J. steroid Biochem. Vol. 22, No. 5, pp. 673–676, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR PLASMA CORTISOL

J. G. LEWIS[1] and P. A. ELDER
Steroid Unit, Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

(*Received* 27 *March* 1984)

Summary—A direct ELISA for plasma cortisol is described which is carried out in a standard 96 well microtitre plate. In this ELISA cortisol-thyroglobulin conjugate is immobilised to the microtitre plate and competes with cortisol in the standard or plasma sample for antibody binding sites. Following washing the rabbit cortisol antibody bound to immobilised cortisol is incubated with peroxidase labelled goat antirabbit IgG. Following further washing *o*-phenylenediamine is added, colour developed, and the plate read at 492 nm on a standard ELISA plate reader. This ELISA shows good agreement with RIA and its sensitivity, specificity and precision allow its use in the routine steroid laboratory.

INTRODUCTION

The determination of plasma cortisol is of established clinical value and constitutes one of the most frequently requested steroid assays. For these reasons the development of an inexpensive, rapid, simple and reliable assay for plasma cortisol is highly desirable. Various radioimmunoassays (RIAs) and fluoroimmunoassays have been described for plasma cortisol [1–5] as well as enzyme immunoassays [6–9]. These methods all have the disadvantages of the synthesis of either radiolabelled- or enzyme labelled-cortisol tracers as well as centrifugation steps.

The present paper describes, to our knowledge, for the first time an inexpensive, simple, rapid and reliable ELISA for plasma cortisol. The method is potentially applicable for other steroid or steroid metabolite determinations.

EXPERIMENTAL

Cortisol (Sigma) standards were prepared in assay buffer by dilution from a stock solution of 2.759×10^{-3} mol/l (1 mg/ml) in ethanol. Cortisol-3-(*o*-carboxymethyl)oxime was synthesised [10] and its purity confirmed by thin-layer chromatography in toluene–methanol–acetone (1:1:1, by vol).

Preparation of cortisol-thyroglobulin conjugate

Cortisol-3-(*o*-carboxymethyl)oxime, 25 mg, was solubilised in 1 ml dimethylsulphoxide (DMSO, (Fisons AR grade); 1,4 dioxane, Baker AR grade, (1:1, v/v) followed by 1 ml of water and combined with bovine thyroglobulin (Sigma) solution (25 mg in 1 ml of water followed by 1 ml DMSO/dioxane). *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodimide hydrochloride (Sigma), 100 mg, was dissolved in 2 ml of water, and slowly added to the cortisol-3-oxime thyroglobulin mixture at pH 5–6. The reaction mixture was stirred overnight at 4°C, dialysed, using visking dialysis membrane, against phosphate buffered saline (PBS) containing 0.1% sodium azide (w/v) and stored as a stock solution at 4°C with a final thyroglobulin concentration of 3.73×10^{-6} mol/l (2.5 mg/ml). The amount of cortisol coupled to thyroglobulin was determined by RIA, following pronase digestion of the conjugate, and was found to be between 1200 and 1400 moles of cortisol per mole of thyroglobulin. Briefly, 100 μl of pronase (Sigma) solution in PBS (1 μg/ml) was added to 10 μl of conjugate stock solution and digested for 24 h at 37°C. Digestion was terminated by heating, 70°C for 20 min, and samples diluted 1:200 and 1:1000 with PBS for RIA determinations. Digestion of the conjugate was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate by the absence of high molecular weight bands compared to the undigested control. Based on the amino acid composition of bovine thyroglobulin the theoretical coupling would be 2000 moles cortisol per mole of thyroglobulin. This determined the molar starting ratio for the coupling reaction.

Buffers

PBS containing 0.05% Tween 20 (v/v), pH 7.4 was used for washing the microtitre plates, PBS containing 0.05% Tween 20 (v/v) and 0.1% gelatin (w/v) was used as the assay buffer.

Antisera

Lyophilised rabbit antiserum to cortisol was purchased from Diagnostic Products Corporation (lot COD 1031) and reconstituted in 10 ml distilled water containing 0.05% Tween 20 (v/v). Affinity purified peroxidase labelled goat antiserum to rabbit IgG was obtained from Tago Inc., U.S.A., and used at 1:6000 dilution in the assay buffer.

Cortisol-free plasma was obtained from patients with undetectable cortisol levels, as measured by RIA, following dexamethasone suppression.

[1]To whom correspondence should be addressed.

Enzyme substrate solution was freshly prepared by dissolving 40 mg of *o*-phenylenediamine (Sigma) in 100 ml of 50 mM Na_2HPO_4, 25 mM citric acid buffer, pH 5.0. Immediately prior to use, 60 μl of H_2O_2 (Fisons, AR) was added.

