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Determination of Cortisol in Human Plasma by Isotope Dilution-Mass Spectrometry

Definitive Methods in Clinical Chemistry, I

By L. Siekmann and H. Breuer † ¹⁾

Institut für Klinische Biochemie, Universität Bonn

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Summary: A definitive method is described for the determination of cortisol in human plasma. The method is based on the principle of isotope dilution-mass spectrometry.

The analytical procedure comprises the following steps:

- (1) Addition of [$4\text{-}^{14}\text{C}$]cortisol to the plasma sample;
- (2) extraction of the ^{14}C -labelled and of the non-labelled cortisol by dichloromethane;
- (3) purification of the cortisol fraction by column chromatography on Sephadex LH-20;
- (4) formation of the trimethylsilyl ether of cortisol methoxime;
- (5) selected ion monitoring at m/z -values 605, 607, 636 and 638 during gas liquid chromatography.

The accuracy of the method is based on the high specificity of mass spectrometry and on the exact control of recovery employing the principle of isotope dilution. The precision of the method was evaluated by calculation of the coefficient of variation from day to day; this varied from 0.7–2.3% in the range of 256–845 nmol/l. The lower limit of detection (ratio of signal to noise 3:1) is 200 pg cortisol per sample.

Bestimmung von Cortisol im menschlichen Plasma mit Hilfe der massenspektrometrischen Isotopenverdünnungsanalyse

Definitive Methoden in der Klinischen Chemie, I

Zusammenfassung: Es wird eine definitive Methode zur Bestimmung von Cortisol im menschlichen Plasma beschrieben, die auf dem Prinzip der massenspektrometrischen Isotopenverdünnungsanalyse beruht.

Das analytische Verfahren umfaßt folgende Schritte:

- (1) Zugabe von [$4\text{-}^{14}\text{C}$]Cortisol zu der Plasmaprobe;
- (2) Extraktion des ^{14}C -markierten und des nachzuweisenden nicht-markierten Cortisols mit Dichlormethan;
- (3) Reinigung der Cortisol-Fraktion durch Säulenchromatographie an Sephadex LH-20;
- (4) Bildung der Trimethylsilylether des Cortisol-methoxims;
- (5) massenspektrometrische Registrierung ausgewählter Ionen bei den m/z -Werten 605, 607, 636 und 638 während der Gaschromatographie.

Die Richtigkeit der Methode beruht zum einen auf der hohen Spezifität des massenspektrometrischen Nachweises, zum anderen auf der Tatsache, daß Substanzverluste während des Analysenganges durch die Anwendung des Isotopen-Verdünnungsprinzips korrigiert werden. Die Präzision, ermittelt aus den Variationskoeffizienten von Tag zu Tag, lag zwischen 0,7 und 2,3% im Bereich von 256 und 845 nmol/l Cortisol. Die untere Nachweisgrenze (bei einem Signal-zu-Rausch-Verhältnis von 3:1) ist 200 pg Cortisol pro Probe.

¹⁾ Prof. Dr. H. Breuer, member of the Editorial Board of our journal, died on August 20, 1982.

Introduction

"Definitive Methods" have been defined as "Methods which after exhaustive investigation are found to have no known source of inaccuracy or ambiguity" (1).

In the field of hormone determinations, the accuracy of results obtained by the methods currently in use cannot be proven, since none of these methods has been checked by a definitive method. Only when this is achieved will it be possible to determine which methods in routine use produce results with a high degree of accuracy. Without such assurance, laboratories will not be ready to concentrate on selected methods for measuring a specific hormone, and therefore interlaboratory, and international differences will be perpetuated.

It is now generally accepted that the technique of isotope dilution-mass spectrometry may serve as a powerful analytical tool for the highly accurate determination of low molecular substances in biological fluids. In view of the above considerations it seemed reasonable to apply this technique to the specific and accurate determination of steroid hormones in human plasma. The general principles of the analytical procedures involved have already been outlined previously (2, 3). In the present paper, a definitive method for the determination of cortisol in human plasma is described in detail.

Principle of the Method

A definite amount of isotopically labelled cortisol is equilibrated with the endogenous non-labelled cortisol in the plasma sample. The steroids are extracted, purified by column chromatography and converted to a derivative suitable for gas liquid chromatography-mass spectrometry. In order to measure the isotope ratio of the non-labelled to the labelled cortisol, the steroid derivatives are subjected to selected ion monitoring. The mass spectrometer is adjusted to record simultaneously the molecular ion and a fragment ion of the cortisol derivative as well as of the corresponding isotopically labelled internal standard. The amount of endogenous non-labelled cortisol in the plasma sample is calculated from the isotope ratio.

