Measurement of Serum Free Cortisol Using Equilibrium Dialysis-Liquid Chromatography-Tandem Mass Spectrometry: Revisiting the Use of Free Cortisol Measurement for Adrenal Insufficiency in Critically Ill Patients

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Abstract

Background: Measurement of total cortisol (tC) is used in the detection of Cushing's syndrome. Due to changes in levels of binding proteins in the critically ill, free cortisol (fC) provides a better measure of their adrenal function.

Methods: Equilibrium dialysis (ED) of 250ul serum was carried out against a dialysis buffer solution and the dialysate was measured for fC using an LC-MS/MS method [1] on an AB Sciex™ Triple Quad 5500 mass spectrometer. fC, tC and albumin were measured in 100 serum samples from critically ill patients. 48 AM and 44 PM samples were collected from healthy donors to establish reference intervals for fC.

Results: Lower limit of detection (LOD) and quantitation (LOQ) for the new method were 0.03 and 0.06 µg/dL respectively. Upper limit of linearity (ULOL) was 60 µg/dL. The method agreed poorly with an ED-electrochemiluminescent immunoassay (ED-ECLIA, RED device, Thermo Fisher Scientific; Roche Cobas e601) (slope=0.41, r=0.52) but well with another ED-LC-MS/MS method (slope=0.82, r=0.98). Time-specific transformed parametric reference intervals (RI) were: 0.21–1.04 µg/dL (8-10 AM) and 0.10–0.63 µg/dL (4-6 PM). 100 samples from critically ill patients showed loss of diurnal variation of total cortisol with mean fC in samples with low albumin concentrations being 3.1 times higher than healthy samples.

Conclusion: We developed a reliable, sensitive and specific ED-LC-MS/MS method for measurement of fC in human serum and established time specific reference intervals in a healthy adult population. Measuring fC rather than tC during critical illnesses could prevent the unnecessary use of glucocorticoid therapy (GC).

Introduction

Measurement of cortisol in serum is essential for the diagnosis of hypercortisolism and adrenal insufficiency. Approximately 90% of circulating cortisol in human serum remains bound to cortisol binding globulin (CBG) and serum albumin. Significant changes in CBG and albumin concentrations occur during critical illnesses when glucocorticoid secretion is maximal. It is therefore recommended that measurement of free or unbound cortisol in serum is a better measure of the hypothalamic-pituitary-adrenal status than measurement of tC in situations where CBG or albumin changes are predictably significant. We have developed and validated a simple and effective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to analyze fC in human serum and established reference intervals in a healthy population of adults.

Methods

- 250 µl of serum samples and controls were dialyzed against 200 µL of buffer at 37ºC for 20± 1h. 200 µl of dialysate was removed and mixed with 100 µL of d^4-cortisol solution in a 96 well plate and subjected to solid phase extraction (SPE) using Strata X SPE cartridges according to the procedure previously published by our laboratory (Ray et al, Clin Chimica Acta 412 (2011) 13-14).
- LC-MS/MS analysis was performed on an API TripleQuad™5500 Mass Spectrometer in positive ion mode using electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode of acquisition with declustering and entrance potentials maintained at 90 and 10 V respectively. Quantitative MRM transitions monitored for cortisol and d4-cortisol were 363.2/121.1 and 367.2/121.1 respectively. Collision energies for the transitions of cortisol and d^4-cortisol were 28 and 31V and exit potential was 15V for both. Ion source was maintained at 500ºC, IS voltage was 4000 V with curtain gas maintained at 30 psi while gas 1 and 2 were 40 and 60 psi respectively.
- RI for fC were established in a heathy population of adults (8-10 AM ; n=48 and 4-6 PM; n=44). Donors had no history of adrenal or pituitary diseases, history of neuroendocrine tumors, no use of glucocorticoid therapy or recent events of stress, hyperglycemia, alcoholism or uncontrolled diabetes, and not pregnant.
- 100 serum/plasma samples (64 morning (8-10 AM) and 36 evening (4-6 PM); 34 male and 66 female) were collected from patients admitted to the intensive care unit (ICU) in the University of Utah Hospital using IRB-approved protocols. Median male and female ages were 33 (age range 18-81) and 49 (age range 18-85) years respectively. Specimens were tested for tC, fC using the LC-MS/MS method and albumin was measured using a colormetric assay (bromcresol green) on the Roche c702 (Roche Diagnostics, Indianapolis, IN).
- ED-LC-MS/MS and ED-ECLIA methods for measurement of fC were compared.

Results

Figure 1: MRM chromatogram for primary transitions of cortisol *m/z* 363/121 in patient serum sample with a concentration of free cortisol 0.068 µg/dL

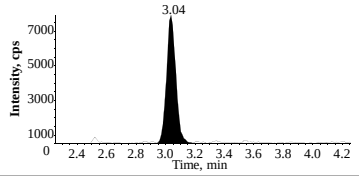


Table 1. fC Assay Imprecision: 3 replicates per run over 20 days; CV, coefficient of variation; to convert from µg/dL to nmol/L multiply fC concentration by 27.6.