Coating of cortisol-thyroglobulin conjugate to ELISA plates

ELISA plates (Falcon 3912 Microtest III, Becton Dickinson, U.S.A.) were coated by the addition of 200 μl of conjugate solution/well and the plate left overnight at 4°C. Conjugate solution was prepared by the addition of stock cortisol-thyroglobulin (4 μl) to 20 ml aqueous 6 M guanidine hydrochloride. Unadsorbed conjugate was removed by three washes. Following washing any remaining binding sites were blocked by the addition of assay buffer (200 μl) for 2 h at 20°C. Six M guanidine hydrochloride was used to improve antigen coating efficiency, presumably by preventing aggregation [11].

ELISA procedure

Following blocking the plates were shaken dry and 25 μl of cortisol standard or assay buffer dispensed into each well. Cortisol free plasma (25 μl) was then dispensed into wells containing standard and 25 μl of sample plasma dispensed into the wells containing assay buffer. Cortisol antibody, 50 μl, was immediately dispensed into all wells and the plate incubated for 2 h at 20°C. Following three washes, the plates were shaken dry and 100 μl of peroxidase-labelled goat antirabbit IgG added to each well for a further 2 h incubation at 20°C. Plates were again washed three times and shaken dry prior to the addition of enzyme substrate solution (100 μl). After 10 min at 20°C the colour reaction was terminated by the addition of 2.5 M H_2SO_4 (50 μl) and the absorbance read on a standard ELISA plate reader at 492 nm (Titertek Multiskan, Finland). Standards and plasma samples were measured in duplicate.

Cross reactivity

Cross reactivity studies were performed in a similar manner except assay buffer (25 μl) was used instead of cortisol-free plasma. Each steroid was tested at five different doses from 0.5 to 5000 ng.

Heat treatment of plasma

To ascertain whether cortisol binding globulin (CBG) interferes in the ELISA, plasma samples were divided in two, one aliquot received no heat treatment and the other heated at 60°C for 20 min. After cooling the samples were assayed in parallel.

Recovery

Recovery was carried out using normal plasma samples (25 μl) spiked with either 35, 8.75, 3.5, 0.88, 0.35 or 0 ng of cortisol. Recovery was calculated by expressing the net cortisol determined by ELISA as a percentage of added cortisol.

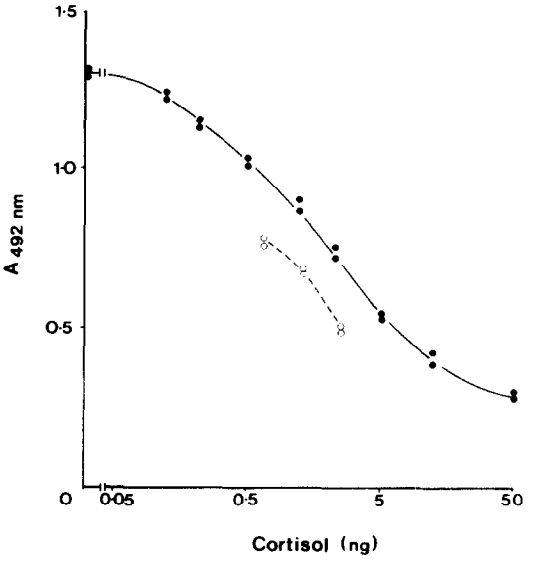

Fig. 1. Standard curve for plasma cortisol ELISA. A plasma sample serially diluted in cortisol-free plasma is also shown ○---○.

Radioimmunoassay

Plasma cortisol was measured using the ^{125}I double antibody radioimmuniassay kit obtained from the Diagnostic Products Corporation, U.S.A.

RESULTS

A typical standard curve prepared in cortisol-free plasma together with dilutions of a plasma sample is shown in Fig. 1.

The specificity of the antibody is shown in Table 1. Cross-reactivity was determined as the amount of steroid, relative to cortisol, to cause a 50% reduction in the absorbance change at 492 nm over the range 0–5000 ng of cortisol.

