Mass spectrometry: an essential tool to be used in discrimination between causes of congenital adrenal hyperplasia, and its benefits versus radioimmunoassay

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Abstract

Background: Measurement of multiple steroids, 17 hydroxyprogesterone, 11 deoxycortisol, and 21 deoxycortisol, is required to discriminate between congenital adrenal hyperplasia due to 21 hydroxylase deficiency and that due to 11 beta hydroxylase deficiency. This work aims at the selection of the more appropriate, cost-effective method among either mass spectrometry or radioimmunoassay for the quantitation of the previous steroids. In this study, blood samples were collected from 31 patients that were newly diagnosed with congenital adrenal hyperplasia; 17 hydroxyprogesterone and 21 deoxycortisol were assayed using tandem mass spectrometry. Eleven deoxycortisol was assayed using 2 methods: radioimmunoassay and tandem mass spectrometry.

Results: Measuring 11 deoxycortisol using tandem mass spectrometry could significantly discriminate patients with 11 beta hydroxylase deficiency from those with 21 hydroxylase deficiency ($p = 0.002$), whereas radioimmunoassay failed ($p = 0.095$). Moreover, the former was highly predictive of 11 beta hydroxylase deficiency at a cutoff $\geq 11$ ng/ml with 100% sensitivity and 92.3% specificity. Simultaneous measurement of 21 deoxycortisol and 11 deoxycortisol and their enrollment in an equation yielded an overall predictive accuracy 96.8% for diagnosis of CAH due to both enzymatic deficiencies.

Conclusions: Measurement of 11 deoxycortisol using mass spectrometric approach is mandated as a part of work up to differentiate types of congenital adrenal hyperplasia.

Keywords: Congenital adrenal hyperplasia, Mass spectrometry, 11 deoxycortisol, 21 hydroxylase deficiency

1 Background

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders leading to deficient cortisol production due to defect in one of the enzymes in the pathway of its synthesis [1]. The most common enzyme deficiency is 21 hydroxylase deficiency (21OHD), which accounts for more than 90% of all cases of CAH [2]. In the classic form of 21OHD, the new born presents with salt losing crisis due to aldosterone deficiency, a commonly fatal condition if undiagnosed immediately after birth [3]. Another presentation is the ambiguity of female external genitalia [4]. In its non-classic form, 50% of the enzyme activity is retained, which leads to milder symptoms and later presentation of hirsutism, polycystic ovary syndrome, or menstrual irregularities [5].

Only 5-8% of cases of CAH are due to 11 beta hydroxylase deficiency (11βOHD) [6], an enzyme immediately downstream of the 21 hydroxylase; thus, it shares the same clinical presentation of 21OHD regarding
androgen excess, but peculiarly presents with hypertension due to accumulation of deoxycorticosterone instead of salt wasting [7]. Missed cases of 11βOHD will suffer the longstanding complications of hypertension as cardiomyopathy and retinal vein occlusion [8].

Measurement of 17 hydroxyprogesterone (17OHP) plays a role in diagnosis of CAH; however, measurement of 11 deoxycortisol that specifically increases in 11βOHD, and 21 deoxycortisol which is produced due 11 beta hydroxylation of 17OHP in case of 21OHD can aid in differentiation between these two types of CAH.

2 Methods

2.1 11 deoxycortisol detection by RIA

The kit was supplied by DIAsource Immunoassays SA, Belgium, catalog number KIPI20000. Twenty-five microliters of each of the calibrators, controls, and patients’ samples was used to perform the assay. The calibration curve was linear up to 56 ng/ml. Minimum detection limit was 0.4 ng/ml.

2.2 Standards and reagents of liquid chromatography tandem mass spectrometry (LC-MS/MS)

Gestodene (a progestogen hormonal contraceptive) was used as an internal standard (IS) stock solution prepared in methanol with concentration of 50 ng/ml. Stock solution of 21 deoxycortisol was prepared in methanol with concentration 200ng/ml. Eleven deoxycortisol and 17OHP were prepared in methanol with concentration 300ng/ml each. All stock solutions were stored at 2-8°C.

