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A DIRECT RADIOIMMUNOASSAY FOR 6β-HYDROXYCORTISOL IN HUMAN URINE

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SUMMARY

A specific radioimmunoassay has been developed for the direct measurement of 6β-hydroxycortisol in human urine. Preliminary purification of the steroid by either chromatography or extraction was not necessary. The assay had a sensitivity of 25 pg, an intra-assay variation of 7.3%, and an inter-assay variation of 8.6%. The specificity of the assay was checked using chromatography. The mean 24 hr urinary output of 6β-hydroxycortisol measured in normal males was 286 μg and in normal females 233 μg but the difference was not statistically significant. The effect of rifampicin and idomethacin on the urinary excretion of 6β-hydroxycortisol was investigated.

INTRODUCTION

6β-Hydroxycortisol (6β-OHF) is a polar metabolite of cortisol formed in the endoplasmic reticulum of heptatocytes by the mixed function oxygenases which are also responsible for the metabolism of many drugs [1]. The activity of these enzymes may be stimulated by inducing agents such as drugs, polycyclic hydrocarbons and pesticides. Therefore changes in the urinary output of 6β-OHF may provide an index of induction of hepatic drug-metabolising enzymes [2]. To date, extensive investigations of urinary 6β-OHF concentrations have been restricted by the methods of measurement [3]. Assays developed previously have employed either extraction and chromatography prior to a non-specific colour reaction [4] or oxidation of the sample prior to gas–liquid chromatography [5].

In order to facilitate our studies of enzyme induction in man we have developed a specific radioimmunoassay for 6β-OHF in urine and defined its specificity, sensitivity and accuracy.

MATERIALS

Tritiated 6β-hydroxycortisol (S.A. 52 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Scintillant (NE 260) was obtained from Nuclear Enterprises. General reagents were purchased from BDH, Poole, U.K. and all solvents were redistilled prior to use.

METHODS

The preparation of antisera specific for 6β-OHF has been described previously [6]. The antiserum used in the present work was that previously described as "R1B2". Standard radioimmunoassay reagents were prepared as described previously [7]. A stock methanolic solution of 6β-OHF (5pg/μl) was used to prepare a standard curve ranging from 25–2000 pg in duplicate. The solvent was evaporated *in vacuo* at 35°C and the standards reconstituted in 50 μl of 0.1% BSA buffer. Urine samples were diluted (1 : 100) in 0.1% BSA buffer and 50 μl duplicates assayed. To all tubes was added antiserum (100 μl of a 1:500 dilution in 0.1% BSA buffer) and 90 pg of tritiated 6β-OHF in 50 μl BSA buffer. The tubes were vortexed and incubated at 4°C overnight.

The final stages of the assay were performed at 4°C. To each tube was added 100 μl of 0.5% BSA buffer followed by a 1 ml suspension of dextran-coated charcoal in PBSM buffer. The tubes were agitated and left to stand for 10 min. After centrifugation at 2500 g for 15 min the supernatants were decanted into scintillation vials containing 4 ml scintillant.

For assessment of assay specificity a pooled urine sample was applied directly to a silica gel plate (20 × 20 cm) in a 6 cm band. Standard 6β-OHF was applied to two outside lanes. The chromatogram was developed in chloroform–methanol (80 : 20, v/v) after Thrasher *et al.*[8]. The plate was divided into bands (17 × 1 cm) and the silica from each band added to ethyl acetate (4 ml) and saturated sodium sulphate (1 ml) in glass conical centrifuge tubes. The tubes were vortexed for 1 min, allowed to stand for 1 h and then centrifuged at 2500 *g* for 10 min. An aliquot of the ethyl acetate layer was taken, evaporated *in vacuo* at 40°C, reconstituted in 0.1% BSA buffer and the 6β-OHF content measured by radioimmunoassay. The experiment was repeated using the solvent system chloroform–methanol–acetic acid (115 : 15 : 3 by vol.). Similar experiments were conducted using the paper chromatography system described by Kuntzman *et al.*[10] for purifying 6β-OHF. Again neat urine was applied to the paper chromatogram. After development the paper chromatogram was divided into 16 × 3 cm bands which were extracted with methanol

Table 1. Cross-reactions of antiserum R1B2 with various steroids

Steroid	Cross-reaction (%)
6β-Hydroxycortisol	100
6α-Hydroxycortisol	1.9
Cortisol	1.4
Cortisone	<0.05
6β-Hydroxycortisone	<0.05
3α, 11β, 17,-21-tetrahydroxy-5β-pregnan-20-one	<0.05
11β, 17, 20α, 21-tetrahydroxy 4-pregnen-3-one	<0.05
Corticosterone	0.09
11-Desoxycortisol	<0.05
21-Desoxycortisol	0.05

(10 ml). An aliquot of the methanol extract was evaporated and its 6β-OHF content measured by radioimmunoassay. The possibility that extraction of 6β-OHF might influence the result of the assay was tested as follows. Samples were diluted 1 : 100 in saturated aqueous sodium sulphate. Duplicate aliquots were pipetted into stoppered tubes and extracted with 1 ml of ethyl acetate. The tubes were placed in an acetone–solid carbon dioxide mixture and the ethyl acetate decanted and evaporated *in vacuo* at 35°C. The residues were reconstituted in 0.1% BSA buffer (50 μl) and used for radioimmunoassay. The values obtained were corrected for recovery which was found to be 95% using [^{3}H]-tracer recovery.

The effect of urine concentration on the assay was investigated by measuring the 6β-OHF content of a pooled urine sample at various dilutions.

RESULTS

The cross-reactions for antiserum "R1B2" are given in Table 1 and a typical standard curve is shown in Fig. 1.

