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THE DETERMINATION OF CORTISOL IN HUMAN PLASMA: EVALUATION AND COMPARISON OF SEVEN ASSAYS

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Summary

In a comparative study, seven different methods for the determination of cortisol in human plasma were evaluated, using routine patient samples. Four of these methods used radioactive steroids (^{125}I- or ^{3}H-labelled) and in three no radioactivity was needed. For the statistical evaluation a direct ^{3}H-radioimmunoassay was arbitrarily taken as the independent variable. It was found that all other methods correlated well with this assay. However, the simplest method, the fluorimetric, cannot be recommended mainly because of its non-specific fluorescence and troublesome interference from some widely-used drugs. Of the methods evaluated a radioimmunoassay is recommended. For laboratories having no equipment for measurements of radioactivity, the more elaborate and time-consuming fluorometric method of Clark may represent a good alternative.

Introduction

A variety of methods for the estimation of cortisol concentrations in human plasma has been described, in which fluorometry, colorimetry, double isotope derivatisation, competitive protein binding and radioimmunological techniques

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have been used, but only a limited number of comparative studies are available [1–6].

In this paper we will describe a comparison of seven different methods, three of which do not rely on the use of radioactivity while four are using radioactive steroids.

The methods compared were:

(1) fluorometric method, as recommended by a working party of the British Medical Research Council [7,8];

(2) modified, more specific fluorometric method, after Clark [9,10];

(3) high performance liquid chromatographic method [11].

(4) competitive protein binding technique, as described by de Jong et al. [12], with ^{3}H-corticosterone as tracer;

(5) solid phase radioimmunoassay [13,14], with ^{3}H cortisol as tracer;

(6) radioimmunoassay using ^{125}I-cortisol; and

(7) direct radioimmunoassay in diluted plasma, according to the method described by Foster and Dunn [15], using ^{3}H-cortisol as tracer.

The latter two methods will be described in more detail, as they have not been published before.

Materials and methods

Reagents

All chemicals were of reagent grade and all organic solvents were redistilled before use. ^{3}H-Cortisol was obtained from the Radiochemical Centre (Amersham, U.K.), specific activity 50 Ci/mmol; ^{125}I-tyrosine-methyl-ether of cortisol was purchased from CEA (Gif-sur-Yvette, France) at a specific activity of about 1000 Ci/mmol.

Radioactivity counting

Tritium was counted after mixing of 1.2 ml of aqueous sample with 10 ml of emulsifier scintillator (special MI-96, Packard Instruments, Brussels, Belgium) in a liquid scintillation counter for at least 10 000 counts. ^{125}I was measured using a Baird Atomic scintillation counter.

Antiserum

The anti-cortisol serum used for Method 6 and 7 was raised in rabbits against a cortisol-21-hemisuccinate-bovine serum albumin conjugate.

During the last three months of the immunisation procedure, which lasted approximately six months, the animals received a daily amount of about 10 μg of dexamethasone per kg body weight in their drinking water, in order to reduce the endogenous production of cortisol which could partially occupy the antibodies raised.

Serum obtained from rabbit number 7438 was finally used for the assays. It showed the following cross-reactions, calculated at 50% of the initial binding:

(1) cortisol	100%
(2) 21-deoxy-cortisol	62%
(3) corticosterone	11%
(4) prednisolone	3%

(5) cortisone	2%
(6) 11-deoxy-cortisol	1.3%
(7) dexamethasone	0.3%
(8) deoxycorticosterone	1.3%
(9) 17-α-OH-progesterone	0.1%

Plasma samples

For the comparison of the different assays, plasma samples obtained during clinical investigations of patients were used. No selection has been made during this procedure, random samples being taken for the tests. After collection and in the time between assays the samples were stored at −20°C; they were transported frozen to the participating laboratories.

Basically the comparative study consisted of 25 samples distributed to all the participating laboratories. These samples were chosen only by their cortisol concentration as measured by Method 7; the range was 0.03—1.41 μmol/l, mean value 0.463 μmol/l. In addition varying amounts of samples were distributed, as indicated by the numbers in Table I. The fluorometric method (No. 1) and Method 7 were both used during the three months in which the random clinical samples were collected. At the time of analysis no information was available about any medication taken by the patients.

Of the methods compared, two have not yet been described in detail, e.g. Methods 6 and 7.