Materials and Procedure

Reagents

[4-¹⁴C]Cortisol (spec. activity 2.035 GBq/mmol) was obtained from the Radiochemical Centre, Amersham, UK; the purity of the labelled substance was checked by selected ion recording and the material proved to be satisfactory for the use as internal standard. Cortisol was supplied as certified reference material (standard reference material 921) from the National Bureau of Standards, Washington, DC, USA. Testosterone 3-enol, 17 β -di-pentafluoropropionic ester was prepared by reacting testosterone (Schering, Berlin-West) with pentafluoropropionic acid anhydride in acetone for 30 min at room temperature. Methoxyamine hydrochloride was purchased from Merck (Darmstadt, FR Germany), N-methyl-N-trimethylsilyl trifluoroacetic amide from Macherey & Nagel (Düren, FR Germany), bromotrimethylsilane from PCR (Gainesville, Florida, USA) and pentafluoropropionic acid anhydride from Pierce (Rotterdam, The Nether-

lands). Sephadex LH-20 was obtained from Pharmacia (Freiburg, FR Germany) and Chromosorb W-HP, coated with 3% Dexsil 300, from WGA (Darmstadt, FR Germany). Dichloromethane used for the extraction of cortisol from the plasma samples was treated with 1 mol/l aqueous NaOH solution prior to use. Pyridine had a water content of less than 0.01% and acetone of less than 0.1% (Merck, Darmstadt, FR Germany). All reagents and solvents were of analytical grade.

Glassware

Volumetric flasks used for the preparation of the cortisol standard solutions (concentrated stock solution and diluted solution for final use) are officially calibrated. For the dilution of the cortisol standard stock solution as well as for the sampling of plasma, enzyme test pipettes (0.5 ml, 1 ml and 2 ml total volume, Blaubrand-Ionodur, Brand, Wertheim, FR Germany) are calibrated prior to use by pipetting and weighing appropriate amounts of water at 20 °C.

For pipetting of the final diluted cortisol standard solution and of the [4-¹⁴C]cortisol standard solution, a 100 μ l and a 25 μ l-syringe (SGE, Melbourne, Australia) equipped with repeating adaptors are used respectively. The 100 μ l syringe is calibrated by sampling and weighing 50 μ l water. The loss of water during the sampling and weighing procedure, due to evaporation of the solvent, is corrected for by weighing the water samples 45 and 90 s after sampling and calculating the original weight at 0 s.

Weighing Procedure

The cortisol certified reference material (National Bureau of Standards, Washington) and the 50 μ l water samples for calibrating the 100 μ l syringe are weighed with an electronic balance (model 4135, Sartorius, Göttingen, FR Germany). For calibrating the 0.5 ml, 1 ml and 2 ml enzyme test pipettes a mechanical balance (model 2432, Sartorius, Göttingen, FR Germany) is used. The accuracy of the balances is checked with officially calibrated weights.

Instruments and Settings

An LKB 9000 combined gas chromatograph-mass spectrometer, equipped with the LKB 2031 computer system is used. The gas liquid chromatography column (130 $\times$ 0.25 cm) is operated by a linear temperature programme from 260 to 290 °C with a temperature rate of 3 °C/min. Helium is used as carrier gas at a flow rate of 15 ml/min. The injection temperature of the gas chromatograph is set to 280 °C and that of the molecular separator to 290 °C. The ion source of the mass spectrometer is operated at 310 °C with an energy of the electron beam of 26 eV and an ionising current of 120 μ A. The high voltage of the electron multiplier is set to 3400 V.

Sample Preparation

To 0.4–1.5 ml plasma is added 25 μ l of an ethanolic solution, containing approximately 110 ng of [4-¹⁴C]cortisol, using the 25 μ l syringe equipped with the repeating adaptor. In order to obtain 1:1 isotope ratios which are most favourable in definitive measurements, the amount of plasma to be used in the experiment is first determined by a preliminary analysis. The plasma sample is equilibrated with the labelled cortisol by gently shaking the test tube for 30 min at room temperature. 15 ml of dichloromethane is added and the mixture mechanically shaken for 30 min at room temperature. The aqueous phase is aspirated; the organic phase is washed twice with 1 ml water and evaporated to dryness under nitrogen at 60 °C.

Column Chromatography

The column is prepared by placing 0.8 g of Sephadex LH-20 into a glass column (30 $\times$ 0.4 cm), equipped with a sintered disc. The column is rinsed with 100 ml of methanol and then equilibrated with 15 ml of a solvent mixture, consisting of dichloromethane, cyclohexane, methanol and water (100 + 80 + 15 + 1, by vol). The residue of the purified plasma extract is taken up twice in 100 μ l of the solvent mixture and transferred to the column. The column is first washed with 7 ml of the solvent mixture and the cortisol fraction eluted with further 2 ml

of the solvent mixture. Since the elution pattern is temperature-dependent and may slightly vary from one batch to another, it is recommended that the exact position of the cortisol fraction be checked by chromatographing a small portion of $[4-^{14}\text{C}]$ cortisol on a separate column and measuring the radioactivity in 0.5 ml fractions. The cortisol fraction obtained after column chromatography is collected in conical test tubes (total volume 2 ml) and the organic phase evaporated under nitrogen at 60 °C.