The assay characteristics are shown in Table 2. The 24 h urinary 6β-hydroxycortisol output of normal male and female volunteers are given in Table 3. Also shown are the 6β-hydroxycortisol values for volunteers before and after the administration of indomethacin (75 mg for 21 days) or rifampicin (1200 mg for 10 days).

The chromatography experiments showed that, in each of the systems used, at least 95% of the immunoreactive material behaved as authentic 6β-OHF. The only other band which consistently contained immunoreactive material coincided with the cortisol

Table 2. Assay characteristics

A. Accuracy

6β-OHF (pg) added to urine (0.5 μl)	6β-OHF (pg) measured	Increment (pg)	Accuracy
0	119	—	
50	177	58	1.16
100	220	101	1.01
250	380	261	1.04
500	620	501	1.00

B. Urine parallelism

Urine volume (μl)	6β-OHF (pg ml)
3	1520
1.5	1536
0.75	1610
0.375	1600
0.195	1760

C. Precision

6β-OHF (pg) in sample	Intra-assay variation % (n = 10)	Inter-assay variation % (n = 6)
100	7.3	8.6
1000	5.9	8.3

D. The correlation coefficient of samples with and without extraction, r = 0.91 (n = 8, P < 0.001) range 90–117%

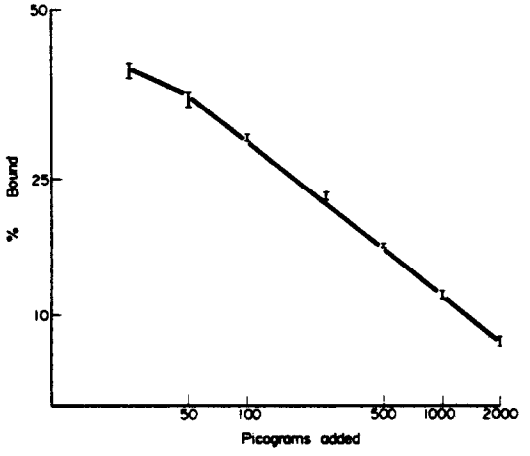

Fig. 1. Standard curve for 6β-hydroxycortisol radioimmunoassay.

standard. This represented approximately 2.5% of the total immunoreactive material. Similar results were obtained with a pooled urine sample from volunteers who had taken rifampicin.

DISCUSSION

We have reported previously the synthesis of an antigen in which 6β-hydroxycortisol was linked through the 3-position and the characterisation of antisera raised against it [6]. Herein we describe the development of radioimmunoassay using such an antiserum. The assay has a sensitivity of 25 pg (distinguishable from zero with 99% certainty) but as this was not on the linear portion of the standard curve 50 pg was considered to be the minimum quantity of 6β-OHF which could be measured reliably. The range of the standard curve (50–2000 pg) was optimised so that all samples could be measured using only 0.5 μl of urine. The urine parallelism experiment indicates that there is no significant dilution effect, although with a very high urine concentration, abnormally low 6β-OHF values were recorded. This is presumably due to urine interfering with the charcoal separation of free from bound steroid in the assay procedure.

The antisera was assessed using the traditional 50% displacement method and found to be highly specific for 6β-hydroxycortisol [6]. Importantly the antisera did not cross-react with cortisol metabolites formed by either reduction of the A ring or reduction of the 20-ketone which are present in high concentrations in urine.

As a further test of assay specificity we examined immunograms of pooled urine. Chromatography systems were selected which has been used previously to purify 6β-OHF prior to its estimation by a non-specific colorimetric assay [8–10]. The results indicate that the assay is sufficiently specific for the measurement of 6β-OHF in human urine. The only material present in urine which was found to interfere was cortisol. The low level of interference (1–2.5%) is consistent with the cortisol cross-reaction, 1.4%. Finally assay of samples with and without extraction indicated that there is no non-specific interference by polar constituents of urine.

Table 3. Urinary 6β-Hydroxycortisol (μg/24 h)

Normal males		
P.R.		275
R.H.		301
M.Y.		218
B.K.P.		306
P.R.		274
M.O.		333
N.S.		216
O.B.		323
W.Z.		293
M.L.		221
		286 ± 54 (Mean ± S.D.)
Normal Females		
E.S.		149
F.C.		226
R.W.		233
S.N.		337
M.Q.		209
S.S.		258
M.K.		340
I.G.		113
		233 ± 80 (Mean ± S.D.)
Subjects before and after indomethacin (75 mg for 21 days)		
F.B.	287	348
J.H.	402	389
B.J.	263	197
G.H.	274	362
	306 ± 64	324 ± 86 (Mean ± S.D.)
Subjects before and after rifampicin (1200 mg for 10 days)		
E.O.	296	1631
M.L.	334	1775
B.K.	365	1638
H.S.	268	855
	290 ± 56	1475 ± 418 (Mean ± S.D.)

Each value represents a mean of 2 samples

The mean 24 h 6β-OHF excretion from normal males 286 ± 54 μg is within the range obtained by other workers using conventional colorimetric techniques [4, 8–11]. Like Thrasher *et al.*[8] we found the mean male excretion of 6β-OHF to be greater than the mean female output but this difference was not statistically significant.

Finally we examined the effect of two drugs on urinary 6β-OHF excretion; rifampicin, which is known

to be an enzyme inducer in man [12] and indomethacin which is not. Indomethacin did not have any significant effect on 6β-OHF excretion whereas rifampicin increased the daily urinary output between 319–551% and this effect was highly significant ($P < 0.001$).

A comparison of 6β-hydroxycortisol excretion with other parameters of enzyme induction in man will be presented elsewhere [13].

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