Method 6

This method was performed in a similar way to Method 7 with the following modifications:

(a) the antiserum was diluted 40 000 times; and

(b) ^{125}I-tyrosine-methyl-ester of cortisol was used in an amount giving 30 000 dpm.

Method 7

Following a number of preliminary tests, the final procedure used was comparable to the one described by Foster and Dunn [15]. Briefly, it consists of diluting plasma 50 times with distilled water, and then 25 μl of the diluted plasma is heated for 10 min at 70°C to inactivate the transcortin; 100 μl of the antiserum diluted 7500 times with 0.01 mol/l phosphate buffered saline at pH 7.4 with 0.1 g/l merthiolate and 100 μl 0.01 mol/l phosphate buffered saline with 0.2 g/l bovine serum albumin (demineralized, Organon, Oss, The Netherlands) containing 15 000 dpm ^{3}H-cortisol are added. After mixing, incubation is carried out for 16 h at 4°C or for 2 h at room temperature. Separation of the free and bound compound is carried out for 15 min with 1.0 ml of dextran-coated charcoal suspension (0.1% norit, 0.05% Dextran-T70). After centrifugation, the supernatant is counted. The standard curve is obtained from cortisol dissolved in cortisol-free plasma, collected after dexamethasone suppression of normal individuals, and checked to confirm cortisol levels were very low (<0.005 μmol/l).

TABLE I

CORRELATION BETWEEN THE VARIOUS METHODS

Method	No. of estimations	Slope (± S.D.) of the regression line	Intercept ± S.D. (μmol/l)	Correlation coefficient	Mean standard deviation per point (μmol/l)	Mean cortisol level of samples as measured by Method 7 mean (range) (μmol/l)
1	288 *	0.98 ± 0.032	0.171 ± 0.018	0.90	0.174	0.400 (0.01—2.51)
2	25	0.95 ± 0.033	0.013 ± 0.012	0.99	0.039	0.293 (0.03—0.80)
3	58	1.05 ± 0.055	0.102 ± 0.023	0.93	0.088	0.463 (0.03—1.41)
4	39	0.80 ± 0.034	0.065 ± 0.022	0.97	0.081	0.508 (0.03—1.41)
5	24	1.10 ± 0.057	0.060 ± 0.034	0.97	0.108	0.457 (0.03—0.80)
6	69	1.13 ± 0.032	0.012 ± 0.016	0.97	0.102	0.309 (0.03—1.41)

Arbitrarily the direct ^{3}H-radioimmunoassay (Method 7 [15]) has been taken as the independent variable.
* Three values have not been included in the calculations (see Fig. 1).
Method 1: fluorometric [7,8].
Method 2: modified fluorometric [9,10].
Method 3: high performance liquid chromatography [11].
Method 4: competitive protein binding [12].
Method 5: solid phase radioimmunoassay [13,14].
Method 6: ^{125}I-radioimmunoassay.

Statistical analysis

From the individual points the regression lines were calculated by a least square method. The fitting function was composed of a constant and a first order term. Furthermore, the mean distance from each individual point to the fitted regression line was calculated (mean standard deviation per point), as were the correlation coefficient and the standard deviation of the fitting coefficients.

Results

I. Evaluation of Method 7

In a series of preliminary experiments, the validity of Method 7 was verified by:

(a) Comparison of the results with those obtained after extraction of the cortisol from the plasma. After evaporation of an ether extract of the plasma, the residue was dissolved in an 0.25% albumin solution and read against a standard curve made up in the same protein solution. For 25 plasma samples the correlation coefficient between the two procedures was 0.99. The slope of the regression line was 0.971 ± 0.022, which is not significantly different from unity. The intercept was 0.03 ± 0.01 μmol/l. The mean cortisol level with the direct assay was 0.57 ± 0.45 μmol/l, and after extraction a level of 0.57 ± 0.49 μmol/l was found.

(b) In 19 plasma samples, the effect of different dilutions of the plasma was studied. In all samples a dilution curve parallel to the standard curve was obtained, confirming the identity of the measured substance was cortisol. The correlation coefficient between the 25 times dilution and the 50 times was 0.98.