Formation of Derivatives

The cortisol-containing residue in the conical tube is reacted with 20 µl of a solution of 10 g/l methoxyamine hydrochloride in pyridine overnight at room temperature. The solvent is evaporated under nitrogen at 60 °C and the residue dried under vacuum in a desiccator. To form the trimethylsilyl ether, 10 µl of a mixture of trimethylsilylbromosilane, pyridine and N-methyl-N-trimethylsilyl-trifluoroacetic amide (5 + 5 + 90, by vol) is added to the residue. The mixture is kept for at least 30 min at room temperature.

Preparation of Standard Solutions

To prepare the ^{14}C -labelled cortisol standard solution, the radioactively labelled cortisol (370 KBq), as obtained from the supplier in 1 ml benzene/ethanol, is transferred to a test tube (2 × 8 cm); the solvent is evaporated and the $[^{14}\text{C}]$ cortisol is dissolved in 12.5 ml ethanol, thus yielding a concentration of about 110 ng/25 µl (4.4 mg/l). The solution is stored at -20 °C and allowed to equilibrate to 20 °C prior to use.

For the preparation of the non-labelled cortisol standard solution, a definite amount in the range from 29 to 31 mg (e.g. 30.187 mg) of the certified reference cortisol (purity 98.9%, estimated inaccuracy 0.2%) is weighed into a 500 ml volumetric flask (officially calibrated, estimated inaccuracy 0.25 ml). The weight is multiplied by the factor 0.989 in order to correct for the impurities as stated in the certificate of the reference material, thus yielding a corrected weight of 29.855 mg. The flask is filled up to the mark with methanol at 20 °C and the contents shaken vigorously for complete dissolution and distribution. The solution is used as concentrated stock solution and stored at -20 °C.

For the preparation of a diluted cortisol solution, 1 ml of the concentrated stock solution is transferred to a 100 ml volumetric flask (officially calibrated, estimated inaccuracy 0.1 ml) by the use of an enzyme test pipette (calibrated by pipetting and weighing 12 samples of 1 ml water at 20 °C, volume = 1.0117 ml ± 0.0017 ml). The 100 ml volumetric flask is filled with methanol at 20 °C to the mark and the content shaken vigorously for distribution. The final diluted cortisol standard solution contains 30.204 ng/50 µl (604.08 µg/l). The solution is stored at -20 °C and equilibrated at 20 °C prior to use.

Preparation of Standards

Three different standards are included in each analytical series:

Standard A, prepared in triplicate, consists of a mixture of 25 µl of the ethanolic $[4-^{14}\text{C}]$ cortisol standard solution (pipetted with the 25 µl syringe equipped with a repeating adaptor) and of 150 µl of the diluted cortisol standard solution. The non-labelled cortisol solution is sampled by pipetting three times 50 µl with the calibrated 100 µl syringe, which is set at 50 µl with the repeating adaptor. The exact volume of the syringe is determined by pipetting and weighing 12 times 50 µl water at 20 °C; the volume adjusted with the repeating adaptor was 49.989 µl ± 0.230 µl (s.d.). Following this calibration the exact amount of non-labelled cortisol in standard sample A was calculated to be 90.593 ng.

Standard B, prepared in triplicate, consists of a mixture of 25 µl of the ethanolic $[4-^{14}\text{C}]$ cortisol standard solution and of 200 µl of the diluted cortisol standard solution. For sampling of the non-labelled cortisol solution, four times 50 µl are pipetted with the calibrated 100 µl syringe. The amount of non-labelled cortisol in standard B is 120.791 ng.

Standard C, prepared in triplicate, is mixed from 25 µl of the ethanolic $[4-^{14}\text{C}]$ cortisol standard solution and 250 µl of the non-labelled cortisol standard solution; the non-labelled material is

pipetted with the calibrated 100 µl syringe (five times 50 µl). Standard C contains 150.988 ng cortisol.

It should be noted that it is not necessary to know the exact weight of $[4-^{14}\text{C}]$ cortisol in standards and samples and therefore the chemical and radiochemical purity of the labelled substance is not critical; however it is important that the amount of $[4-^{14}\text{C}]$ cortisol in the standards is exactly the same as that added to the plasma samples; this is achieved by using the same syringe with the repeating adaptor for the two procedures. Moreover, the amount of non-labelled cortisol in the standard samples must be exactly known.

The standard mixtures are evaporated under nitrogen at 60 °C and the residues are dried under vacuum in a desiccator. The methoxime-trimethylsilyl ether derivatives are prepared as described above.

Gas Chromatography-Mass Spectrometry

20 µg of testosterone 3-enol, 17β-dipentafluoropropionic ester is introduced into the ion source of the mass spectrometer by the use of a direct inlet system. The magnetic field is adjusted to the molecular ion of the testosterone derivative (m/z 580). This molecular ion is used to continuously correct, by means of the computer system, the temperature-dependent drift of the magnetic field strength during the actual recordings of the cortisol-specific m/z -values. The high voltages necessary for the recording of these m/z -values (605, 607, 636 and 638) are calculated by the computer system and adjusted by means of a digital-analogue converter.