Comparison of plasma cortisol levels of random samples analysed by ELISA and RIA are shown in Fig. 2. Cortisol determinations by ELISA in heat treated and non-heat treated plasma samples

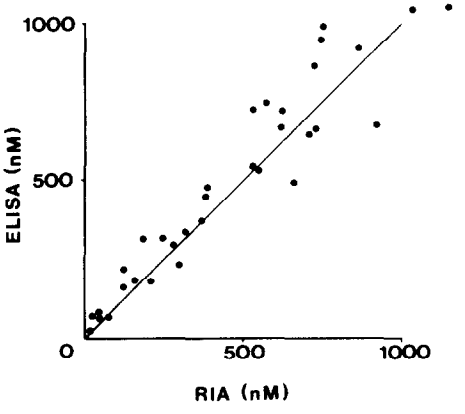

Fig. 2. Comparison between cortisol levels in normal or pathological plasma specimens determined by ELISA and by RIA.

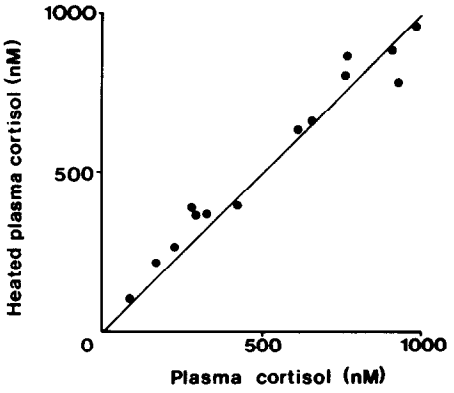

Fig. 3. Comparison of cortisol levels as measured by ELISA between heated and non-heated normal or pathological plasma samples.

are shown in Fig. 3. Recovery results are shown in Table 2.

The normal adult plasma 8 a.m. cortisol range was determined by both ELISA and RIA and found to be 300–850 and 300–770 nmol/l respectively ($n = 20$). Plasma cortisol determinations by both ELISA and RIA were compared before and 1 h following synacthen (0.25 mg, i.m.). The results for 14 consecutive patients are shown in Table 3. Plasma 4 p.m. cortisol levels were determined by both ELISA and RIA 16 h after dexamethasone (1 mg) administration. The results for 14 consecutive patients are shown in Table 4.

The precision was assessed using 3 pools of sample plasma and each was measured 6 times in duplicate and gave mean results of 144, 251 and 587 nmol/l with within-assay variation of 9.3, 10.1 and 10.2% respectively. Between-assay variation for each of the 3 pools did not exceed 10.4%. Precision was further assessed using lyphochek immunoassay control serum levels I, II and III (BioRad). Level I always gave cortisol values <28 nmol/l; level II within-assay variation 469 ± 30 ($n = 5$), between-assay variation 424 ± 21 ($n = 5$) and level III within-assay variation 1650 ± 248 ($n = 5$), between-assay variation 1645 ± 187 ($n = 5$). Values are nmol/l, mean ± 1 standard deviation and within those determined by other methods. The smallest amount of unlabelled cortisol which differs from zero by 2 standard deviations was less than 0.25 ng which corresponds to less than 28 nmol/l.

Table 1. Cross reactivity of anti-cortisol serum

Compound	Cross reaction %
Cortisol	100
11-Deoxycortisol	2.2
21-Deoxycortisol	6.5
6β-Hydroxycortisone	6.5
Corticosterone	10.8
11-Dehydrocorticosterone	0.06
17α-Hydroxyprogesterone	0.03
Testosterone	<0.006
Estradiol	<0.006
Predisone	0.9
Prednisolone	40
Dexamethasone	0.01

Table 2. Recovery from normal plasma

Amount added (ng/25 μl plasma)	Amount assayed by ELISA (ng/25 μl plasma)	% Recovered
0	5.0	100
0.35	5.4	114
0.88	6.0	114
3.5	8.5	100
8.75	14.0	91
37.5	40	93

Table 3. Plasma cortisol levels (nmol/l) before and 1 h following synacthen determined by RIA and ELISA

	RIA		ELISA	
Patient	Pre-	Post synacthen	Pre-	Post synacthen
A.I.	116	315	220	341
J.W.	525	1039	550	1045
M.N.	617	1142	715	1045
M.W.	122	375	176	374
A.B.	276	630	297	660
S.C.	591	853	484	660
E.R.	671	924	660	1100
N.S.	18	63	46	198
P.M.	392	522	418	572
V.O.	194	540	198	418
R.S.	496	745	462	880
F.W.	342	600	352	550
N.R.	488	844	484	737
M.C.	274	617	407	990

DISCUSSION

Although enzyme immunoassays for cortisol have been described, all require long incubation times, centrifugation as well as the synthesis of enzyme-cortisol conjugates [6–9]. Relatively few ELISA methods have been described for measuring steroids or steroid metabolites [12] and to our knowledge, this is the first time a plasma cortisol ELISA has been reported. The reliability criteria are comparable to other methods of cortisol determination [5], and the assay has been validated. It is specific, shows good recovery from spiked plasma and exhibits parallelism. This plasma cortisol ELISA agrees well with radioimmunoassay determinations, not only on random samples, Fig. 2, but also before and after synacthen administration (Table 3), as well as with patients given dexamethasone (Table 4).