(c) Addition of cortisol to plasmas with very low cortisol levels and measurement of the recovered cortisol. At all concentrations tested (0.06—5.5 μmol/l = 10—1000 pg/tube) the recovery was quantitative within a 10% variation.

(d) During routine use over a period of six months, the interassay variation was calculated for three sera, which were analysed 25 times by four technicians. The results were:
control serum 1: 0.47 ± 0.04 μmol/l; 7.5% coefficient of variation;
control serum 2: 0.74 ± 0.05 μmol/l; 7.0% coefficient of variation;
control serum 3: 1.05 ± 0.07 μmol/l: 6.3% coefficient of variation.

Method 7 has been in routine use now for a period of more than two years. With this assay the following normal values for cortisol have been obtained:
cortisol level in plasma in the morning between 8 and 9 h:
0.39 ± 0.14 μmol/l (95% range of values 0.18—0.66 μmol/l; n = 150)
in the afternoon between 16 and 17 h:
0.23 ± 0.09 μmol/l (95% range of values 0.08—0.36 μmol/l; n = 32).

After suppression of the cortisol production with 1 mg dexamethasone orally at 22—23 h the previous night, a morning level of <0.08 μmol/l was found (n = 109). Stimulation with 0.25 mg of synthetic tetracosactide intramuscularly (Synacthen ® or Cortrosyn ®) resulted in an increase of the cortisol level to 0.47 ± 0.23 μmol/l (95% range of values 0.19—0.97 μmol/l; n = 50) one hour later. Stimulation with 1 mg of the depot-preparation of the tetracosactide

intramuscularly gave an increase of 0.99 ± 0.25 μmol/l (95% range of values 0.69—1.52 μmol/l; n = 27) after eight hours.

II. Comparison of assays

In the comparative study, using routinely obtained samples from patients, no absolute method was available to measure the true cortisol content of each sample. Because the direct ^{3}H-radioimmunoassay (Method 7) has been compared with the other assays using a varying series of plasma samples, arbitrarily the values obtained with Method 7 were chosen as the "independent variable" in the statistical evaluation.

In Table I the results of the comparative studies are shown. In this table the number of plasma samples assayed by the two techniques, the correlation coefficient, the intercept (± S.D.) and the slope (± S.D.) are given, as compared to Method 7. In addition, the calculated mean standard deviation per point is given together with the mean cortisol level of the samples tested as measured by Method 7.

The figures given in the table indicate a fairly good correlation between the results obtained with the commonly used fluorometric method (*Method 1*) and Method 7. The slope of the calculated regression line is not significantly different from unity. However, even though a relatively large number of samples was assayed, the estimated mean standard deviation per point is large. In addition, there is a very large intercept of 0.17 ± 0.02 μmol/l of the ordinate. This intercept emphasises the problems encountered with the fluorometric method in which, apart from the fluorescence given by other steroids, e.g. corticosterone, interference by many widely-used pharmaceutical preparations may be serious. The extent of the problem is illustrated in Fig. 1 with the cortisol values obtained after suppression by dexamethasone, as estimated by Method 1 and Method 7.

In three cases (shown as ↑ in Fig. 1) the interference is evident from the much higher levels given by fluorometry, but in a number of cases the interference is fairly large, yet the disturbance cannot easily be judged from the values obtained. At the time of analysis no information was available as to whether potentially interfering drugs were being taken by some of the patients. Interference in the fluorometric estimation is frequently caused by the commonly used tranquillizers derived from diazepam. The properties of these substances are such that under the conditions used for cortisol assay they show a clear fluorescence [16].

The more specific and more time-consuming fluorometric method described by Clark (*Method 2*) shows a more satisfactory correlation and a good regression with the ^{3}H-RIA. The slope and intercept are not significantly different from unity and zero, respectively.

With the *third* method, a liquid chromatographic method, the slope of the regression line is not different from 1.0. The calculated intercept is puzzling in view of the separation ability of the chromatographic step and the selective UV detection method employed. The correlation coefficient with the ^{3}H-RIA was satisfactory, lying between the correlations seen with Methods 1 and 2.

