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A Sensitive ELISA for 6β-Hydroxycortisol in Urine using Enzyme Penicillinase (β-Lactamase)

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A sensitive and specific, enzyme labelled immunosorbent assay (ELISA) for 6β-hydroxycortisol in diluted urine using penicillinase was developed. 6β-Hydroxycortisol-21-hemisuccinate was conjugated with enzyme penicillinase. Antibody immobilized on a polyvinylchloride ELISA plate (Dynatech) was used for separation of bound from free ligand. The sensitivity of the assay was between 2.0–3.0 pg per well and recovery of 6β-hydroxycortisol from urine ranged between 85.0–108.0%. The assay is simple, rapid and precise.

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INTRODUCTION

Cortisol is excreted by the kidney in human urine as 6β-hydroxycortisol which constitutes approx. 1–2% of the total daily cortisol secretion. It is formed in the endoplasmic reticulum of hepatocytes by the mixed function oxygenases (cytochrome *P*-450 enzyme) which are also responsible for the metabolism of many drugs [1, 2]. Its measurement is therefore, useful for monitoring the effects of hormones and drugs acting as inducers of microsomal enzymes.

Drugs metabolized by the liver appear to be cleared from the plasma at a faster rate in children than in adults. These quantitative differences between the rates of drug metabolism in children and adults suggest that data concerning the effect of drugs on hepatic drug metabolism in adults cannot automatically be applied to children.

A study was undertaken by our department to investigate whether minute quantities of chronically ingested maternal drugs by nurslings can alter their hepatic drug metabolizing activity pattern. Hence, a penicillinase (β-lactamase) linked ELISA was developed for estimation of 6β-hydroxycortisol from urine. The present paper details the development of a simple, rapid and economical ELISA for the estimation of 6β-hydroxycortisol from urine.

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MATERIALS AND METHODS

Materials

6β-Hydroxycortisol was purchased from Steraloids Inc (Wilton, NH). 6β-Hydroxycortisol-21-hemisuccinate and antisera to 6β-hydroxycortisol-21-hemisuccinate-bovine serum album (BSA) were a gift from Dr Hosoda (Pharmaceutical Institute Tohoku University, Japan). Penicillinase (EC 3.5.2.6), sp. act. 66000 μ/mg protein and penicillin V were obtained from Hindustan Antibiotics Ltd. (Pune, India). Dimethyl formamide was purchased from E. Merk (India). Isobutyl chloroformate, *n*-methyl morpholine, polyoxyethylene sorbitan (Tween 20) and BSA were purchased from Sigma Chemical Co (St Louis, MO). Soluble starch was purchased from Reanal (Budapest, Hungary). Disposable flexible flat bottomed polyvinylchloride microtitre ELISA plates were purchased from Dynatech (Virginia). All the other chemicals and salts used were of analytical grade.

Method

Coating buffer:	50 mmol/l carbonate-bicarbonate buffer pH 9.6.
Phosphate buffer:	200 mmol/l sodium phosphate buffer, pH 7.0
Phosphate buffer saline:	100 mmol/l sodium phosphate buffer containing 0.8% NaCl, pH 7.2

Assay buffer:	100 mmol/l sodium phosphate buffer saline pH 7.2, containing 0.1% sodium azide and 1.0% BSA.
Washing solution:	Normal saline containing 0.05% Tween 20.
Iodine reagent:	2.0% of crystalline iodine in 3325 mmol/l potassium iodide.
Hydrolysed starch solution:	2.0% starch in 200 mmol/l phosphate buffer.
Starch–iodine solution:	To 25 ml of 200 mmol/l phosphate buffer, 5 ml of hydrolysed starch solution and 0.05 ml of iodine reagent were added with vigorous shaking.

The buffers were checked for the required pH at room temperature (30 ± 2°C) and then stored at 4°C.

Preparation of 6β-hydroxycortisol-21-hemisuccinate–penicillinase conjugate

6β-Hydroxycortisol-21-hemisuccinate was conjugated to enzyme penicillinase by using a modified mixed anhydride method as described previously [3].

Internal quality control pools for three different concentrations of 6β-hydroxycortisol were prepared as follows: *Low pool*, was prepared from urine samples collected from 90-day-old infants belonging to the control group. *High pool*, was prepared by pooling urine samples from normal healthy mothers not on any medication. *Medium pool*, was prepared by mixing equal quantities of urine from the low and high pools. Urine samples from each pool were further aliquoted in 0.2 ml quantities, labelled and preserved with sodium azide at −20°C.

