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Determination of cortisol production rates with contemporary liquid chromatography-mass spectrometry to measure cortisol-d₃ dilution after infusion of deuterated tracer

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Abstract

Objectives—Measurement of 24-hour cortisol production rate (CPR) using steady-state infusion of deuterated cortisol and analysis of stable-isotope dilution by MS is a valuable tool to examine hypothalamic-pituitary-adrenal axis activity in humans. We have developed and validated an improved method for measuring cortisol dilution with contemporary LC-MS technology.

Design and Methods—Plasma samples and calibrators were extracted with ethylacetate. LC-MS was performed with a Surveyor HPLC and TSQ Quantum triple-quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source.

Results—Selectivity was improved over previous methods *via* elimination of an interferent identified as 20 β -dihydrocortisol. The LLOQ for cortisol-d₃ was 2.73 nmol/L and LOD 1.37 nmol/L. Plasma calibrators were linear over the concentration range 1.5%-10% cortisol-d₃, with correlation coefficients >0.995.

Conclusions—This APCI LC-MS method offers simplified sample work-up and analysis and enables selective detection of the low concentration of cortisol-d₃ infused for determination of 24-hour CPR.

Keywords

Cortisol production rate; Plasma; Atmospheric pressure chemical ionization; Liquid chromatography-mass spectrometry; 20 β -dihydrocortisol

1. Introduction

Measurement of hypothalamic-pituitary-adrenal (HPA) axis activity has found increasing application across many areas of clinical research, although methods for assessing HPA axis activity are not consistent. Determination of 24-hour cortisol production rate (CPR) by steady state stable-isotope tracer infusion and dilution analysis offers advantages compared to other measures of HPA axis activity [1]. This technique takes into account changes in the circadian variation in cortisol and is independent of differences in cortisol binding globulin between individuals, enabling sensitive detection of alteration in total daily cortisol production within the normal range in humans. In addition, the low level of stable-isotope tracer used allows the technique to be an effective tool for the study of HPA axis activity with negligible impact on endogenous cortisol physiology.

Dilution of cortisol by deuterated tracer for calculation of 24-hour CPR is measured using mass spectrometry (MS) techniques [1,2], predominantly gas chromatography (GC)-MS [1]. GC-MS generally requires laborious clean up of biological samples or frequent column maintenance, along with derivatization of samples for increased volatility. In contrast, analysis by liquid chromatography (LC)-MS is possible without sample derivatization. Indeed, cortisol derivatization was not required for 24-hour CPR determination with thermospray ionization LC-MS to measure stable-isotope dilution [2]. However, contemporary ionization techniques like atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) have succeeded thermospray ionization over the last decades [3] and are now widely used coupled with LC for measurement of steroids by isotope dilution MS. Although APCI and ESI LC-MS and tandem MS (MS/MS) methods for quantification of cortisol from serum or plasma possess acceptable accuracy and precision [4-10], deuterated internal standard analogues are typically spiked at levels 10-100 fold higher than the method lower limit of quantification. In contrast, calculation of 24-hour CPR by cortisol-d₃ infusion requires measurement of dilution within the range 1.5-10% cortisol-d₃ to cortisol-d₀. At the low level of deuterated tracer utilized, plasma interferences are significant that do not play a role at the concentrations of cortisol-d₃ used as internal standard for other published methods [4-6].

In this article we describe development and validation of an APCI LC-MS method with the selectivity to enable accurate and reproducible determination of plasma cortisol-d₃ at the concentration observed in subjects infused to enable calculation of 24-hour CPR. We report separation of a novel interferent in the detection of cortisol-d₃, allowing for improved selectivity over previous LC methods [2].

