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SHORT COMMUNICATION

ENZYMIC FLUOROMETRIC METHOD FOR THE DETERMINATION OF CORTISOL IN PLASMA

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SUMMARY

A simple enzymic fluorometric method for plasma cortisol determination has been developed. After a rapid Sephadex LH-20 column chromatography cortisol is measured with 3α,20β-hydroxysteroid dehydrogenase using NaOH-induced fluorescence for NAD^+ determination. Cortisol in 100 μl of plasma can be measured (sensitivity approx. 0·020 μmol/l) and when compared with the fluorometric method of Clark the results were very similar, the correlation coefficient being 0·92.

Three main principles have been used in the determination of plasma cortisol: Photometric measurement after the Porter–Silber reaction, fluorometric measurement in conc. H_2SO_4 and protein binding assay. Pirke and Stamm [1, 2] have compared the existing methods in these three main categories and concluded that the specificity and precision of the fluorometric methods of Clark *et al.* [3] and Graef and Staudinger [4] are comparable with that of the protein binding method [5], but their sensitivity is much lower (1 ml of plasma is needed). Fluorometric methods are easily disturbed by non-specific fluorescence from, e.g. various drugs; this drawback does not affect the protein binding method. We have used a modified Clark *et al.*'s procedure in our routine analyses and we feel that the only drawbacks of this method are that it is time-consuming (10 duplicates in 5 h) and produces a lot of glass-ware to be acid washed. For the protein binding method a liquid scintillation counter is necessary and therefore this method is not suitable for small laboratories.

We have developed an enzymic fluorometric method for plasma cortisol, which would be useful for routine analyses in most laboratories equipped with a simple filter fluorometer. Heparinized plasma, 0·5 ml (0·1 ml is enough for a single determination), is extracted twice with 1·5 ml of dichloromethane, the extract is evaporated to dryness in a 5 ml tube and the dry residue is dissolved in approx. 100 μl of petroleum ether (b.p. 50–70°C):chloroform (1:3, v/v). The extract is transferred to a 5 × 0·5 cm Sephadex LH-20 column (Pasteur pipette) and the tube is washed twice with the same volume (100 μl) of the solvent which is transferred to the column. The column is eluted with petroleum ether: chloroform (1:3, v/v), the first 6·5 ml is discarded and the next 6·5 ml is collected in a graduated tube. This fraction is evaporated to dryness and the residue is dissolved in 250 μl of the eluting solvent. Two 100-μl aliquots are transferred to separate 3-ml Pyrex tubes for duplicate assay. After evaporation of the solvent 5 μl of ethanol is added to dissolve the dry cortisol extract and subsequently, 100 μl of a reagent containing 0·1 mol/l K-phosphate buffer, pH 6·7, 0·2 mmol/l dithiothreitol, 0·01 mmol/l NADH (5 mmol/l stock solution of NADH in 0·1 mol/l carbonate buffer, pH 10·6 is heated for 10 min at 60°C to destroy contaminating NAD^+) and 25 μg/ml 3α,20β-hydroxysteroid dehydrogenase (Boehringer, Mannheim, Germany). The tubes are incubated at room temperature for 30 min and then 10 μl of 5 mol/l HCl is added to destroy NADH [6]. After a few min 1 ml of 6·6 mol/l NaOH is added from an automatic syringe in dim light and heated for 10 min at 60°C. Under these conditions an intensely fluorescent compound of NAD^+ is formed [7]. The tubes are wiped and the fluorescence measured, e.g. with a Farrand model A-4 Fluorometer (the primary filter Corning No. 5840 and the secondary filter a combination of Corning No. 4303 and 3387).

The standard curve and the curve obtained after adding various amounts of cortisol to plasma is shown in Fig. 1. Recoveries from plasma were reasonably constant, the mean value being 75·5 ± 6·3% (S.D.M.). Usually two standards dissolved in water were taken through the whole procedure to correct the values for procedural losses. We compared the plasma cortisol values obtained with this method with those obtained using the procedure of Clark *et al.*, in 18 patients. Values of 0·36 ± 0·05 μmol/l (mean ± S.E.M.) were obtained with the method described and 0·36 ± 0·06 μmol/l with the method of Clark *et al.* Regression analysis gave the formula $y = 1{\cdot}02\ x - 0{\cdot}01$ where y is the method of Clark *et al.* and x the method described. The correlation coefficient, r, was 0·92. The precision of the whole method was calculated from routine duplicate plasma analyses and was found to be ±8% (coefficient of variation). The sensitivity is approx. 0·020 μmol/l.

The chromatographic purification of cortisol is necessary because there are several other steroids in plasma which react with 3α,20β-hydroxysteroid dehydrogenase, e.g. progesterone, 17α-hydroxy-progesterone and 11-deoxycortisol. These are all eluted in the first 6·5 ml. All solvents used were redistilled using all-glass apparatus and fractionating

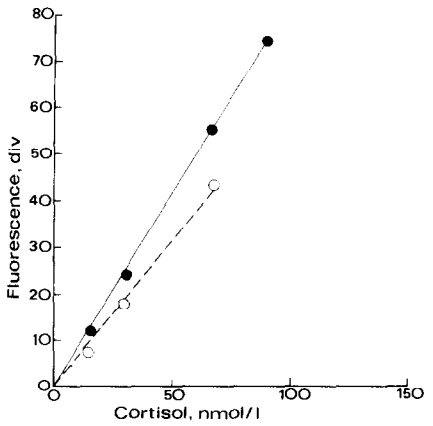

Fig. 1. Fluorescence curve for set of cortisol standards (●—●) and recovery curve for plasma with cortisol standards (○—○).

columns. The Sephadex LH-20 was pre-washed several times with the solvent used for elution. One technician can carry out 30 determinations (with duplicate enzyme assays) in 6 h and only four tubes per sample need be washed.

Rick [8] has also described an enzymic method for the determination of 20-ketosteroids in plasma by means of NAD^+ fluorescence in strong alkaline solution. However, it is obvious that our method which includes a chromatographic step and treatment of the alkaline NADH solution by heat represents a considerable refinement of the latter procedure and is more sensitive and specific for cortisol measurement.

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