Passive adsorption of antibody to 6β-hydroxycortisol onto the ELISA plate

200 μl of the appropriate dilution of antiserum (1:10,000) in coating buffer was dispensed into each well of microtitre ELISA plate and left overnight at 4°C. Unadsorbed antibody was then washed off with the washing solution.

Preparation of standard

6β-Hydroxycortisol powder was dissolved in absolute ehthanol to give a stock solution of 0.1 mg/ml. This stock solution was kept at −20°C until used. From the stock solution, working standards (8000 pg–31.25 pg/ml) were prepared in immunoassay buffer.

Preparation of urine samples

Urine samples were diluted 1:100 with the assay buffer. The quality control pools, low (A), medium (B) and high (C) were diluted 1:50, 1:100 and 1:200, respectively.

ELISA procedure

To each well of microtitre ELISA plate previously adsorbed with antibody, 100 μl of any one of the following was added: standard, buffer, excess standard, or diluted urine samples. Then 100 μl of the appropriately diluted conjugate (1:15,000) was added. The plate was incubated at 37°C for 2 h, and then washed with washing solution. The enzyme activity in the bound fraction was measured as follows: to 25 ml of starch–iodine solution, 4.0 mg of penicillin V (phenoxymethyl penicillinic acid) was added and mixed well, 0.25 ml of this solution was dispensed in each well of the plate. After incubation at room temperature for 40 min the plates were read on a microtitre ELISA Reader using a 620 nm filter [4].

RESULTS

The developed ELISA was validated using the general validatory criteria required for immunoassays, such as sensitivity, specificity, accuracy and precision.

Sensitivity

The sensitivity of the assay, defined as the least amount of the 6β-hydroxycortisol distinguishable from zero concentration at the 95% confidence limit ranged between 2.0 to 3.0 pg/well (20–30 pg/ml) (Fig. 1).

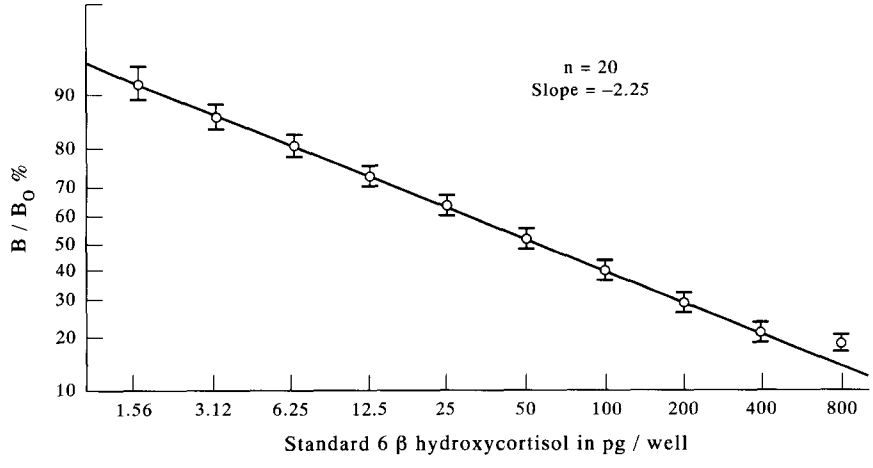

Fig. 1. Composite standard curve of ELISA for 6β-hydroxycortisol (mean ± SD).

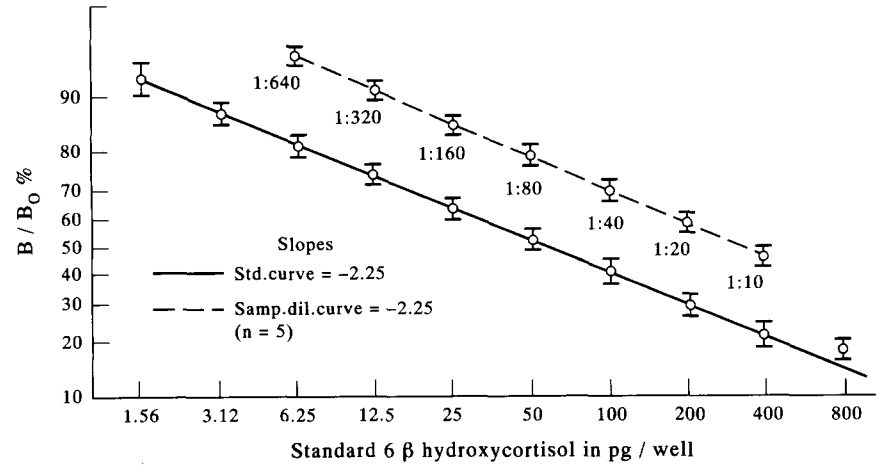

Fig. 2. Parallelism between the standard curve and sample dilution curve.