2. Experimental

2.1. Chemical and reagents

Dexamethasone was obtained from Sigma (St. Louis, MO, USA). Fenofibrate (Lofibra™) from Gate Pharmaceuticals (Sellersville, PA, USA). Cortisol (98% purity), 20 β -dihydrocortisol (DHC), prednisolone and prednisone were purchased from Steraloids (Newport, RI, USA). A 2.75 mmol/L dilution in ethanol of 9,12,12-²H₃-cortisol (99.8%) from Cambridge Isotope Labs (Andover, MA, USA) was prepared for human administration by the Oregon Health & Sciences University (OHSU) research pharmacy according to the code of federal regulations for good manufacturing process [1]. HPLC grade ethyl acetate, methanol and water were purchased from Burdick and Jackson (Muskegon, MI, USA). Volume 0.5 mL Ultrafree-MC centrifugal filters (0.45 μ m) were from Millipore (Bedford, MA, USA). Ethyl acetate was added with a dispensette from Brinkmann Instruments Inc. (Westbury, NY, USA). The Vx2500 multi-tube vortexer was from VWR (West Chester, PA,

USA). Centrifuges used were an Allegra 6R from Beckman (Fullerton, CA, USA) and a model 5402 from Eppendorf (Westbury, NY, USA).

2.2. Sample collection

Twenty seven subjects were consented and admitted to the General Clinical Research Center at OHSU the evening prior to beginning study procedures. Samples were collected as previously described [1] according to OHSU Institutional Review Board approved policies and procedures. On admission, two separate peripheral intravenous lines were started, one for infusion of deuterium-labeled cortisol isotope, and the other for drawing blood. Starting at 02:00 hour, a continuous infusion of deuterium-labeled cortisol (54.6 nmol/hour) was administered at a constant rate over the next 30 hour (until 08:00 hour of the final study day). Blood samples (5 mL) were drawn every 30 minutes from the second IV line during the final 24 hour of the deuterated cortisol infusion. The total amount of deuterated analog infused over 24 hour (1.31 μ mol) was less than 5% of normal endogenous 24-hour CPR. Plasma was separated, and 50 μ L from each sample was combined into a 24-hour pooled sample for mass spectral analysis. The samples were stored at -80°C. GC-MS sample preparation and analysis was performed as previously described [1].

2.3. Preparation of calibrators and samples

A stock dilution of cortisol-d₀ was prepared at 2.75 mmol/L in ethanol. Further working dilutions of cortisol-d₀ and cortisol-d₃ were prepared in methanol at 13.7 mol/L and 1.37 μ mol/L respectively. Extracted with each set of samples were plasma method calibrators spiked to generate cortisol-d₃/d₀ ratios ranging from 1.5% to 10% cortisol-d₃. These were made using drug-free Na EDTA male/female pooled plasma from Biological Specialty Corp (Colmar, PA, USA). After addition of cortisol, plasma samples were equilibrated for 1 hour. Prior to spiking, plasma cortisol-d₀ levels were determined in the OHSU General Clinical Research Center Core Laboratory by automated Immulite chemiluminescent assay from Diagnostic Products Corporation (Los Angeles, CA, USA). Cortisol-d₃ was spiked at various concentrations from 2.73-18.0 nmol/L (allowing for measurement of cortisol down to 27.5 nmol/L; although concentrations determined in plasma from healthy volunteers at different time points provide a lower cut-off for physiologic cortisol of around 110-165 nmol/L [9]). After transferring 0.5 mL of the pooled plasma aliquots to glass tubes, 5 mL of ethyl acetate was added. The tubes were shaken on a multi-tube vortexer then centrifuged at 200 times g for 15 minutes -. The upper organic layer in each case was transferred to a new glass tube and the aqueous phase extracted with a further 2.5 mL ethyl acetate. Double extraction was necessary for LC-MS detection of cortisol-d₃ with TSQ Quantum Discovery instrument After storage at -20°C for up to 48 hours the combined organic extracts were dried under vacuum at 40°C. The dried extracts were reconstituted in 80 μ L of HPLC mobile phase. They were briefly vortexed and agitated at room temperature for 15 minutes, then centrifuged and filtered using centri-filters at 4°C to remove any sample particulate present in order to prevent back-pressure issues with sub-2 μ m particle size HPLC column. Samples were transferred to auto-sampler vials with 200 μ L inserts and kept at 4°C until injection.

