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The Estimation of 6β-Hydroxycortisol in Urine — A Comparison of Two Methods: High Performance Liquid Chromatography and Radioimmunoassay

By *E. Gerber-Taras, B. K. Park* and *E. E. Ohnhaus*

Department of Medicine, University of Berne, Switzerland

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Summary: In the present study two recently developed methods, a high performance liquid chromatography (HPLC) and a radioimmunoassay (RIA) for the estimation of 6β-hydroxycortisol in urine were compared. Both methods showed a very reliable intraassay variation of about 5.2 or 7.2% respectively. Using the baseline values of excretion during a 24-hour collecting period in human urine, and after induction of the liver microsomal enzyme system by different substances, both methods gave identical values. The HPLC-method still needs an extraction step at the beginning of the analytical procedure, while a direct measurement in the urine can be performed with the RIA. Furthermore, the RIA is not time consuming and is easy to perform. As the antiserum is highly specific there is no cross-reaction with other cortisol metabolites.

Zur Bestimmung von 6β-Hydroxycortisol im Urin:
Ein Methodenvergleich zwischen Hochdruckflüssigkeitschromatographie und Radioimmunoassay

Zusammenfassung: Zur Bestimmung von 6β-Hydroxycortisol im Urin stehen neuerdings zwei repräsentative Methoden zur Verfügung, eine mittels Hochdruckflüssigkeitschromatographie, die andere durch Radioimmunoassay. In der vorliegenden Studie wurden beide Methoden miteinander verglichen, wobei zwei 24-Stunden-Urine gesunder Versuchsprobanden vor und nach Induktion des mikrosomalen Leberenzymsystems zur Analyse verwendet wurden. Dabei ergab sich für beide Methoden eine nahezu identische Intra-assayvariation von 5.2 bzw. 7.2%. Auch waren die mit beiden Verfahren gemessenen Werte nicht signifikant voneinander verschieden und ergaben eine sehr gute Korrelation. Trotzdem muß bei Verwendung der Hochdruckflüssigkeitschromatographie vorher ein Extraktionsverfahren vorgenommen werden, während beim Radioimmunoassay der Urin direkt verwendet werden kann. Außerdem ist der Radioimmunoassay leicht und in kurzer Zeit durchzuführen, eine Kreuzreaktion mit anderen Cortisolmetaboliten ist wegen der hohen Spezifität des Antiserums ausgeschlossen.

Introduction

6β-hydroxycortisol, a polar metabolite of cortisol is formed in the smooth endoplasmic reticulum of the hepatocytes by the mixed function oxygenase system and excreted unconjugated by the kidneys (1, 2). 6β-hydroxycortisol is estimated together with the total 17-hydroxycorticosteroids excreted in the urine, and it represents 2–6% of this mixture. Following the administration of certain agents known to induce the metabolism of other substances and drugs, an increased 6β-hydroxycortisol urinary excretion was observed (3–7). Therefore it was thought that 6β-hydroxycortisol excretion might be used as an in vivo parameter of enzyme induction. This suggestion was confirmed in a recent investigation comparing three different drugs known to be potent enzyme inducers in man (8).

Extensive investigations of urinary 6β-hydroxycortisol concentrations have been restricted by the available methods of measurement. Assays developed previously have employed extraction from urine, followed by thin layer or paper chromatography, then quantitative estimation by a non specific colour reaction (9, 10, 11). In addition, gas-liquid-chromatography has been utilized following derivatisation of the sample (12, 13). The results published using these methods show wide variations in daily excretions, with mean values between 185 and 534 μg per 24 hours. Recently two new methods became available for measuring 6β-hydroxycortisol, a radioimmunological method (14) and a method using high performance liquid chromatography (15). Having used the radioimmunoassay quite extensively (8) we were interested to compare both methods.

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Methods

Measurement of 6β-hydroxycortisol by high performance liquid chromatography (HPLC)

The estimation of 6β-hydroxycortisol by HPLC was performed according to the method described by *Roots* et al. (15). A 1 to 5% aliquot of the total urine volume collected over a period of 24 hours was used for estimation. In order to correct losses during the extraction procedure [^{3}H]6β-hydroxycortisol was added to the urine and 20 µl of the final extract was counted in a liquid scintillation counter Beckman LS-133. Calibration curves were obtained by adding pure 6β-hydroxycortisol to the urine.

