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Free cortisol method comparison: Ultrafiltation, equilibrium dialysis, tracer dilution, tandem mass spectrometry and calculated free cortisol

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ABSTRACT

Background: Free cortisol (FC) can be calculated from measurements of total cortisol and binding proteins or measured after mechanical separation of unbound and bound fractions by equilibrium dialysis or ultrafiltration. FC can then be measured indirectly by ^{3}H-cortisol dilution or directly by immunologic or tandem mass spectrometry assays.

Methods: We compared FC measured with ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC MS/MS) with ^{3}H-cortisol dilution in ultrafiltrates and dialysates and also with calculated FC (Coolens equation). An adult FC reference interval was established.

Results: The UHPLC MS/MS and ^{3}H-cortisol dilution methods were non-linearly related (Cusum linearity test $p<0.001$) but well correlated ($R^2 = 0.984$). FC calculated with Coolens equation agreed with the UHPLC MS/MS method. Impurity of ^{3}H-cortisol and non-specific adsorption were excluded as causes on non-linearity. Ultrafiltration was linearly related to equilibrium dialysis, simpler to perform and more repeatable. A gender non-specific FC reference interval of 2.1–19.1 nmol/L was established.

Conclusions: In view of the non-linearity between measuring techniques and the variability of reported reference ranges, care should be exercised in adopting a reference range. The ultrafiltration UHPLC MS/MS method we described is robust and suitable for use in a routine laboratory.

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1. Introduction

Cortisol is produced by the adrenal gland under the control of adrenocorticotrophic hormone and it is transported in blood more than 90% protein bound, approximately 70% with high affinity to corticosteroid binding globulin (CBG) and 20% with low affinity to albumin. Routine laboratory methods measure total plasma or serum cortisol (TC), however it is unbound free cortisol (FC) which is physiologically active, able to diffuse across cell membranes, bind to the cytosolic glucocorticoid receptor (NR3C1) and modulate transcription of target genes [1–3]. Thus changes in albumin and CBG, due to the effects of acute inflammation or intercurrent disease, confound the interpretation of both baseline and stimulated TC measurements [2–4]. Similarly the effect of oestrogens on CBG production will increase TC while the FC remains relatively unaffected. Although FC levels are considered a better indicator of glucocorticoid status, not all authors have demonstrated a clear clinical benefit from measurement of free vs. total cortisol in the setting of infection and sepsis [5,6].

Free cortisol can be estimated indirectly with the Coolens or other defined equations incorporating measurements of TC, CBG and albumin [3,7]. Methods that measure FC depend on the mechanical separation of the unbound and bound fractions before directly or indirectly measuring the cortisol concentration. Separation can be achieved by equilibrium dialysis [8–10], ultrafiltration [7,11], gel-filtration chromatography [12] and immuno-capture procedures [13]. Equilibrium dialysis and gel-filtration are both cumbersome and time consuming techniques. Temperature control is critical and small variations markedly influence the FC [11,14,15]. Indirect measurement of FC in the ultrafiltrate or dialysate can be achieved by tracer dilution where the fraction of ^{3}H-cortisol recovered and the measured TC is used to calculate FC [6,10,15]. Measurement of FC in the dialysate or filtrate by immunologic or tandem mass spectrometry assays with appropriate analytical sensitivity presents an alternative direct approach [9–11,14].

Reference intervals reported in most papers were established on small numbers of individuals and differ substantially between authors. This complicates the application of reported findings to local patient populations. Given the range of methods available to separate the free from bound fractions, directly or indirectly measure FC and calculate FC, it is not surprising that there are systematic differences in results due to variations in analytical specificity and the challenges involved in accurately measuring very low levels of cortisol. A second obstacle is the dynamic nature of free cortisol that complicates quality control of the separation steps due to a lack of stable commercial quality control material.

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We recently described an ultra high performance liquid chromatography – tandem mass spectrometry method (UHPLC MS/MS) capable of selectively measuring cortisol with good analytical sensitivity (0.6 nmol/L) which is suitable for measurement of both TC and FC in a routine laboratory [16] We used ultrafiltration and equilibrium dialysis separation of bound and free cortisol fractions, to compare direct measurement of FC with UHPLC MS/MS with indirect ^{3}H-cortisol tracer dilution method and a calculated FC derived from the Coolens equation. We also established a FC reference interval in apparently healthy adult volunteers.

2. Materials and methods

2.1. Sample collection

We used excess plasma samples submitted to the laboratory for routine analysis of plasma cortisol to create 50 sample pools with sufficient volume to complete all tests in duplicate and that covered a range of cortisol values encountered in the routine practice [17]. Routine samples were collected into lithium heparin vacutainer tubes (Greiner Vacuette, Greiner Bio-one, Graz, Austria), separated at 2200 g for 10 min at room temperature and stored at 4 °C. Pooled samples were stored at −20 °C until analysis. To establish a FC reference interval we collected plasma samples between 08:00 and 10:00 am from volunteer laboratory staff with no reported acute medical conditions and not on glucocorticoid therapy.

2.2. Ultracentrifugation

Samples for plasma free cortisol were prepared by equilibrating 500 μL of plasma at 37 °C for 15 min in Amicon Ultra-4 regenerated cellulose 30,000 molecular weight cut-off centrifugal filter devices (Millipore, Billerica, USA) before centrifugation at 3040 g for 20 min at 37 °C.