2.4. LC-MS

The method was validated using a Thermo TSQ Quantum Discovery triple-quadrupole mass spectrometer (San Jose, CA, USA) equipped with an APCI source. The ionization interface was operated in the positive mode using the following settings: source vaporizer temperature, 450°C; corona discharge current, 4 kV; sheath and aux gas flow rates, 40 and 5 respectively; tube lens voltage, 150 V; capillary voltage, 35 V; and capillary temperature, 250°C. The MS method was optimized using infusion experiments to examine cortisol ionization. A syringe pump was used to provide a constant analyte stream from 1-10 ng/

minute into the HPLC flow using a T-connection. A selected ion monitoring instrument method was created to monitor for m/z 363.2 and 366.2, corresponding to the $[M+H]^+$ ions for cortisol- d_0 and cortisol- d_3 respectively. Scan event settings were scan width 1.5 m/z , scan time 0.5 s, and Q1 peak width 0.7. The LC-MS system was composed of an in-line Thermo Surveyor auto-sampler and HPLC pump. Cortisol isotopes were resolved from other plasma components using a 50×2.1mm, 1.9 μ m Hypersil Gold C₁₈ column with an in-line C₁₈ 10×2.1mm, 3 μ m Javelin guard. The column temperature was 20°C and the isocratic mobile phase consisted of methanol and water (45:55 by volume) delivered at a flow rate of 0.25 mL/minute. The injection volume was 20 μ L. Between each sample injection the needle was flushed and washed with methanol. Sample runs included a 15 minute column wash at 95% methanol 0.1% acetic acid every 5 extracts. The first 2 minutes of the HPLC run were diverted to waste and cortisol isotopes eluted within 12 minutes with acceptable peak shape. Data acquisition and quantitative processing were accomplished with Thermo Xcalibur software, Version 2.0. Quantification was by integration of peak area. Daily CPR was calculated from the product infusion rate (IR) and the ratio of the isotopic enrichment to isotopic dilution in plasma ($CPR = IR/(d_3/d_0)$) [1,11]. This calculation assumes that the absorption, distribution, and metabolism of cortisol- d_3 and cortisol- d_0 are equal.

3. Results

3.1. LC-MS

Maximum ion intensity for cortisol was achieved with APCI, with no occurrence of in-source cortisol fragmentation, as previously described with LC-thermospray MS [2]. Cortisol-9,11,12,12- d_4 was reported to undergo deuterium/hydrogen exchange with water forming unlabeled steroid with APCI [12]. This was not a significant process for cortisol-9,12,12- d_3 at the concentrations employed for determination of CPR. An MS profile recorded in the positive mode demonstrates the $[M+H]^+$ ions detected for cortisol- d_3 and cortisol- d_0 authentic standards (at 10% cortisol- d_3 , Figure 1). The isotope dilution ratio of cortisol- d_3 /cortisol- d_0 was determined in plasma samples with LC-MS experiments monitoring for the ions m/z 366 and 363. Extracted ion chromatograms are shown that demonstrate detection of cortisol- d_3 at 9.8 minutes from various plasma extracts (pooled-plasma blank and representative calibrator at 1.5% cortisol- d_3 , respectively, Figure 2 Panels A and B, and plasma from study subjects where significant 20 β -dihydrocortisol (DHC) A+1 isotope signal was detected Figure 2 Panel C). Separation of cortisol from 20 β -DHC (retention 8.9 minutes) was required as 20 β -DHC A+1 isotope signal detected would otherwise have significantly contributed to variation in the method. Use of a 1.9 μ m particle size Hypersil Gold™ C₁₈ column (50×2.1mm) enabled resolution of cortisol- d_3 and 20 β -DHC within 12 minutes with acceptable peak shape. Cortisol carry-over was <0.1% when a methanol needle wash was included between each sample injection.

3.2. Method validation and comparison

The cortisol- d_3 signal-to-noise value for the lowest plasma calibrator (1.5% cortisol- d_3) was >5:1 (Figure 2 Panel B). The lower limit of quantification for cortisol- d_3 (determined as the lowest ratio at which CV was 15% or less) was for the 1.5% calibrator at 2.73 nmol/L plasma cortisol- d_3 . The limit of detection for cortisol- d_3 was 1.37 nmol/L plasma (where signal-to-noise ratio was 3:1). For all plasma calibrators the intra- and inter-assay precision (CV) for calculated percent cortisol- d_3 across the range 2% to 10% cortisol- d_3 was <15% and accuracy was within $\pm 15\%$ (detailed precision and accuracy information for low and high QCs provided in Table 1). Calibrators were extracted from commercially available drug-free pooled plasma. A least-squares linear regression of peak area ratio (cortisol- d_3/d_0) versus percent cortisol- d_3 was used for calibration. Plasma calibrators were included with each sample set and monitored over 6 months. Acceptable linearity was observed with