Preparation of working standard solutions in plasma from stock solutions for; 21 deoxycortisol certified reference material (Cerilliant, Round Rock, TX, USA) at concentrations 20, 30, 40, 80, 120, 140, 180, and 200 ng/ml and for 11 deoxycortisol and 17OHP (Steraloids, Newport, RI, USA) at concentrations 30, 45, 60, 120, 180, 210, 270, and 300 ng/ml.

Fifty microliters of each of the working standard solutions and 50 μl of IS were spiked into 450 μl of blank plasma, vortexed for 10 s so that the final concentrations of calibration standards were 2, 4, 8, 12, 14, 18, and 20 ng/ml respectively for 21 deoxycortisol and 3, 4.5, 6, 12, 18, 21, 27, and 30 ng/ml respectively for both 11 deoxycortisol and 17OHP.

There levels of quality control (QC) were prepared for each steroid from the stock solution, so that the final concentrations of high, medium, and low QC for 21 deoxycortisol were 16, 10, and 6 ng/ml, respectively and that for 11 deoxycortisol and 17OHP were 42, 15, and 9, respectively.

For each analyte, a calibration curve was drawn, and a regression line was calculated. The correlation coefficient, y-intercept and the slope of regression line were used to assess the linearity.

2.3 Sample preparation

Patient’s serum (500 μl) was spiked with 50 μl of IS, vortexed for 10 s. Liquid-liquid extraction was carried out by adding 3.5 ml tert-butyl methyl ether, samples were vortexed for 4 min and then were centrifuged at 4000 rpm (1789× g) at 5 °C for 10 min [10]. The clear supernatant was transferred into clean Wassermann tube, evaporated then reconstituted with 400 μl methanol. Calibration standards and quality control samples were extracted in the same way.

2.4 LC-MS/MS

The column used for chromatographic separation was Zorbax SB column C18 (5 μm, 4.6 × 50 mm), the mobile phase used was isocratic methanol and 0.1% formic acid, pH 2.8 (85:15, v/v) at flow rate 0.6 ml/min at 40 °C, the injection volume was 7 μl.

The mass spectrometer used was Model API 4000, AB Sciex, Framingham, USA. The ion polarity was set in positive mode, and the source was TurboIon Spray. Standard solutions of 21 deoxycortisol, 11 deoxycortisol, 17OHP, and Gestodene IS (50.00 ng/mL of each in methanol) were directly infused into the mass spectrometer, and the operating conditions were optimized as summarized in Table 1. The nebulizer gas was air (zero grade), whereas nitrogen was used as the auxiliary, curtain, and collision gas. The source/gas-dependent parameters for 21 deoxycortisol, 11 deoxycortisol, and 17 OHP determination were as follows: curtain gas, 20 psi;
collision gas, 10 psi; medium temperature, 500 °C; ion spray voltage, 5500 V; ion source gas one, 45 psi; and gas two, 45 psi. A chromatogram of a calibration standard for each steroid parameter and the internal standard and their corresponding retention times are illustrated in Fig. 1.

### 2.5 Statistical method

The nonparametric Kruskal-Wallis test was used to compare independent samples. Median values and ranges were determined. Spearman rank correlation analysis for variables and the correlation coefficient ($r$) was calculated.

Logistic regression analysis was done to evaluate the joint discriminatory power of both 11 deoxycortisol and 21 deoxycortisol when simultaneously considered for diagnosis. Diagnosis of 21OHD was used as the (binary) dependent variable (where 1 is 21OHD and 0 is 11βOHD). Eleven deoxycortisol and 21 deoxycortisol levels were entered as independent (predictor) variables. Cox and Snell pseudo $R^2$ square was 0.49 indicating that the model accounted for 49% of the total variance in the dependent variable (diagnosis). Logistic regression analysis yielded the following equation:

$$\text{Logit}(P) = 2.961 \times (21-\text{deoxycortisol}) - 0.327 \times (11-\text{deoxycortisol}) + 3.917$$

Where, logit $(P)$ is the natural log of the odds of diagnosis of 21 hydroxylase deficiency. Logit $(P)$ can be converted into probability $(P)$ of 21 hydroxylase deficiency by using the following equation:

$$P = \frac{1}{1 + e^{-\text{logit}(P)}}$$

A $P$ value < 0.05 was considered statistically significant. Data were statistically analyzed using IBM SPSS v. 20.