Specificity

The antiserum used in this assay system was highly specific. It showed 100% cross-reactivity with 6β-hydroxycortisol at 50% binding [5]. Urine samples ($n = 5$) of varying concentration were serially diluted (from 1:10 to 1:640) and analysed by the above mentioned ELISA technique. The dose–response line of each of the urine samples was found to be parallel to that of the standard 6β-hydroxycortisol (Fig. 2). However, the estimates obtained with the developed ELISA could not be correlated with RIA as radiolabelled analyte was not available (custom made and hence very expensive).

Accuracy

Accuracy of the ELISA method was validated by spiking 1.0 ml of infant's urine sample with varying quantities of 6β-hydroxycortisol (75–600 ng). These samples along with the original infant's sample were diluted 1:100 before being assayed. The recovery of the added 6β-hydroxycortisol ranged between 83.0 to 108.0%.

Precision

Three different pools low (A), medium (B) and high (C) were assayed several times in the same assay and in different assays to determine the intra- and inter-assay coefficient of variation (Table 1).

Table 1. Intra- and inter-assay coefficient of variation for the measurement of 6β-hydroxycortisol (6β-OHF) in 3 urine pools

Type of pool	Mean (ng/ml)	SD	Coefficient of variation%
	Intra-assay (n = 12)		
A	71.75	2.36	3.28
B	175.00	5.77	3.29
C	285.00	10.00	3.50
	Inter-assay (n = 20)		
A	70.75	6.36	8.98
B	163.12	16.79	10.29
C	285.00	21.21	7.44

DISCUSSION

Estimation of 6β-hydroxycortisol activity generally reported in the literature has been either by RIA or high performance liquid chromatography (HPLC). The former, besides requiring custom made radioisotope adding to the cost, also poses a major health hazard. The latter is a time consuming method requiring highly sophisticated instruments. The ELISA method on the other hand only requires a simple spectrophotometer for detecting the enzyme activity. Also the shelf life of enzyme labelled penicillinase is >2 years at 4°C. This method like the HPLC procedure, requires no prior extraction but only simple dilution of the urine sample. The ELISA method is also more sensitive than the other two methods. The sensitivity reported for the HPLC method was 40 ng [6]and 0.05–0.5 μg [7] and for the radioimmunoassay method it was 25 pg/ml [8] and 50 pg [9]. The sensitivity reported for the ELISA method was 10 pg [5, 10]. Hence the ELISA method of estimation of 6β-hydroxycortisol while eliminating the disadvantages of radioimmunoassay and HPLC offers a simple, cheap, rapid and sensitive method tailor made for routine use.

Hosoda *et al.* [5] and Zhiri *et al.* [10] estimated 6β-hydroxycortisol from adult urine by the ELISA method using β-galatosidase and horse radish peroxidase, respectively. Horse radish peroxidase substrate is photosensitive hence as well as carrying out the reaction in the dark, care has to be taken in handling these reagents. Measurement of β-galactosidase activity is a tedious and time consuming procedure. Sauer *et al.* [11] had reported improved assay sensitivity in the order of penicillinase > peroxidase > β-galactosidase. The advantages of using penicillinase in the assay system has been exhaustively documented by Khatkhatay *et al.* [12].

As the currently developed enzyme immunoassay for 6β-hydroxycortisol using enzyme penicillinase is simple, rapid, sensitive, economical and satisfies all the validatory criteria it appears to be an ideal method for routine use. Estimation of 6β-hydroxycortisol may be useful for monitoring the effects of hormones and drugs

[7, 13–15] acting as inducers of microsomal enzymes, in pathological conditions like Cushing syndrome [6] cancer [16] and effect of environmental agents, pesticides etc. [17].

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