Using the same procedure as for thin layer chromatography, the urine was extracted to remove lipophilic compounds (9, 16). After adding and dissolving 200 g/l sodium sulphate (anhydrous, Firma Merck) at 35 °C, the urine was extracted once with three times its own volume of ethyl acetate for three minutes at room temperature using a shaking device. The aqueous phase was discarded and the ethyl acetate phase was washed twice for 30 s with 1/20 volume of 0.25 mol/l sodium hydroxide saturated with sodium sulphate. After discarding the aqueous phase, the ethyl acetate was evaporated to dryness in a rotary vacuum evaporator. The residue was dissolved in 6 ml ethanol, transferred to a conical evaporation tube and again completely evaporated. The addition of 100 µl ethanol gave an extract ready for injection of 20 µl into the HPLC apparatus.

High performance liquid chromatography was performed with a Waters Associated Model 440 in conjunction with an UV-detector and an integrator system (Infotronics). Absorbance was measured at 240 nm. The chromatographic conditions were: stainless steel (V4A) column 30 cm × 4 mm i.d. filled with silica-gel (Lichrosorb SI 60), particle size 5 µm. The eluents consisted of methylene chloride (410 ml), *n*-hexane (470 ml) and ethanol (112 ml), with the addition of about 20 ml water until turbidity persisted. The eluents were stirred overnight to reach equilibrium. The residual water was removed by a separating funnel or filtration. The mobile phase had a pH between 5 and 6. The pressure was about 80 bar, the flow rate 2.0 ml/min.

Estimation of 6β-hydroxycortisol by radioimmunoassay (RIA)

The radioimmunoassay was performed according to the method published by *Park* (14). 6β-hydroxycortisol was dissolved in methanol solution and used to prepare a standard curve ranging from 25 to 2000 pg in duplicate. The solvent methanol was evaporated at 35 °C in vacuo. Bovine serum albumin 1 g/l solved in a sodium phosphate merthiolate buffer solution was used to inhibit adsorption, onto the inside of the test tubes, of the components necessary for the reaction. The urine samples were diluted (1:100) in the bovine serum albumin buffer solution and 50 µl duplicates were taken for estimation. To all test tubes 100 µl of a specific antiserum for 6β-hydroxycortisol (1:100 dilution in bovine serum albumin buffer) and 90 pg of [^{3}H]6β-hydroxycortisol in 50 µl buffer solution were added. The test tubes were vortexed and incubated for a period of about 12 hours at 4 °C. After incubation 100 µl of a more concentrated bovine serum albumin buffer (5 g/l) and 1 ml suspension of dextran-coated charcoal in sodium phosphate merthiolate buffer solution were added to each sample. The tubes were agitated and left to stand for about 10 minutes. After centrifugation at 4 °C and 2500 *g* the supernatant in the test tubes were decanted into scintillation vials containing 4 ml scintillation cocktail (Micellar Scintillator NE 260, Nuclear Enterprise Ltd. Edinburgh/Scotland). Subsequently all samples were measured in a liquid scintillation counter (Beckman LS-133).

Study design

The urine samples assayed in the present study by both methods were obtained from healthy volunteers. Two different groups were investigated before and after induction of the liver microsomal enzyme system. Two 24-hour urine collections were measured before and after the induction period. Antipyrine, phenobarbital and rifampicin were used as enzyme-inducing drugs. A daily dose of 1200 mg antipyrine and 600 mg rifampicin was administered to the first group, while 1200 mg antipyrine and 100 mg phenobarbital was given to the second. A period of 14 days was used as an induction period.

Statistical analysis

The mean and standard deviation of the measurements made in each group of volunteers were calculated. The variances of the means were found to be homogeneously distributed, as tested by the F-Test. Therefore all values were compared by a t-test for paired observations. In addition, the values obtained by HPLC or RIA were compared by means of linear regression using the method of least squares (18).

