN URINE

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SUMMARY

White New Zealand rabbits were immunized with 21-succinate-bovine serum albumin conjugates of 20β-cortol and 20α-cortolone. The resulting 20β-cortol antiserum shows 100% crossreaction with 20β-cortol, 26% with 20α-cortol and with tetrahydrocortisol and 52% with tetrahydrocortisol-21-glucosiduronate. The 20α-cortolone antiserum shows 100% crossreaction with 20α-cortolone, tetrahydrocortisone and tetrahydrocortisone-21-glucosiduronate and 60% with 20β-cortolone. We describe the application of both antisera for radioimmunoassays in human urine.

INTRODUCTION

The determination of the excretion of cortisol metabolites give a good reflection of the secretion rate of cortisol. We present radioimmunoassays (RIAs) for two major groups of cortisol metabolites, the cortols and cortolones.

EXPERIMENTAL

Nonradioactive steroids were obtained from Sigma, St. Louis, MO., U.S.A. and from Ikapharm, Ramat-Gan, Israel. Steroid-3/21-glucosiduronates (steroid-3α/21-yl-β-D-glucosiduronates) were synthesized and kindly supplied by Prof. Dr. V. R. Mattox, Mayo Clinic, Rochester, MN., U.S.A.

Tritiated 20β-cortol and 20β-cortolone were prepared by enzymatic reduction of [1,2-^{3}H]-THF and [1,2-^{3}H]-THE (New England Nuclear Corp., Boston, MA., U.S.A.), using the NADH dependent 3α, 20β-hydroxysteroiddehydrogenaše from Streptomyces hydrogenans (Sigma).

Tritiated 20α-cortolone is not readily available, so we used [^{3}H]-20β-cortolone in the cortolone radioimmunoassay.

The preparation of the antigens and the immunization were carried out as described previously [1] according to the method of Erlanger *et al.*[2].

Human urine samples were prepared by three different methods as follows:
(a) by dilution of native urines; (b) by glucuronidase splitting; and (c) by glucuronidase splitting and subsequent paper chromatography: After glucuronidase treatment samples were labelled with 10,000 d.p.m. of tritiated 20β-cortol or 20β-cortolone and extracted with ethyl acetate. The extracts were fractionated by paper chromatography in a modified system of Eberlein *et al.*[3]: iso-octaine/tert. butanol/0.1 M borate buffer, pH 9, (10:5:9, by vol.) on Whatman chromatography paper No. 1, which were impregnated with the same borate buffer. In this system the C20 epimers of cortol and cortolone separated very well. 20α-cortol and 20α-cortolone could not be recovered on the chromatograms of urine extracts because these compounds were not available in tritiated form.
The radioimmunoassay was carried out as described previously [1].

Total urinary corticoid (C21-α-ketolic steroid) excretion was measured by the tetrazolium-blue method.

The cortisol secretion rate was estimated as described by Vecsei *et al.*[5].

THE and THF-radioimmunoassays were carried out as described by Will *et al.*[1].

RESULTS

1. Titer

About 16 weeks after the first immunization, all animals showed antibody titers between 1:5000 and 1:14000. 20β-cortol antiserum No. 9/2/3 was diluted 1:10000 and 20α-cortolone antiserum No. 13/3/3 was diluted 1:4600. All experiments were performed with these two antisera.

2. Sensitivity

The c.p.m. bound were plotted against the logarithm of the dose of standard steroid added for the standard curve. In both assays 10 pg of standard steroid differed significantly from zero.

3. Specificity

The specificity of both assays was tested by cross reaction studies and by immunological analyses of paper chromatograms of human urines. The results of the cross reaction studies with various steroids and conjugates are shown in Table 1. The percentage of cross reaction was calculated taking account of the differences in the molecular weights between cross reacting and standard steroid.

The immunological analyses of chromatograms of human urine samples were carried out in a system of Schneider *et al.*[6]: *n*-buthylether–butanol–acetic acid–water (13:7:6:14, by vol.) on Whatman No. 1 chromatogram sheets. These analyses confirmed the results of the cross reaction studies.