characteristic correlation coefficients (r^2) greater than 0.995. Calibration curves were reproducible with a typical linear regression equation of $y = 0.005 + 0.01x$. Plasma calibrators were generated ranging from 1.5-10% cortisol- d_3 to encompass the stable-isotope dilution ratios obtained after steady-state infusion of cortisol- d_3 [1]. To date, >99% of more than 300 samples we analyzed for a variety of clinical research studies fell within the range 1.5-10% cortisol- d_3 , confirming this range is adequate for measuring CPR in diverse patient populations (data not shown). Initial isolation of cortisol by SPE with methanol elution [1,13] demonstrated large variation for analyte signal recovery. A comparison of SPE with methanol or ethyl acetate elution [14], liquid-liquid extraction using methyl-*tert*-butyl ether [7], dichloromethane [12,15], or ethyl acetate [14] found the most consistent signal recovery for cortisol was obtained with liquid-liquid extraction using ethyl acetate (data not shown). After generating plasma with cortisol- d_3 across the range 1.5% to 7.5%, values for cortisol- d_3 signal recovery from replicate experiments were 110-116% at 1.5% cortisol- d_3 , were 96-127% at 3% cortisol- d_3 , were 116-119% at 4.5% cortisol- d_3 , were 106-110% at 6% cortisol- d_3 and were 92-100% at 7.5% cortisol- d_3 . Mean absolute signal recovery averaged 109%, indicating an ionization enhancement effect from matrix.

Cortisol isotope dilution ratios were stable after three plasma freeze (-80°C)/thaw cycles with the peak area ratios differing from a baseline value by an average of 0.8%. Ratios were stable for at least a year when plasma was stored at -80°C . Ethyl acetate extracts were stored at -20°C for up to 96 hours with no difference from baseline in peak area ratio. Storage stability for re-constituted extracts in the auto-sampler was evaluated at 4°C by analysis at time zero and after 24 hours. The cortisol isotopes exhibited up to a 40% loss in absolute signal but demonstrated no variation in cortisol- d_3/d_0 isotope dilution ratios for up to 24 hours. Extracts in mobile phase were stable after storage at -80°C for up to one month. The ethanol and methanol standard stocks were stable at -80°C for at least a year.

Examination of commonly encountered synthetic corticosteroids reported as possible method interferents (including prednisolone, prednisone, and dexamethasone) revealed prednisolone A+2 isotope signal at the retention time of cortisol when monitoring for m/z 363 ion [15,16]. Prednisolone signal was >5% that of cortisol at a 5:1 ratio of prednisolone:cortisol, a ratio that is attainable with prednisolone treatment. Therefore method interference can be expected and prednisolone-treated subjects should be excluded from determination of 24-hour CPR with this method. The drug fenofibrate was also reported as a potential interferent for detection of cortisol by LC-MS/MS [17,18]. Detection of m/z 363 ion for fenofibrate was also >5% the signal for cortisol at a 750:1 ratio of fenofibrate:cortisol (a ratio attained in plasma after treatment [19]), therefore interference from this drug can be expected and subjects treated with fenofibrate should be excluded.

The CPR values from measurements obtained by negative ion chemical ionization GC-MS [1] and APCI LC-MS from identical samples demonstrated acceptable correlation (Bland-Altman plot Figure 3 Panel A) [20,21]. To account for imprecision in both methods the results were also evaluated by Deming regression [22] (Figure 3 Panel B), revealing a correlation coefficient $r = 0.916$ ($p < 0.001$). The Bland-Altman difference plot and regression analysis show a constant systematic inter-method CPR difference of $2.5 \mu\text{mol/day}$. Random variation around the mean difference is constant across the range of measurement. Although the sample size was small ($n=27$ subjects) and limited sample availability did not allow replicate sample analysis by both methods, the method comparison was acceptable. The method demonstrated acceptable accuracy with a regression analysis for the method comparison that closely followed the line of identity.