The mixture containing the cortisol derivative is injected into the gas liquid chromatography column. During gas chromatography the signals obtained at the four cortisol-specific m/e -values are continuously recorded by the computer and stored on a magnetic disc. The signal for each of the m/z -values is integrated over a period of 75 ms. For the final inspection of the peak shapes, the selected ion monitoring chromatograms are reconstructed by the computer and made visible on a display. The quantitative evaluation of peak heights is carried out either manually with a cross hair cursor or automatically by the computer programme. A photograph of the computer terminal display after processing a plasma sample is shown in figure 1. Each standard and plasma sample is analysed in two separate selected ion monitoring runs. The mean isotope ratio obtained after the two injections is used for the calculation of the analytical results. It should be noted that the plasma and standard samples are injected alternately in order to keep the measurement system under continuous control.

Calculation Procedure

The concentration of cortisol in the plasma sample is calculated from the ratio of the peak heights measured at m/z -values 605 and 607. The recordings at m/z -values 605 and 607 are checked by measurements at m/z -values 636 and 638; calculation is carried out accordingly.

For the calculation of the analytical results it must be taken into account that the radioactively labelled cortisol is not available as isotopically pure material; it contains 19.1% of non-labelled steroid. On the other hand, due to the natural isotope composition of the chemical elements, the non-labelled steroid to be determined contributes to a certain extent to the recording of the ^{14}C -labelled cortisol derivative. Moreover, there may be a small alternate interference depending on the resolution of the instrument. On the basis of these considerations the following equation is formulated:

$$X[\text{ng}] = \frac{I - f_1 I^*}{I^* - f_2 I} f_0 S[\text{ng}] \quad (\text{Eq. 1})$$

whereby

X = amount of cortisol to be calculated,
I = intensity of the non-labelled cortisol in the plasma sample,
I* = intensity of the labelled cortisol in the plasma sample,
f₁ = ratio I/I* in the labelled cortisol,
f₂ = ratio I*/I in the non-labelled cortisol,
f₀ = proportional factor,
S = amount of labelled cortisol present in the plasma sample.

START RETENTION TIME	SECONDS AND END RETENTION TIME		500 SECONDS	
RUN NUMBER 111				
6 WHO-POOL IV-12				
MASS 6380 AMPL.	1 SCALE FAC.	1 MIN.	113 MAX.	1244
MASS 6360 AMPL.	1 SCALE FAC.	1 MIN.	106 MAX.	1179
MASS 6070 AMPL.	1 SCALE FAC.	1 MIN.	106 MAX.	4383
MASS 6050 AMPL.	1 SCALE FAC.	1 MIN.	107 MAX.	4245

x

Fig. 1. Selected ion recording of the 3,20-methoxime,11 β ,17,21-trimethylsilyl ether of cortisol and of [4- 14 C]cortisol; the figure shows a photograph of the computer terminal display after processing a plasma sample. First trace (lowest on the scale): m/z 605 (M^+-31 of the cortisol derivative); second trace: m/z 607 (M^+-31 of the 14 C-labelled cortisol derivative); third trace: m/z 636 (molecular ion of the cortisol derivative); fourth trace: m/z 638 (molecular ion of the 14 C-labelled cortisol derivative). The beginning of the scale at the left side indicates the time of injection into the Dexsil 300 column; the peaks of the non-labelled and labelled cortisol are eluted 360 s after injection.

It is obvious that the amount of steroid to be calculated is proportional to the ratio I/I^* of the non-labelled to the labelled materials as measured by multiple ion detection and to the amount of labelled standard material S which is added to the serum samples. The terms $-f_1I^*$ and $-f_2I$ are necessary to correct for the alternate contribution of the isotope fractions.

The above mentioned formula is simplified to

$$X[\text{ng}] = \frac{R - f_1}{1 - f_2 R} f_0 S[\text{ng}] \quad (\text{Eq. 2})$$

whereby

$R = I/I^*$ (intensity ratio of non-labelled to labelled in the plasma sample as measured by isotope dilution-mass spectrometry)

Due to the alternate isotope contribution of the non-labelled and the labelled cortisol, the standard curve is not linear and in addition shows an intercept at the origin. The parameters f_1 and f_2 of the formula are directly measured as isotope ratios in the labelled and in the non-labelled cortisol respectively. The third parameter of the formula f_0S is obtained after measuring the isotope ratio R of a standard mixture and insertion of the known amount of non-labelled cortisol (X) as well as f_1 and f_2 into the formula.

So far the calculation procedure is based on theoretical grounds and therefore produces accurate results as discussed earlier (3). However, it should be noted that the precision of the measurement of the low isotope ratios f_1 and f_2 for the labelled and the

non-labelled cortisol is significantly lower as compared to the precision of the measurement of a standard mixture which has a ratio of about one. The lower precision of the f_1 - and f_2 -measurements may contribute to a small ambiguity of the position of the standard curve. Although the resulting imprecision of the standard curve would be in the order of 1% only, an improved method for calculating the parameters f_1 , f_2 and f_0S was developed.