2.3. Equilibrium dialysis

Equilibrium dialysis was undertaken with 10,000 molecular weight cut-off Slide-A-Lizer MINI Dialysis Units, (Thermo Scientific, Rockford, USA) contained in Cobas analyser sample cups. Serum (740 μL) was dialysed against 370 μl of 1% gelatin in phosphate buffered saline at 37 °C for 24 h.

2.4. UHPLC MS/MS method

The method has been described in detail elsewhere [16]. In brief we used an Acquity Ultra Performance Liquid Chromatography system coupled with a Micromass Quatro Premier XE mass spectrometer (Waters, Milford, USA) with electron spray ionisation in positive ion detection mode. Deuterated internal standard (d4-cortisol) was obtained from Cambridge Isotopes Laboratories (Novachem Pty Ltd, Victoria, Australia). 250 μL ultrafiltrate or dialysate was mixed with 100 μL internal standard and 500 μL H_2O before solid phase extraction on Oasis HLB 1 cc (30 mg) cartridges (Waters, Milford, USA). Samples were eluted with 1 mL 100% ethyl acetate, dried down at 50 °C and reconstituted in 100 μL of mobile phase before injecting 20 μL. The total analytical run time on the UHPLC MS/MS was 3 min.

2.5. Tracer dilution, purification of ^{3}H-cortisol and estimation of free cortisol

[1,2,6,7-^{3}H(N)]-cortisol (MW 362) with a specific activity of 72.4 Ci/mmol and purity greater than 97% was purchased from PerkinElmer Life (PerkinElmer Life and Analytical Sciences, Boston, USA). A 100 μL aliquot of stock ^{3}H-cortisol in ethanol (approximately 2×10^{5} dpm) was evaporated in a glass tube before addition of 1 ml plasma, mixed and allowed to equilibrate for 60 min at room temperature before removing 100 μL to determine the total count. The estimated amount of ^{3}H-cortisol added to the samples was 1.3 nmol/L. The balance of the sample was used for ultrafiltration, equilibrium dialysis and measurements of total cortisol. The free counts were determined on 100 μL of ultrafiltrate and dialysate respectively. FC was calculated from the measured TC and the free fraction (FC = TC × free count/total count). We purified the ^{3}H-cortisol by HPLC reverse phase chromatography (5 μm C18 column, Waters 2695 Separation Module and 996 Photodiode Array) as described elsewhere [18].

2.6. Calculated free cortisol

TC was measured with a Beckman Unicel DxI800 analyser (Beckman Coulter Diagnostics, Brae, USA) and UHPLC MS/MS. CBG (Transcortin) was measured by radioimmunoassay (IBL International GMBH, Hamburg, Germany). The inter-assay precision for the assay was 6.2% at 30 mg/L and 5.1% at 111 mg/L. FC was calculated with the method of Coolens [3].

2.7. Statistical methods

Method comparison was performed according to CLSI guideline EP9-A2 with 50 samples [17]. The ultrafiltration and equilibrium dialysis procedures were performed in duplicate with two separately labelled sample aliquots and measurement of FC in each ultrafiltrate or dialysate performed once only. Reference intervals were established according to CLSI guideline C28-A3 [19].

3. Results

The pooled samples had a TC mean (range) measured with UHPLC MS/MS of 413 nmol/L (35–1678). In three samples a duplicate result for equilibrium dialysis was not available due to technical failure of the dialysis apparatus and in five more cases insufficient dialysate was available to measure cortisol directly. The FC in the pools ranged between 1 and 329 nmol/L and the percentage FC was between 1.9 and 19.6% with the various methods. In one sample with a TC of 1678 nmol/L the CBG result was not available to calculate FC. Short term repeatability (%CV determined from duplicates) with ultrafiltration followed by direct measurement with UHPLC MS/MS was 3.3% and 2.3% with tracer dilution. Short term repeatability for equilibrium dialysis with UHPLC MS/MS was 15.3% and 6.7% with tracer dilution. In comparison calculated free cortisol had a short term repeatability of 6.4%.

The comparison between free cortisol measured with UHPLC MS/MS after ultrafiltration and equilibrium dialysis separation techniques is presented in Fig. 1. The relationship was linear (Cusum linearity test $p>0.1$) with a slope (95% confidence interval) of 1.08 (1.02–1.14). The relationship for free cortisol determined with tracer dilution in the same samples (data not shown) was also linear with a slope of 1.31 (1.22–1.38). The UHPLC MS/MS and tracer dilution measurements on ultrafiltrates are compared in Fig. 2 and it is clear that the relationship is non-linear (Cusum linearity test $p<0.001$). Fitting of a quadratic curve yielded a R^2 of 0.984 compared to 0.968 of a simple linear regression with a reduction in the standard error of the regression from 10.60 to 7.55. The non-linear relationship was also evident when the FC is expressed as a fraction relative to TC. The lowest percentage FC measured by tracer dilution was 7.5% compared to 1.9% with UHPLC MS/MS. Comparing FC in equilibrium dialysates resulted in a similar finding (data not shown). Comparing the direct measurement of FC in ultrafiltrates by UHPLC MS/MS with a FC calculated with Coolens equation from CBG and TC measured by UHPLC MS/MS

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