Results

The results are seen in table 1 and figure 1. As seen from table 1 baseline values in a range between 200 and 400 µg 6β-hydroxycortisol per 24 hours were found in both groups of volunteers. After the enzyme induction period, following two different drug regimens, increases up to 2000 µg/24 hours occurred. In these experiments, the above values measured by RIA and HPLC showed no significant differences. A very good correlation was found between the two methods for all values of urinary 6β-hydroxycortisol in healthy volunteers (figure 1), irrespective of drug status (before or after the induction period), or nature of the drug (rifampicin plus antipyrine, or phenobarbital plus antipyrine). Using the method of least squares, a correlation coefficient of 0.99 ($p < 0.00001$) was calculated. The slope of the regression line calculated was not significantly different from the line of identity between both methods. The calculated values for the intraassay variation were similar for both methods, 5.2% for the HPLC and 7.2% for the RIA-method.

Tab. 1. Estimation of 6β-hydroxycortisol in urine by HPLC and radioimmunoassay in healthy volunteers before and after induction of the liver microsomal enzyme system. The figures given in this table are the means of two 24 hour samples.

	6β-Hydroxycortisol (µg/24 h)			
	before induction		after induction	
	HPLC	RIA	HPLC	RIA
Rifampicin + antipyrine				
W.C.	186	229	1004	1243
R.S.	255	270	1707	1940
P.M.	349	322	2076	2470
R.H.	321	252	1469	1733
G.E.	434	382	2786	2909
U.E.	361	324	1414	1379
Mean ± SD	318 ± 87	297 ± 56	1743 ± 621	1946 ± 642
Phenobarbital + antipyrine				
B.A.	171	231	583	574
M.A.	164	179	711	892
F.D.	219	199	574	611
O.E.	188	238	459	473
S.H.	204	174	442	471
K.B.	274	283	424	519
Mean ± SD	203 ± 40	217 ± 40	532 ± 111	590 ± 158

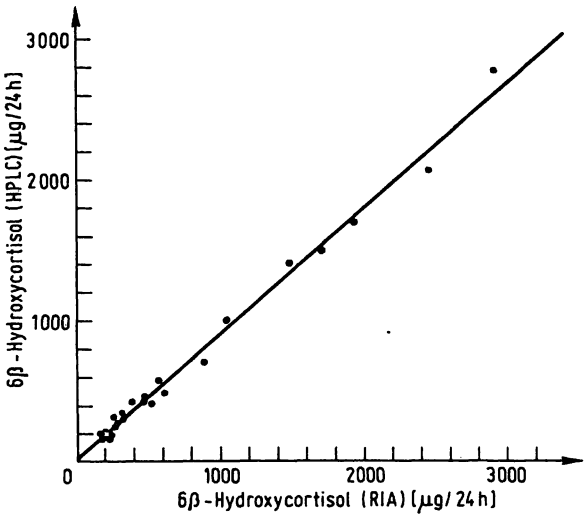

Fig. 1. The 6β-hydroxycortisol values of 24 urine collections measured by HPLC and RIA are plotted by means of linear regression. The calculated regression line was $y = 22.03 + 0.89 \cdot x$ showing a correlation coefficient of 0.99 ($p < 0.00001$).

Discussion

In contrast to other methods reported in the literature (15) both methods give reliable and consistent values showing no large variation in the baseline values between 72 µg to 445 µg. However, an extraction is still necessary in the HPLC-method. In the RIA the urine can be used without any previous treatment. The antiserum is very specific and showed no cross reactions with other metabolites of cortisol, formed by reduction of the A ring or reduction of the 20-ketone, which are present in high concentrations in the urine (17). In addition, the RIA is easy to perform and is not time consuming, compared with the HPLC-method. Therefore, based on the present data, the RIA is easy to perform and reliable. It gives consistent results for urine and can be used for investigations of endocrinology and drug metabolism.

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Professor Dr. E. E. Ohnhaus
Medizinische Universitätsklinik
Hufelandstraße 55
D-4300 Essen