The results with a competitive protein binding method (*Method 4*) give a correlation coefficient almost equal to those found for *Methods 5 and 6*, both

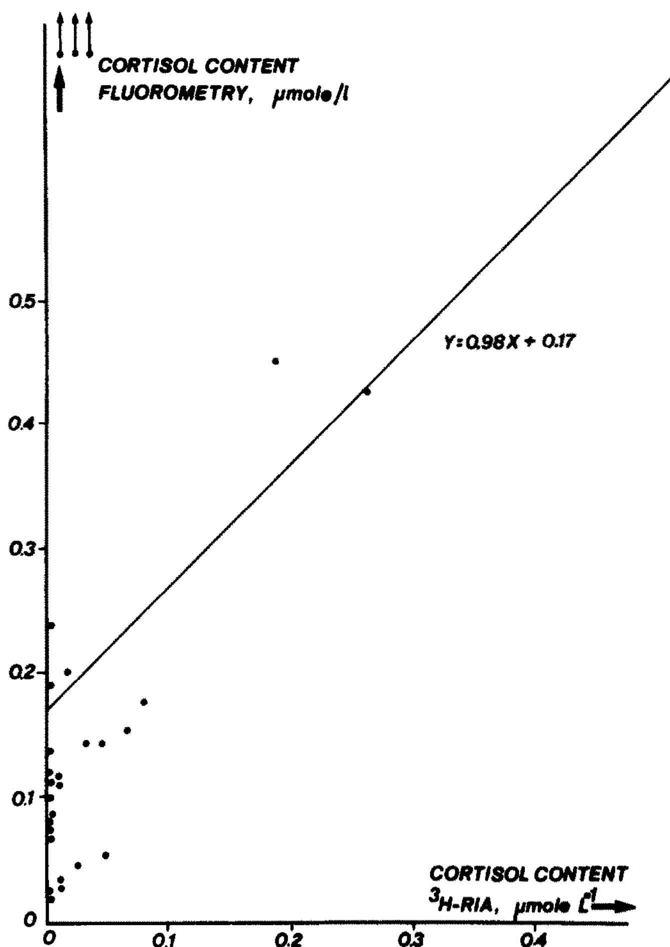

Fig. 1. The concentration of cortisol in plasma as measured by fluorometry vs. measurement by ^{3}H-radioimmunoassay. The plasma samples were obtained from patients after suppression of the cortisol secretion with dexamethasone. The regression line is drawn from the data of Table I. Three points, indicated with ↑ in the figure have not been used for the calculations.

the slope and the intercept of the regression line being significantly different from unity and zero, respectively. Again, these results are different from those expected, for, with the protein used for the estimation, more cross-reacting steroids may be present in plasma samples obtained from patients. The results of the two other radioimmunoassays, which were investigated, agree well with those of ^{3}H-RIA. Intercepts were not significantly different from zero. With the ^{125}I-cortisol estimation, a slope, slightly different from unity was found.

Discussion

One of the purposes of this study was to evaluate the practicability of various methods which can be used for the estimation of cortisol in human plasma. The first practical decision that must be made in the laboratory is whether radio-

active labels may or may not be used. If one can use radioactive tracers, the choice is obvious.

Performing a radioimmunoassay offers several advantages, i.e. small plasma sample required, simplicity and usually high specificity, depending on the antiserum used. Although the competitive protein binding is a good alternative, the larger variability of this method and lower specificity are disadvantages. Making a choice between radioimmunoassays based on the use of ^{3}H-cortisol or of ^{125}I-methyl-tyrosine-cortisol depends on: (a) availability of equipment for the measurement of radioactivity; (b) ^{125}I-cortisol cannot be prepared easily.

Therefore commercially available tracer usually is used. At least in Europe, the quality of this tracer did show large variations, furthermore its stability is low, partly due to the relatively short half-life of the ^{125}I.

For laboratories which are not planning to use radioactive tracers for the estimation of cortisol, the results of three different methods are offered. The widely used relatively simple fluorometric method can hardly be recommended because: (a) the need to use aggressive chemicals; (b) moderate specificity towards cortisol; (c) non-specific fluorescence due to plasma components; and (d) potential interference by several drugs in the assay. The latter especially can lead to highly misleading results. The more specific fluorometric method of Clark also has two disadvantages: the use of aggressive chemicals and the time-consuming character of the analysis. The liquid chromatographic method offers an estimation for only those laboratories with the technical facilities to perform this type of chromatography. Its separating power gives the method special value, and, in addition, it offers the possibility of assaying several compounds (e.g. prednisolone) during the same analysis.

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