Free Cortisol	Mean, µg/dL	Within run, CV%	Between run/day, CV%	Total Imprecision %
Level 1	0.45	8.84	4.06	9.73
Level 2	1.49	6.25	6.26	8.84
Level 3	23.27	1.93	2.61	3.25
Level 4	42.95	1.89	1.43	2.37

Table 2. Limit of Quantitation (LOQ) and Upper Limit of Linearity (ULOL)

LOQ µg/dL	ULOL µg/dL
0.06	60.0

Figure 2a, b: Histogram with distributions of concentrations of AM (2a) and PM (2b) fC (µg/dL)

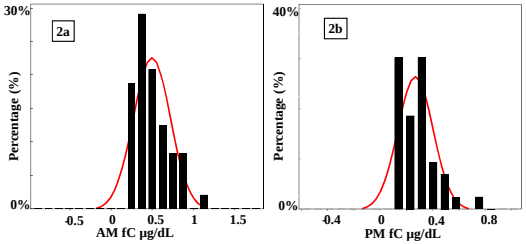


Table 3. Transformed parametric time-specific RIs for serum free cortisol

8-10 AM (n = 48)	4-6 PM (n= 44)
0.21–1.04 µg/dL	0.10 –0.63 µg/dL

Figure 3: Comparison of experimental method with an ED-LC-MS/MS method from another reference laboratory. The red line is the line of identity.

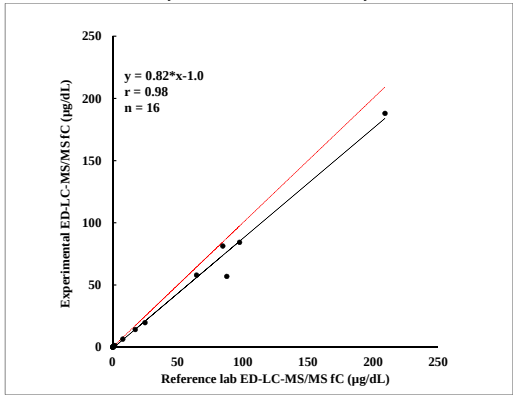


Figure 4a: Comparison of experimental ED-LC-MS/MS method vs ED-ECLIA; Inset figure 4b: Comparison of experimental ED-LC-MS/MS method vs ED-ECLIA between fC concentrations of 0.1-0.4 µg/dL

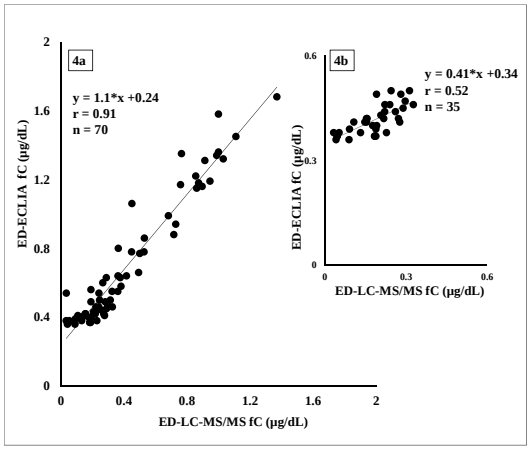
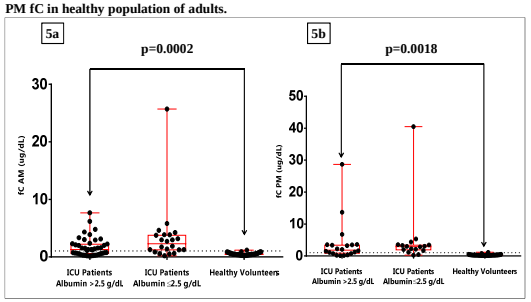


Figure 5a, b: Box plot representation of elevated AM (5a) and PM (5b) fC in two groups of ICU patients (with normal and low albumin) and healthy volunteers. Significantly different p values 0.0002 (fC AM) and 0.0018 (fC PM) between ICU patients with normal albumin and healthy volunteers. Dotted lines show reference interval of AM and PM fC in healthy population of adults.



Conclusions

During critical illnesses, the hypothalamic–pituitary–adrenal axis undergoes severe changes where the 12-hour diurnal profile of cortisol is lost, corticosteroid insufficiency occurs, and GC therapy is the preferred method of treatment. In our study both AM and PM samples from critically ill patients were associated with high levels of fC and low or normal tC. The need for GC therapy in these patients may be more accurately established by the measurement of fC, rather than tC, along with measurement of cortisol binding proteins. The method described here demonstrates that fC can be measured in an effective and reliable manner using ED-LC-MS/MS. Establishing reference intervals in a healthy adult population shows the utility of reliable fC measurement for improved test interpretation and patient care, particularly in critically ill populations of patients.

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References

Analysis of cortisol, cortisone and dexamethasone in human serum using liquid chromatography tandem mass spectrometry and assessment of cortisol: Cortisone ratios in patients with impaired kidney function, Ray et al, Clin Chimica Acta 412 (2011) 13-14)