### 3 Results

The median age of the patients was 6 (0.2-14) years. The most common presentation was ambiguity of external genitalia 73%, 38.4% were presented with salt losing crisis, and 16% presented with precocious puberty. None of the patients had hypertension at the time of presentation. The karyotype of 8 patients (25.8%) was 46 XY although 22 patients (70.9%) were recognized as males at birth, and that of the other 23 patients (74.2%) was 46 XX.

#### 3.1 Comparison of the RIA method to LC-MS/MS

Thirty-one samples were measured for 11 deoxycortisol using both methods, the median value by RIA was 23.4 ng/dl (6.4-56 ng/dl) and by LC-MS/MS was 1.6 ng/dl (0-76 ng/dl). LC-MS/MS method was able to discriminate between patients with 21OHD and 11βOHD ($p$ value = 0.002), whereas RIA failed ($p$ value = 0.095). Pearson’s correlation showed significant positive correlation between both methods ($r = 0.666, p = 0.000$).

#### 3.2 Simultaneous steroid analysis by LC-MS/MS

Elevation of 11 deoxycortisol was highly predictive of 11βOHD. Receiver operating characteristic (ROC) curve analysis demonstrated an area-under-curve (AUC) of 0.973 (standard error = 0.052, $p = 0.0001$). At a cutoff for ≥ 11 ng/ml, all the 5 patients with 11βOHD were detected (sensitivity = 100.0%). Only two of the 26 patients with 21OHD were falsely diagnosed with 11βOHD (specificity = 92.3%) (Fig. 2).
Although the median value for 21 deoxycortisol was 6 ng/dl (0.70 ng/dl), it showed significant positive correlation with 17OHP ($r = 0.805, p = 0.000$). Elevation of 21 deoxycortisol was poorly predictive of 21OHD. ROC curve demonstrated an area-under-curve of 0.588 (standard error = 0.135), $p = 0.512$. At a cutoff $\geq 6.4$ ng/ml, only 11 of the 21OHD patients were correctly diagnosed (sensitivity = 42.3%). None of the 5 patients with 11βOHD were falsely positively diagnosed (specificity = 100.0%) (Fig. 3).

When 11 deoxycortisol and 21 deoxycortisol were simultaneously considered and enrolled in the following equation for discrimination between 21OHD and 11βOHD diagnosis,

![Fig. 2](image-url)

**Fig. 2 a** ROC curve of 11 deoxycortisol measured by LC-MS/MS in diagnosis of CAH due to 11βOHD. **b** A dot plot chart shows that at cutoff $\geq 11$ ng/ml for 11 deoxycortisol measured by LC-MS/MS, sensitivity was 100% and specificity 92.3% for diagnosis.
Logit \( P \) = \(2.961 \times (21\text{-deoxycortisol}) - 0.327 \times (11\text{-deoxycortisol}) + 3.917\)

Then, the predicted probability of diagnosis was calculated for the 31 studied patients (had previous genetic testing) and was compared against their actual diagnosis, where \( P \) values > 0.5 predict 21OHD, while values < 0.5 suggest 11\( \beta \)OHD. Thirty out of 31 patients were correctly diagnosed, yielding an overall predictive accuracy of 96.8%. It was better for predicting 11\( \beta \)OHD, 100.0% were correctly diagnosed (5 out of 5 patients) than for predicting 21OHD, 96.2% were correctly diagnosed (25 out of 26 patients).

4 Discussion
In this study, we examined two methods used in steroid assay, RIA and LC-MS/MS, to find out the better method or declare that there is no obvious difference between them. We tried to set a cutoff for these methods that could help in future diagnosis, and to shed some light on the practicability of each method.

It was noticed that the median value for 11 deoxycortisol measured by RIA was 23.4 ng/dl (6.4-56 ng/dl),
according to the kit we used in this study which was certified for in vitro diagnostic, values greater than 2.55 ng/ml were considered positive. Thus, 100% of patients were considered positive according to this cutoff. When compared to LC-MS/MS, the median value of 11 deoxycortisol was 1.6 ng/ml (0-76 ng/dl), and 11 ng/ml was used as a cut off at which 26.9% of cases were suggestive of 11ßOHD.