Clearly, more precise results are obtained when the three parameters are calculated from standard samples with isotope ratios near to unity. For the determination of the parameters f_1 , f_2 and f_0S the measurement of the three standard mixtures A , B , and C is required. In the present investigation isotope ratios of the non-labelled to the labelled cortisol in the range of 0.75:1, 1:1 and 1.25:1 were selected for the standard samples A , B and C respectively. Let the amounts of cortisol in the standard mixtures be X_1 , X_2 and X_3 and the isotope ratios, as measured by isotope dilution-mass spectrometry be R_1 , R_2 and R_3 . Then three equations are obtained:

$$X_1 = \frac{R_1 - f_1}{1 - f_2 R_1} f_0 S \quad (\text{Eq. 3})$$

$$X_2 = \frac{R_2 - f_1}{1 - f_2 R_2} f_0 S \quad (\text{Eq. 4})$$

$$X_3 = \frac{R_3 - f_1}{1 - f_2 R_3} f_0 S \quad (\text{Eq. 5})$$

From these equations, the parameters f_1 , f_2 and f_0S are calculated as follows:

From Eq. 3:

$$f_1 = \frac{-X_1 + f_2 R_1 X_1 + R_1 f_0 S}{f_0 S} \quad (\text{Eq. 6})$$

From Eq. 4:

$$f_1 = \frac{-X_2 + f_2 R_2 X_2 + R_2 f_0 S}{f_0 S} \quad (\text{Eq. 7})$$

From Eq. 6 and Eq. 7:

$$-X_1 + f_2 R_1 X_1 + R_1 f_0 S = -X_2 + f_2 R_2 X_2 + R_2 f_0 S \quad (\text{Eq. 8})$$

$$f_2 = \frac{X_1 - X_2 + f_0 S(R_2 - R_1)}{R_1 X_1 - R_2 X_2} \quad (\text{Eq. 9})$$

After developing Eq. 4 and Eq. 5 in a similar manner to Eq. 2 and Eq. 4:

$$f_2 = \frac{X_2 - X_3 + f_0 S(R_3 - R_2)}{R_2 X_2 - R_3 X_3} \quad (\text{Eq. 10})$$

From Eq. 9 and Eq. 10:

$$f_0 S = \frac{(X_2 - X_3)(R_1 X_1 - R_2 X_2) - (X_1 - X_2)(R_2 X_2 - R_3 X_3)}{(R_2 - R_1)(R_2 X_2 - R_3 X_3) - (R_3 - R_2)(R_1 X_1 - R_2 X_2)} \quad (\text{Eq. 11})$$

$f_0 S$ is calculated from Eq. 11 and inserted into Eq. 10, in order to obtain the parameter f_2 .

After f_2 and $f_0 S$ have been calculated they are inserted into Eq. 3:

$$f_1 = R_1 + \frac{f_2 R_2 X_1 - X_1}{f_0 S} \quad (\text{Eq. 12})$$

It should be noted that the term $f_0 S$ mainly reflects the amount of labelled cortisol S ; the proportional factor f_0 depends on the isotope distribution in the labelled and the non-labelled substance and usually has a value which is close to one. Since it is difficult to measure the isotope distribution pattern in the labelled compound with high accuracy and precision, as necessary for the definitive methodology, it is of great advantage to calculate the term $f_0 S$ from standard mixtures as described above.

The BASIC computer programme by which the parameters of the theoretical curve and the results of the analyses are calculated is given in the Appendix.

Accuracy

The accuracy of the method described here for the determination of cortisol is based on the high specificity of mass spectrometry and on the exact control of recovery employing the principle of isotope dilution.

Precision

The coefficients of variation from day to day as determined in five different plasma pools are summarized in table 1. With one exception the coefficients of variation for a given concentration of cortisol in plasma are lower when calculated from measurements at m/z -values 605/607, as compared to those calculated from measurements

at m/z -values 636/638. This is due to the fivefold higher intensity of the M-31 fragment ion recorded at m/z 605.

The imprecision of the analytical results which is less than 2.4% is a result of several non-systematic errors in the measurement and calibration procedure. The coefficient of variation for pipetting the serum samples in the range from 0.4 to 2.0 ml is about 0.12%; the imprecision of sampling the labelled internal standard solution with the 25 μ l syringe was found to be 0.48% (C.V.); the coefficient of variation for pipetting the diluted cortisol standard solution with the 100 μ l syringe was measured to 0.46% (C.V.). The imprecision of the mass spectrometer for the isotope ratio measurement at the m/z values 605 and 607 was calculated from duplicate measurements of the same samples. The coefficient of variation varied from day to day depending on the performance of the instrument and was in the range from 0.5 to 1.5%.

Tab. 1. Parameters of precision of the isotope dilution-mass spectrometry method for the determination of cortisol in human plasma.

Plasma pool	Measurement at m/z -values	Cortisol ($\bar{x} \pm s$) [nmol/l]	n	C.V. [%]	C.V.* (A - B) [%]
P.A.	605/607	453 $\pm$ 11.0	7	2.4	1.8
	636/638	455 $\pm$ 11.5	7	2.5	
P.B.	605/607	844 $\pm$ 11.0	7	1.3	0.5
	636/638	845 $\pm$ 7.0	7	0.8	
R.O.	605/607	243 $\pm$ 1.8	7	0.7	2.2
	636/638	247 $\pm$ 3.6	7	2.3	
S.I.	605/607	317 $\pm$ 3.5	7	1.1	2.2
	636/638	324 $\pm$ 8.7	7	2.7	
C.A.	605/607	256 $\pm$ 5.9	6	2.3	1.9
	636/638	259 $\pm$ 9.1	6	3.5	

* Coefficient of variation calculated from the duplicate measurements at m/z (605/607 (A) and 636/638 (B)).