4. Discussion

Over the last decades it has been demonstrated that measurement of cortisol with isotope dilution MS offers multiple advantages over HPLC-UV and immunoassay methods that suffer from a lack of specificity and consequent positive method bias. Concurrently the benefits of MS coupled to HPLC have increased the popularity of LC-MS methods in clinical research laboratories. A number of LC-MS and MS/MS methods for measuring cortisol in plasma or serum have been described [4-10]. Unlike quantification of cortisol with isotope dilution MS, calculation of CPR by infusion of deuterated tracer requires measurement of much lower concentrations of cortisol-d₃ [1]. This is the first detailed study examining endogenous interference for LC-MS detection of plasma cortisol-d₃ at the low concentrations we infused for CPR measurement.

We describe elimination of the endogenous method interferent 20 β -DHC utilizing HPLC, with chromatographic run times achieved comparable to those for negative ion chemical ionization GC-MS [1]. LC-MS/MS can also be used to improve method selectivity, and selected reaction monitoring (SRM) methods for quantification of cortisol have been described that monitor for the transition from cortisol and deuterated analog precursor ions to a CID m/z 121 product ion [5,7,8,12,¹³,15,17,18,23], a relatively non-specific ion originating from the A ring [24]. In our hands the m/z 366 ions of cortisol-d³ and 20 β -DHC A+1 isotope both generated m/z 121 product ion leading to little gain in method selectivity with SRM (supplementary data). Other LC-MS/MS methods for cortisol quantification monitor for the transition from [M+H]⁺ precursor ion to [M+H-2H₂O]⁺ product ion [4,14], which is also not useful as the m/z 366 ions of cortisol-d³ and 20 β -DHC A+1 isotope both generated m/z 330 product ion under our method conditions. For these reasons we chose not to pursue an MS/MS method but utilized chromatographic separation of 20 β -DHC from cortisol to minimize endogenous interference.

The LC-MS method reported here for determination of 24-hour CPR by infusion and measurement of cortisol-d₃/cortisol-d₀ dilution was compared to a prior negative ion chemical ionization GC-MS method, which involved conversion of plasma cortisol to fluoracyl derivative [1]. Values for identical samples analyzed by GC-MS and LC-MS demonstrated acceptable method correlation. Greater selectivity is provided by the LC-MS method compared to previous methods where separation from the endogenous interferent 20 β -DHC was not ensured [2]. At least 150 plasma samples have been analyzed by duplicate injection with minimal column maintenance. For healthy subjects, the measured CPR values were within the range previously reported [1], with a mean body surface area adjusted CPR of 18.7 ± 0.8 $\mu\text{mol}/24\text{-hr}/\text{m}^2$.

As the method presented in this manuscript measures total cortisol production rate, one limitation is the inability to differentiate between cortisol produced via the HPA axis, and that which may be produced peripherally by the enzyme 11- β hydroxysteroid dehydrogenase type 1. 11- β hydroxysteroid dehydrogenase type 1 and type 2 (HSD1 and HSD2) are isozymes that interconvert cortisol and cortisone differentially within various tissues. Upon infusion of cortisol-d₃, peripheral tissue interconversion of cortisol-d₃ and cortisone-d₃ by HSD1 and HSD2 may occur, which would reach steady state after approximately 4 hours of infusion [25-27]. The equilibrium reached would be expected to be equivalent to the ratio of whole body cortisol and cortisone interconversion. Thus, this method is able to take into account production of cortisol from multiple sources, and provide an accurate measure of whole body cortisol production.

In summary, we have developed an APCI LC-MS method suitable for measuring cortisol-d₃/cortisol-d₀ dilution in plasma after deuterated tracer infusion to enable calculation of 24-

hour CPR. The LC-MS method can be used to very selectively determine plasma cortisol-d₃ down to 2.73 nmol/L; at least 10-fold lower than the cortisol-d₃ concentrations previously utilized for isotope dilution MS [4-6]. The availability of an APCI LC-MS method is a valuable addition as a clinical research tool and simplified sample work-up and ease of analysis make this the MS method of choice for analysis of cortisol-d₃/cortisol-d₀ dilution after deuterated tracer infusion to enable calculation of 24-hour CPR.