The difference between the previous values reveals one of the disadvantages of RIA which is interference; it may be due to cross reaction between 11 deoxycortisol and other metabolites that increase in CAH as 21 deoxycortisol and have structural similarity to the target steroid molecule against which the assay antibodies were generated. This was in agreement with Travers et al. [11], who attributed this overestimation to a lack of specificity of antibodies by cross-reactivity, and to potential miscalibration resulting from differences in calibrator designs and biological matrix of calibrators and Fiet et al. [12] who referred this problem to lack of standardization of calibrators among the RIA kits.

However, applying Pearson’s correlation between 11 deoxycortisol measured by RIA and that measured by LC-MS/MS showed that there was positive correlation between the 11 deoxycortisol measured by the two methods, $r = 0.666$ and $p$ value = 0.000. So, it deserves trials to be done to find out easy methods for sample extraction and purification before applying RIA. Sample treatment before RIA would increase its specificity [13], and makes it a more available substitute for LC-MS/MS, as the latter needs a high starting cost of instrumentation and more importantly needs well experienced users and operators.

The biggest problem with analyzing steroids by mass spectrometry is the isobaric interferences: 11 deoxycortisol and 21 deoxycortisol in this study and the similar fragmentation patterns of some endogenous steroids [14]. Therefore, the use of chromatography before mass spectrometry becomes critical to the unambiguous measurement of the various steroids.

An add-on advantage to LC-MS/MS is that one sample subjected to same steps of sample preparation, single injection to LC column for separation, and detection of multiple steroids, as long as their calibrators are available and their retention times are known. On the other hand, on using RIA, a kit should be available for each parameter, each prepared and measured separately. Pitt [15] agreed on the practicability of LC-MS/MS versus RIA and added that LC-MS/MS was fast, reliable method that needed small sample volume allowing diagnosis of adrenal diseases in newborns.

In the study in hand, measuring 11 deoxycortisol using LC-MS/MS was beneficial in stratifying cases of CAH according to the deficient enzyme whether 21 hydroxylase or 11 beta hydroxylase ($p$ value = 0.002), its median value was 49.3 ng/ml among patients with 11 beta hydroxylase deficiency and was only 0.1 ng/ml in patients with 21 hydroxylase deficiency. Twenty-one deoxycortisol failed to stratify cases of CAH according to the deficient enzyme, though it was 100% specific but with low sensitivity, this was in agreement with Boelen et al. [16]; they found that 21 deoxycortisol was discriminative for 21 hydroxylase deficiency and its addition to 17OHP could improve the sensitivity of screening programs.

Better results were found when both 11 deoxycortisol and 21 deoxycortisol were used simultaneously and incorporated in the equation obtained by logistic regression analysis with overall predictive accuracy 96.8%. This was in concordance with the study of Janzen et al. [17, 18] which showed that the analysis of 17OHP, 11 deoxycortisol, 21 deoxycortisol, cortisol, and androstenedione in one sample using LC–MS/MS reduced the number of false-positive results to almost zero and was able to precisely discriminate between 21OHD and 11ßOHD.

Patients exceeded the cutoff suggested for diagnosing 11ßOHD when 11 deoxycortisol was used alone, comprised 22.5% of the cohort but with specificity 92.3%. But when both 11 deoxycortisol and 21 deoxycortisol were used in the previous model, patients probably diagnosed with 11ßOHD were 19.3%. Putting into consideration that the prevalence of 11ßOHD among CAH patients is 5-8%. The observed higher prevalence in this study cohort may be related to the increase of this form of enzymatic deficiency among people with African descent, as there is a lack of reporting on the prevalence of CAH in countries with poor resources, and lack of screening facilities. Only 4 countries in the Middle East have newborn screening programs, not necessarily including a screening test for CAH [19].

Although the sample size of this study is small but it is important as there are only few studies concerning this perspective in the Middle East; however, it showed a high level of significance not affecting its reliability.

4.1 Study limitation
The limited funding resources, the refusal of many parents to share in the study, and the selection of patients that had not started steroid treatment were among the reasons of the small sample size. The study needs to be complemented with others: after increasing the sample size, taking samples from different regions in Egypt, and even increasing the number of the studied parameters. That could help to have reliable data base on the Egyptian population regarding the prevalence of CAH and its types.
5 Conclusions
In conclusion, LC-MS/MS is a better method than RIA in diagnosis of CAH, although the initial cost is high, its cost effective as it simultaneously measures multiple parameters that could help in diagnosis and differentiation of types of CAH, especially in screening programs, ending up in proper diagnosis, treatment, avoiding many complications, and saving time using only one sample.