Sensitivity

The lower limit of detection, defined as a 3:1 ratio of signal to noise, is 200 pg per sample, when measured at m/z value 605.

Discussion

The development of a definitive method requires the availability of a primary reference material of the analyte of high and accurately known purity. The cortisol preparation obtained as certified reference material from the National Bureau of Standards is, according to the certificate, a well characterized substance of known purity which fulfils all criteria of a primary standard and therefore is used as standard material in the present investigation.

With respect to the selection of a suitable isotope label for the internal standard the possibility cannot be excluded that the isotope may undergo exchange in steroids, tritiated or deuterated in the ring skeleton. Therefore ^{14}C -labelled cortisol was selected in the present study as internal standard material since carbon atoms of the steroid molecule are very stable and not exchanged during the analytical procedure.

However, ^{14}C -labelled cortisol may be replaced in future by cortisol which is labelled either with carbon 13 in the ring skeleton or with deuterium in an angular methyl group.

The methoxime trimethylsilyl ether of cortisol proved to be the only derivative suitable for gas liquid chromatography-mass spectrometry because of its thermal stability. This derivative was originally described by *Åringer & Gustafsson* (4) and applied to mass fragmentography by *Björkhem et al.* (5) and by our group (2). The method for the formation of the cortisol derivative by the use of a mixture containing bromotrimethylsilane and N-methyl-N-trimethylsilyl-trifluoroacetic amide, as described in this paper, was found to be advantageous, since the reaction mixture can be applied directly to the gas liquid chromatograph-mass spectrometer when packed Dextsil 300 columns are used. For the injection into a glass capillary column the reaction mixture may be easily evaporated under nitrogen at 60°C and transferred to the tip of a moving needle inlet system (6) with a few microliters of cyclohexane.

It should be pointed out that during methoxime formation syn-anti isomeres of steroid ketones are formed (7). These isomeric derivatives of cortisol are partially separated when an OV-101 capillary column of about 25 m length is used. Due to this incomplete separation, the quantitative results after use of the long capillary column are of lower precision. No advantage with respect to specificity or accuracy was observed for the cortisol analysis when the long glass capillary column was used in a few preliminary experiments. Therefore the packed Dextsil 300 or a short capillary column (6 m OV-101) are used, since with these columns the two syn-anti isomeres are eluted as one single well-defined peak, as shown in figure 1.

In the mass spectra of the cortisol derivative as well as the corresponding ^{14}C -labelled compound the peaks of highest intensities (base peaks) were found at m/z 605 and 607; they were used for selected ion monitoring. Simultaneously, an additional pair of ions was recorded at the m/z -values of the molecular ions (636, 638). Measurements of these m/z -values were performed to check the results obtained at m/z 605 and 607.

As already mentioned, the determination of cortisol by isotope dilution-mass spectrometry is highly specific. This statement is supported by the agreement of the results obtained by measurements at m/z -values 605/607 and 636/638, respectively. It can be seen from the table

that the variation of the differences between the two measurements (C.V. in the last column of tab. 1) is relatively low as compared to the mean variation of each of the two measurements.

In the present method for the measurement of a definitive value, the plasma sample to be analysed should contain cortisol in an amount which exceeds the lower limit of detection (200 pg per sample) by a factor of at least two hundred. This means in practice that between 0.4 and 2 ml of normal plasma is required to measure a definitive value.

In order to optimize the precision of the isotope dilution measurement, it is necessary to select isotope ratios for standards and samples which are not too low and not too high. Therefore, the final measurement of the definitive values is carried out at an isotope ratio which is close to one; this ratio is obtained by adjusting the volume of the plasma sample rather than the amount of internal standard ($[4\text{-}^{14}\text{C}]\text{cortisol}$) added.

Furthermore it seems to be important that the isotope ratios in the standard samples are close to that of the plasma sample; thus the three standard samples used for the calibration of the parameters of the standard curve are mixed from non-labelled and labelled cortisol with ratios of about 0.75:1, 1:1 and 1.25:1; these standard mixtures are prepared with high precision by the use of a syringe equipped with a repeating adaptor.

It should be emphasized that all volumetric measurements are based on weighing procedures since pipettes and syringes are calibrated by weighing appropriate amounts of water. This method improves the accuracy of the determination since the systematic errors associated with the weighing procedure are much lower as compared to volumetric measurements.

The calculation procedure, as described in the present paper, is developed on well-founded theoretical fundamentals and therefore may be superior to other methods of reading the analytical results from an empiric calibration curve or of calculations by regression analysis. Calculation of the theoretical curve from three standard mixtures makes it unnecessary to measure the exact amount, the purity or the isotope distribution pattern of the labelled internal standard; by calculating rather than weighing the amount of labelled standard the total calibration procedure is based on the well characterized certified reference cortisol; thus, the present method avoids several sources of error incurred in other definitive measurement techniques that use a weighing procedure for the internal standard (8).