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References

1. Brandon DD, Isabelle LM, Samuels MH, Kendall JW, Loriaux DL. Cortisol production rate measurement by stable isotope dilution using gas chromatography-negative ion chemical ionization mass spectrometry. *Steroids*. 1999; 64:372–8. [PubMed: 10433173]
2. Esteban NV, Yergey AL, Liberato DJ, Loughlin T, Loriaux DL. Stable isotope dilution method using thermospray liquid chromatography/mass spectrometry for quantification of daily cortisol production in humans. *Biomed Environ Mass Spectrom*. 1988; 15:603–8. [PubMed: 3293669]
3. Lim CK, Lord G. Current developments in lc-ms for pharmaceutical analysis. *Biol Pharm Bull*. 2002; 25:547–57. [PubMed: 12033491]
4. Tai SS, Welch MJ. Development and evaluation of a candidate reference method for the determination of total cortisol in human serum using isotope dilution liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. *Anal Chem*. 2004; 76:1008–14. [PubMed: 14961732]
5. Tenhola S, Turpeinen U, Halonen P, Hamalainen E, Voutilainen R. Association of serum lipid concentrations, insulin resistance index and catch-up growth with serum cortisol/cortisone ratio by liquid chromatography tandem mass spectrometry in children born small for gestational age. *Pediatr Res*. 2005; 58:467–71. [PubMed: 16148058]
6. Vogeser M, Briegel J, Jacob K. Determination of serum cortisol by isotope-dilution liquid-chromatography electrospray ionization tandem mass spectrometry with on-line extraction. *Clin Chem Lab Med*. 2001; 39:944–7. [PubMed: 11758607]
7. Kushnir MM, Neilson R, Roberts WL, Rockwood AL. Cortisol and cortisone analysis in serum and plasma by atmospheric pressure photoionization tandem mass spectrometry. *Clin Biochem*. 2004; 37:357–62. [PubMed: 15087250]
8. Guo T, Taylor RL, Singh RJ, Soldin SJ. Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. *Clin Chim Acta*. 2006; 372:76–82. [PubMed: 16707118]
9. Huang W, Kalhorn TF, Baillie M, Shen DD, Thummel KE. Determination of free and total cortisol in plasma and urine by liquid chromatography-tandem mass spectrometry. *Ther Drug Monit*. 2007; 29:215–24. [PubMed: 17417077]
10. Carvalho VM, Nakamura OH, Vieira JG. Simultaneous quantitation of seven endogenous c-21 adrenal steroids by liquid chromatography tandem mass spectrometry in human serum. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008; 872:154–61.
11. Purnell JQ, Brandon DD, Isabelle LM, Loriaux DL, Samuels MH. Association of 24-hour cortisol production rates, cortisol-binding globulin, and plasma-free cortisol levels with body composition, leptin levels, and aging in adult men and women. *J Clin Endocrinol Metab*. 2004; 89:281–7. [PubMed: 14715862]
12. Taylor RL, Machacek D, Singh RJ. Validation of a high-throughput liquid chromatography-tandem mass spectrometry method for urinary cortisol and cortisone. *Clin Chem*. 2002; 48:1511–9. [PubMed: 12194928]