4. Precision

The intra and inter assay variance was evaluated by duplicate measurements of 20 samples in the same and

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Table 1. Crossreactions of various steroids and glucuronic acid conjugates with antisera 9/2/3 (β-cortol antibodies) and 13/3/3 (α-cortolone antibodies) at titer dilution; the values are based on molecular ratio

Steroids	% Crossreaction 9/2/3	13/3/3
20α-Cortol	26.3	0.4
20β-Cortol	100.0	1.3
20α-Cortolone	0.6	100.0
20β-Cortolone	<0.1	60.0
Cortisol	<0.1	<0.1
11β,17α,21-Trihydroxy-5β-pregnan-3,20-dione	0.6	0.7
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one (THF)	26.4	2.8
cortisone	<0.1	0.7
17α,21-Dihydroxy-5β-pregnan-3,11,20-trione	<0.1	10.7
3α,17α,21-Trihydroxy-5β-pregnan-11,20-dione (THE)	0.3	100.0
3α,21-Dihydroxy-5β-pregnan-11,20-dione (THA)	0.6	4.3
3α,11β,21-Trihydroxy-5β-pregnan-20-one (THB)	7.9	<0.1
3α,21-Dihydroxy-5β-pregnan-20-one (THDOC)	<0.1	<0.1
3α,17α,21-Trihydroxy-5β-pregnan-20-one (THS)	1.9	4.1
THF-3-Glucuronate	1.35	0.45
THF-21-Glucuronate	78.0	15.0
THE-3-Glucuronate	<0.1	<0.1
THE-21-Glucuronate	7.5	149.0
THA-21-Glucuronate	0.38	7.1
THB-21-Glucuronate	5.8	0.55
3α,17α,20β-Trihydroxy-11-oxo-5β-pregnan-21-oic acid	<0.1	<0.1
3α,11β,17α,20-Tetrahydroxy-5β-pregnan-21-oic acid	<0.1	<0.1

in two different assays as described by Abraham *et al.*[7]. The resulting variation coefficients were:

α-cortolone radioimmunoassay: intra: 8.4%, inter: 9.3%.
β-cortol radioimmunoassay: intra: 10.4%, inter: 11.9%.

5. Clinical results

20β-cortol and cortolones levels were determined in eleven urines of patients without any endocrine disease before and after glucuronidase treatment (methods a and b).

The values obtained [mg/24 h ± S.DEV.] are: cortol RIA before: 0.497 ± 0.231; after: 0.991 ± 0.366; cortolone RIA before: 3.082 ± 1.584; after: 5.35 ± 2.38. From nineteen other urines, which had shown normal results in the THE and THE routine RIAs, 20β-cortol and 20β-cortolone were determined by method c: 20β-cortol: 0.347 ± 0.155; 20β-cortolone: 0.661 ± 0.393. The THF and THE values of these urines were 1.548 ± 0.908 and 2.291 ± 1.185 respectively [mg/24 h ± S.DEV.].

6. Comparative studies

A great number of clinically uncharacterized urines were tested for cortol and cortolone by assay of diluted urine and the results were compared either with the cortisol secretion rate (SR F), the total urinary corticoid excretion or the values obtained by THE- and THF-radioimmunoassays. The values correlate well with the cortisol secretion rate (cortolone/SR F: $r = 0.88$; cortol/SR F: $r = 0.95$; $n = 12$) and with the THE- and THF-RIAs (cortolone/THE: $r = 0.98$ and cortol/THF: $r = 0.91$; $n = 85$). They correlate to a lesser extent with the corticol excretion (cortolone/corticoid: $r = 0.67$; cortol/corticoid: $r = 0.67$; $n = 34$).

DISCUSSION

Two radioimmunoassays for the determination of cortisol metabolites from human urine are presented. The results obtained from measurement in unprocessed urines give a good reflection of the cortisol secretion. Both radioimmunoassays are not suitable for measuring the cortol- or the cortolone levels in diluted urine without further purification steps. In these cases a glucuronidase splitting and a subsequent chromatographic fractionation are necessary.

Using our 20α-cortolone antiserum, the sum of the cortolone and THE concentrations can be determined simultaneously; this is the first antiserum described which is able to bind two metabolites of the same steroid hormone specifically. It is the first step in the development of methods by which a great spectrum of cortisol metabolites can be determined simultaneously. The high correlation between the cortol- and cortolone-assays and the THF- and THE-radioimmunoassays indicate the acceptability of the new methods. The THE- and THF-RIAs have proved to be very practical for clinical routine diagnosis of adrenocortical function for several years.

The mean value for total β-cortol in human urine determined by radioimmunoassay is 0.347 ± 0.155 mg/24 h and for total β-cortolone 0.661 ± 0.393. These values cannot be compared to any published data obtained by spectrophotometric methods, because in those publications the 20α- and 20β-isomers were not separated from each other.

Romanoff[8] gave as the sum of 20α- and 20β-cortolone for young men 1.44 mg/24 h and for older men 0.98 mg/24 h. Husmann *et al.*[9] obtained the following mean values: 20α- and 20β-cortol: 2.38 mg/24 h; 20α- and 20β-cortolone: 2.45 mg/24 h.

The new radioimmunological methods will lead to more reliable data.

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