The hierarchy concept of methods in clinical chemistry as summarized in the approved recommendation of the International Federation of Clinical Chemistry (1, 9) comprises definitive methods, reference methods and methods of known or unknown bias for routine use.

Following this concept (9) the accuracy of methods which are in routine use in clinical chemical laboratories should be confirmed by comparison with reference methods. The accuracy of reference methods must be established by comparison with definitive methods which reside at the highest level of the hierarchy scheme. The definitive values represent "the best known approximation to the true value".

On theoretical reasons there exists no final proof that a clinical chemical method, not even a definitive method, yields the "true" concentration value of an analyte in the biological matrix of a human body fluid. Therefore, the definition for definitive methods (1) includes the restriction that "no *known* source of inaccuracy" should be found; this implicates that the definition refers to the current state of art. In regard to this, the technique of isotope dilution-mass spectrometry, at present, provides the most suitable procedure for the development of a definitive method for the measurement of cortisol in human plasma.

Furthermore, it should be noted that exhaustive investigations are carried out in the present procedure in order to achieve optimal calibration and to confirm the accuracy of the results of the cortisol measurements. Therefore, the analytical procedure described here may be regarded as a definitive method.

On the basis of definitive values obtained by isotope dilution-mass spectrometry it will be possible to develop

reference methods, e.g. by using high performance liquid chromatography, for the measurement of cortisol and finally to transfer accuracy to the level of routine methods. This will improve the reliability of radio-immunoassay and protein binding techniques which are currently in use for the measurement of cortisol in human plasma.

Despite the well proven accuracy of the isotope dilution-mass spectrometry procedure as described here, there still remain some small sources of uncertainty of the analytical results. In addition to the contribution of imprecision which is below 2.4% for the plasma samples tested, there may be a small undetected bias due to systematic errors associated with the weighing procedure. This concerns the preparation of the cortisol stock solution as well as the calibration of the enzyme test pipettes used for measuring the plasma volume. This undetected error which is mainly due to the inaccuracy of the balances and of the officially calibrated test weights is assumed to be less than 0.5%. A further source of undetectable error is the possible inaccuracy of the cortisol content in the certified reference material (98.9%) which is estimated to be less than 0.2% as stated in the certificate of the National Bureau of Standards.

The present method has been used during the last four years for the determination of definitive values for cortisol in plasma pools for accuracy control in collaborative surveys.

Appendix

BASIC computer programme for the calculation of isotope dilution-mass spectrometry results.

```
10 DIM N$(50)
20 DIM R(50)
30 DIM N(50)
40 DIM N1(50)
50 DIM N2(50)
60 DIM M(50)
70 DIM Z(50)
100 PRINT #0: "PROGRAM FOR CALCULATION OF IDMS RESULTS"
200 PRINT
201 PRINT
202 PRINT
203 PRINT #0: "ANALYSIS: (TYPE A HEADLINE OF YOUR CHOICE!)", "/"
210 INPUT #0: A$
220 PRINT #0: "DATE: (TYPE THE DATE OF ANALYSIS!)", "/"
230 INPUT #0: D$
231 PRINT
232 PRINT
233 PRINT
240 PRINT #0: "TYPE OF ANALYSIS"
250 PRINT #0: "ANSWER WITH 'Y' FOR YES AND 'N' FOR NO !"
255 PRINT #0:
260 GO TO 2000
261 PRINT
262 PRINT
263 PRINT
264 PRINT
```