Table 4. Plasma cortisol levels (nmol/l) following dexamethasone (1 mg) determined by RIA and ELISA

Patient	RIA	ELISA
N.E.	12	<28
C.H.	326	242
S.J.	28	77
C.C.	18	<28
J.H.	404	308
A.C.	33	<28
A.M.	11	<28
M.W.	50	30
S.D.	23	<28
J.C.	99	<28
M.B.	77	83
B.M.	20	<28
A.W.	9	<28
H.I.	37	<28

As 80% of cortisol in plasma is bound to CBG or transcortin [13], it was necessary to heat denature CBG in plasma samples, 60°C for 20 min [2] and compare with the non-heat treated plasma samples. The results demonstrate that CBG does not interefere in this ELISA and therefore heat treatment of plasma samples is unnecessary.

One feature of the ELISA is that the cortisol-thyroglobulin conjugate is immobilised to the plate rather than the antibody, and hence, there is no requirement for the synthesis of cortisol-enzyme conjugates. In addition, the second antibody, peroxidase-labelled goat antirabbit IgG, could be used in other steroid or steroid metabolite ELISAs providing the first antibody is of rabbit origin. These could be seen as advantages in using an immobilised antigen system. With the recent availability of high amplification systems such as the biotin–antibody, avidin and biotin–enzyme conjugates, the determination of lower levels of other steroid hormones using immobilised antigen should be possible.

Acknowledgements—We thank Mrs Bria Steer for RIA cortisol determinations and Dr C. M. Andre for helpful discussion.

REFERENCES

1. Connolly T. M. and Vecsei P.: Simple radioimmunoassay of cortisol in diluted samples of human plasma. *Clin. Chem.* **24** (1978) 1468–1472.
2. Ellis G. and Morris R.: A one-tube micromethod for radioimmunoassay of plasma cortisol. *Clin. Chem.* **24** (1978) 1954–1957.
3. Riad-Fahmy D., Read G. F., Gaskell S. J., Dyas J. and Hindawi R.: A simple, direct radioimmunoassay for plasma cortisol, featuring a ^{125}I radioligand and a solid-phase separation technique. *Clin. Chem.* **25** (1979) 665–668.
4. Wong P. Y., Mee A. V. and Ho F. F. K.: A direct radioimmunoassay of serum cortisol with in-house ^{125}I-tracer and preconjugated double antibody. *Clin. Chem.* **25** (1979) 914–917.
5. Al-Ansari A. A. K., Smith D. S. and Landon J.: Polarisation fluoroimmunoassay of serum and salivary cortisol. *J. steroid Biochem.* **19** (1983) 1475–1480.
6. Comoglio S. and Celada F.: An immuno-enzymatic assay of cortisol using *E. coli* β-galactosidase as label. *J. immunol. Meth.* **10** (1976) 161–170.
7. Kobayashi Y., Ogihara T., Amitani K., Watanabe F., Kiguchi T., Ninomiya I. and Kumahara Y.: Enzyme immunoassay for cortisol in serum using cortisol 21-amine. *Steroids* **32** (1978) 137–144.
8. Hindawi R. K., Gaskell S. J., Read G. F. and Riad-Fahmy D.: A simple direct solid-phase enzymeimmunoassay for cortisol in plasma. *Ann. clin. Biochem.* **17** (1980) 53–59.
9. Nakao T., Tamamura F., Tsunoda N. and Kawata K.: Double antibody enzyme immunoassay of cortisol in bovine plasma. *Steroids* **38** (1981) 111–120.
10. Janoski A. H., Shulman F. C. and Wright G. E.: Selective 3-(*o*-carboxymethyl)oxime formation in steroidal 3,20-diones for hapten specificity. *Steroids* **23** (1974) 49–64.
11. Wieslander J., Bygren P. and Heingard D.: Isolation of the specific glomerular basement membrane antigen involved in Goodpasture Syndrome. *Proc. natn. Acad. Sci., U.S.A.* **81** (1984) 1544–1548.
12. Shah H. P. and Joshi U. M.: A simple, rapid and reliable enzyme-linked immunosorbent assay (ELISA) for measuring estrone-3-glucuronide in urine. *J. steroid Biochem.* **16** (1982) 283–286.
13. Brien T. G.: Free cortisol in human plasma. *Horm. metab. Res.* **12** (1980) 643–650.