13. Dodds HM, Taylor PJ, Cannell GR, Pond SM. A high-performance liquid chromatography-electrospray-tandem mass spectrometry analysis of cortisol and metabolites in placental perfusate. *Anal Biochem.* 1997; 247:342–7. [PubMed: 9177697]
14. Tang C, Kassahun K, McIntosh IS, Brunner J, Rodrigues AD. Simultaneous determination of urinary free cortisol and 6beta-hydroxycortisol by liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry and its application for estimating hepatic cyp3a induction. *J Chromatogr B Biomed Sci Appl.* 2000; 742:303–13. [PubMed: 10901135]
15. Taylor RL, Grebe SK, Singh RJ. Quantitative, highly sensitive liquid chromatography-tandem mass spectrometry method for detection of synthetic corticosteroids. *Clin Chem.* 2004; 50:2345–52. [PubMed: 15486026]
16. Ionita IA, Fast DM, Akhlaghi F. Development of a sensitive and selective method for the quantitative analysis of cortisol, cortisone, prednisolone and prednisone in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009; 877:765–72.
17. Meikle AW, Findling J, Kushnir MM, Rockwood AL, Nelson GJ, Terry AH. Pseudo-cushing syndrome caused by fenofibrate interference with urinary cortisol assayed by high-performance liquid chromatography. *J Clin Endocrinol Metab.* 2003; 88:3521–4. [PubMed: 12915630]
18. Kushnir MM, Rockwood AL, Nelson GJ, Terry AH, Meikle AW. Liquid chromatography-tandem mass spectrometry analysis of urinary free cortisol. *Clin Chem.* 2003; 49:965–7. [PubMed: 12765998]
19. Keating GM, Ormrod D. Micronised fenofibrate: An updated review of its clinical efficacy in the management of dyslipidaemia. *Drugs.* 2002; 62:1909–44. [PubMed: 12215067]
20. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986; 1:307–10. [PubMed: 2868172]
21. Dewitte K, Fierens C, Stockl D, Thienpont LM. Application of the bland-altman plot for interpretation of method-comparison studies: A critical investigation of its practice. *Clin Chem.* 2002; 48:799–801. author reply -2. [PubMed: 11978620]
22. Martin RF. General deming regression for estimating systematic bias and its confidence interval in method-comparison studies. *Clin Chem.* 2000; 46:100–4. [PubMed: 10620577]
23. Xu R, Sang BC, Navre M, Kassel DB. Cell-based assay for screening 11beta-hydroxysteroid dehydrogenase inhibitors using liquid chromatography/tandem mass spectrometry detection. *Rapid Commun Mass Spectrom.* 2006; 20:1643–7. [PubMed: 16636996]
24. Ronquist-Nii Y, Edlund PO. Determination of corticosteroids in tissue samples by liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal.* 2005; 37:341–50. [PubMed: 15708676]
25. Andrew R, Smith K, Jones GC, Walker BR. Distinguishing the activities of 11beta-hydroxysteroid dehydrogenases in vivo using isotopically labeled cortisol. *J Clin Endocrinol Metab.* 2002; 87:277–85. [PubMed: 11788660]
26. Basu R, Singh RJ, Basu A, Chittilapilly EG, Johnson CM, Toffolo G, et al. Splanchnic cortisol production occurs in humans: Evidence for conversion of cortisone to cortisol via the 11-beta hydroxysteroid dehydrogenase (11beta-hsd) type 1 pathway. *Diabetes.* 2004; 53:2051–9. [PubMed: 15277385]
27. Basu R, Singh R, Basu A, Johnson CM, Rizza RA. Effect of nutrient ingestion on total-body and splanchnic cortisol production in humans. *Diabetes.* 2006; 55:667–74. [PubMed: 16505229]

Figure 1.
An APCI MS profile in the positive mode exhibits $[M+H]^+$ ions for cortisol- d_0 and cortisol- d_3 (m/z 363 and 366, respectively).

Figure 2.

Extracted ion chromatograms for m/z 366 demonstrate chromatographic separation of endogenous 20 β -DHC A + 1 isotope signal from cortisol- d_3 signal (*). As expected the chromatogram for pooled-plasma blank (Panel A) includes signal from naturally occurring cortisol A + 3 isotope (combination of ^{13}C and ^{18}O), which has a constant isotopic dilution ratio contribution (reflected in the y-intercept of the calibration curve). Significant 20 β -DHC A+1 signal is apparent in the 1.5% cortisol- d_3 spiked calibrator (Panel B) as well as in a number of the study subjects for which CPR was determined (representative subjects, Panel C).

Figure 3.

Bland-Altman plot comparison of GC-MS and LC-MS methods for measurement of 24-hour CPR (Panel A). The mean difference between methods of $-2.5 \mu\text{mol/day}$ is indicated by the dashed line, the 95% confidence intervals by dotted lines. Deming regression analysis of 24-hour CPR measured by LC-MS compared to GC-MS (Panel B), $y = -1.2429 + 0.9626x$; $r=0.92$, $p<0.01$.

Table 1

Precision characteristics of LC-MS method

	Intra-run		Inter-run	
<i>Nominal isotope dilution ratio (d_3/d_0) as percent-d_3</i>				
	2.0	10.0	2.0	10.0
<i>Calculated isotope dilution ratio (d_3/d_0) as percent-d_3</i>				
mean	2.1	10.8	2.2	10.8
SD	0.25	0.39	0.31	0.46
CV (%)	11.7	3.7	13.7	4.2
difference (%)	6.1	7.6	12.1	8.2
n	20	20	20	20