BASIC computer programme (continued)

```

270 PRINT #0: "STANDARDS"
280 PRINT
282 PRINT #0: "AMOUNT OF NON-LABELLED SUBSTANCE IN STANDARD MIXTURE 1 = ";
284 INPUT #0: X1
290 PRINT #0: "ISOTOPE RATIO MEASURED BY IDMS IN STANDARD MIXTURE 1 = ";
300 INPUT #0: R1
302 PRINT #0:
303 PRINT #0: "AMOUNT OF NON-LABELLED SUBSTANCE IN STANDARD MIXTURE 2 = ";
304 INPUT #0: X2
306 PRINT #0: "ISOTOPE RATIO MEASURED BY IDMS IN STANDARD MIXTURE 2 = ";
307 INPUT #0: R2
308 PRINT #0:
309 PRINT #0: "AMOUNT OF NON-LABELLED SUBSTANCE IN STANDARD MIXTURE 3 = ";
310 INPUT #0: X3
311 PRINT #0: "ISOTOPE RATIO MEASURED BY IDMS IN STANDARD MIXTURE 3 = ";
312 INPUT #0: R3
320 LET S1=(X2-X3)*(R1*X1-R2*X2)-(X1-X2)*(R2*X2-R3*X3)
322 LET S2=(R2-R3)*(R1*X1-R2*X2)-(R1-R2)*(R2*X2-R3*X3)
324 LET S=S1/S2
325 LET F2=((X2-X3)-S*(R2-R3))/(R2*X2-R3*X3)
326 LET F1=R1+(F2*R1*X1-X1)/S
330 PRINT #0:
340 PRINT #0:
400 PRINT #0: "SAMPLES"
402 PRINT #0:
405 PRINT #0: 'AFTER THE LAST SAMPLE TYPE "NO" FOR NAME OF SAMPLE !'
410 PRINT
420 LET I=3
430 LET I=I+1
431 LET Z(I)=I-3
432 PRINT #0:
433 PRINT #0: Z(I)
440 PRINT #0: "NAME OF SAMPLE =", "/",
450 INPUT #0: N$(I)
451 IF N$(I)="NO" THEN 510
452 IF N$(I)="DE" THEN 454
453 GO TO 456
454 I=I-1\GO TO 431
456 PRINT #0: "ML SERUM =", "/",
457 INPUT #0: M(I)
460 PRINT #0: "ISOTOPE RATIO (IDMS) =", "/",
470 INPUT #0: R(I)
480 N(I)=S*(R(I)-F1)/(1-R(I)*F2)
485 PRINT #0: "NG/SAMPLE", "/",
490 PRINT #0: N(I)
491 LET N1(I)=N(I)/M(I)
492 LET N2(I)=N1(I)*F
500 GO TO 430
510 LET N(2)=0
515 LET K=Z(I)
830 OPEN "LP:" FOR OUTPUT AS FILE #1
831 PRINT #1: A$
832 PRINT #1: T1$
833 PRINT #1: D$
834 PRINT #1:
835 PRINT #1:
836 PRINT #1:
840 PRINT #1: "STANDARDS"
850 PRINT #1:
855 PRINT #1: "AMOUNT OF NON-LABELLED SUBSTANCE IN STANDARD MIXTURE 1 =", X1
860 PRINT #1: "ISOTOPE RATIO MEASURED BY IDMS IN STANDARD MIXTURE 1 =", R1
861 PRINT #1:
862 PRINT #1: "AMOUNT OF NON-LABELLED SUBSTANCE IN STANDARD MIXTURE 2 =", X2
863 PRINT #1: "ISOTOPE RATIO MEASURED BY IDMS IN STANDARD MIXTURE 2 =", R2
864 PRINT #1:
865 PRINT #1: "AMOUNT OF NON-LABELLED SUBSTANCE IN STANDARD MIXTURE 3 =", X3
866 PRINT #1: "ISOTOPE RATIO MEASURED BY IDMS IN STANDARD MIXTURE 3 =", R3

```


BASIC computer programme (continued)

```

867 PRINT #1:
910 PRINT #1:
920 PRINT #1:
930 PRINT #1:
940 PRINT #1: "SAMPLES"
950 PRINT #1:
960 PRINT #1: "NAME OF SAMPLE"; TAB(20); "ISOTOPE", TAB(30); "ML"; TAB(40); "NG/"; TAB(50); "NG/ML"; TAB(60); "NMOL/L"
962 PRINT #1: TAB(21); "RATIO"; TAB(29); "SERUM"; TAB(39); "SAMPLE"
970 PRINT #1:
980 FOR I=4 TO K+2
995 PRINT #1:
1010 PRINT #1: N$(I); TAB(20); R(I); TAB(29); M(I); TAB(39); N(I); TAB(49); N1(I); TAB(59); N2(I)
1015 NEXT I
1040 FOR I=1 TO 60
1050 PRINT #1:
1060 NEXT I
1070 END
2000 PRINT #0: "OESTRADIOL ?", "/",
2002 INPUT #0: T$
2004 IF T$="Y" THEN 3000
2010 PRINT #0: "OESTRIOL ?", "/",
2012 INPUT #0: T$
2014 IF T$="Y" THEN 3010
2020 PRINT #0: "CORTISOL ?", "/",
2022 INPUT #0: T$
2024 IF T$="Y" THEN 3020
2030 PRINT #0: "ALDOSTERONE ?", "/",
2032 INPUT #0: T$
2034 IF T$="Y" THEN 3030
2040 PRINT #0: "TESTOSTERONE ?", "/",
2042 INPUT #0: T$
2044 IF T$="Y" THEN 3040
2050 PRINT #0: "PROGESTERONE ?", "/",
2052 INPUT #0: T$
2054 IF T$="Y" THEN 3050
2090 GO TO 2000
3000 LET F=3.671
3002 LET T1$="OESTRADIOL-17B----"GO TO 261
3010 LET F=3.468
3012 LET T1$="OESTRIOL"GO TO 261
3020 LET F=2.759
3022 LET T1$="CORTISOL"GO TO 261
3030 LET F=2.774
3032 LET T1$="ALDOSTERONE"GO TO 261
3040 LET F=3.467
3042 LET T1$="TESTOSTERONE"GO TO 261
3050 LET F=3.18
3052 LET T1$="PROGESTERONE"GO TO 261
3053 GO TO 270

```

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Prof. Dr. L. Siekmann
Inst. f. Klin. Biochemie
d. Univ. Bonn
Sigmund-Freud-Str. 25
D-5300 Bonn 1