References
1. Alqahtani MA, Shati AA, Zou M, Alsheehl AM, Alhayanii AA, Al-Qahtani SM et al (2015) A novel mutation in the CYP1B1 gene causes steroid 11beta-
hydroxylase deficient congenital adrenal hyperplasia with reversible cardiomyopathy. Int J Endocrinol 2015:595164
2. New M, Yau M, Lekaev O, Lin-Su K, Parra A, Pina C et al (2000) Congenital adrenal hyperplasia. (Updated 2017 Mar 15). In: De Groot LJ, Chrousos G, Dungan K et al (eds) Endotext. MDText.com, Inc., South Dartmouth Available from: https://www.ncbi.nlm.nih.gov/books/NBK279833/
3. Falhammer H, Nordenstrom A (2015) Nonclassic congenital adrenal hyperplasia due to 21-hydroxylase deficiency: clinical presentation, diagnosis, treatment, and outcome. Endocrine 50(1):32–50. https://doi.org/10.1007/s12020-015-0656-0
4. Sharma R, Seth A (2014) Congenital adrenal hyperplasia: issues in diagnosis and treatment in children. Indian J Pediatr 81(2):178–185. https://doi.org/10.1007/s12020-013-0948-5
5. Skepper PW, Azizz R, Baskin LS, Ghizzioli L, Hensle TW, Merke DP, Meyer-Bahlburg HF, Miller WL, Montori VM, Oberfield SE, Ritzen M, White PC, Endocrine Society (2010) Congenital adrenal hyperplasia due to steroid 21-
hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 95(4):1433–4160. https://doi.org/10.1210/jc.2009-2631
6. Munar A, Fraxee C, Garg U (2016) Quantification of dehydroepiandrosterone, 17-deoxycorticosterone, and testosterone by liquid chromatography-tandem mass spectrometry. J Steroid Biochem Mol Biol 165(Pt B):202–211
7. Fett J, Villette JM, Galonis H, Boudou P, Burthier JM, Hardy N, Soliman H, Julien R, Vexiau P, Gourmelen M, Kuttenn F (1994) The application of a new highly-sensitive radioimmunoassay for plasma 21-deoxycorticosteroids to the detection of steroid-21-hydroxylase deficiency. Ann Clin Biochem 31(Pt 1):56–64. https://doi.org/10.1177/00033051940301010
8. Sippel WG, Billigmeaner F, Becker H, Brunig T, Dorr H, Hahn H, Golder W, Hoffmann G, Knor D (1978) Simultaneous radioimmunoassay of plasma aldosterone, corticosterone, 11-deoxycorticosterone, progesterone, 17-
hydroxyprogesterone, 11-deoxycorticosterol, cortisol and cortisone. J Steroid Biochem 9(1):63–74. https://doi.org/10.1016/0022-4731(78)90104-8
9. Honour JW (2010) Liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30(1):19–38. https://doi.org/10.2478/v10139-009-0034-7
10. Boelen A, Ruiter AF, Claasen-van der Grinten HL, Endert E, Ackermans MT (2016) Determination of a steroid profile in heel prick blood using LC-MS/ MS. Bioanalysis 8(5):375–384. https://doi.org/10.4155/bio.16.6
11. Janzen N, Sander S, Terhardt M, Peter M, Sander J (2008) Fast and direct quantification of adrenal steroids by tandem mass spectrometry in serum and dried blood spots. J Chromatogr B Analyst Technol Biomed Life Sci 861(1):117–122. https://doi.org/10.1016/j.jchromb.2007.11.006
12. Janzen N, Sander S, Terhardt M, Steunenberg U, Peter M, Das AM, Sander J (2011) Rapid steroid hormone quantification for congenital adrenal hyperplasia (CAH) in dried blood spots using UPLC liquid chromatography-tandem mass spectrometry. Steroids 76(13):1437–1442. https://doi.org/10.1016/j.steroids.2011.07.013
13. Saadallah A, Rashid M (2007) Newborn screening: experiences in the Middle East and North Africa. J Inherit Metab Dis 30(4):482–489. https://doi.org/10.1007/s10545-007-0660-5

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