Confirmation and Differential Diagnosis of Congenital Adrenal Hyperplasia from Dried Blood Spots by UHPLC–MS/MS

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Abstract

Newborn screening for congenital adrenal hyperplasia (CAH) has high false-positive rates, necessitating confirmation of primary results. We developed a single LC-MS/MS assay for dried blood spots (DBS) that allows concurrent confirmation and differential diagnosis of CAH. All five steroids (cortisol, 21-deoxycortisol, 11-deoxycortisol, 4-androstenedione and 17-hydroxyprogesterone) were baseline resolved and reliably determined (UHPLC: PerkinElmer Flexar FX-10; MS/MS: ABSCIEX QTRAP 5500; column: Phenomenex Kinetex XB-C18). In Hungary-Romania Cross-Border Cooperation Project (HU-RO 0802/008 SCREENGEN), the 21-hydroxylase deficient form of CAH was confirmed in one of a total of 163 samples tested positive in primary screening. Our validated assay can use the same DBS as in primary screening (2[nd]-tier test), eliminating the need for repeated blood sampling and accelerating diagnosis.

Introduction

Congenital adrenal hyperplasia (CAH), a severe inherited disorder of cortisol biosynthesis, can cause death in early infancy (due to disturbed sodium homeostasis) and prenatal virilisation in affected girls and signs of a postnatal androgen excess in both sexes (caused by an accumulation of steroid precursors, metabolized to androgens) [1].

Newborn screening for CAH is generally performed through measurement of the 17-hydroxyprogesterone (17-OHP) level in dried blood spots (DBSs) by means of a fluorescence immunoassay (DELFIA®) [1]. However, the number of false-positives in primary CAH screening is high: CAH is confirmed in only around 1-2 of every 100 positive results [1]. Therefore, primary screening results for CAH based on a 17-OHP assay must be confirmed with a second method, preferably using the same DBS specimen as in the primary screening [1].

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) can effectively decrease the number of false-positives in CAH screening [1, 2, 3]. However, earlier assays could not differentiate between the two main forms of CAH, a 21-hydroxylase deficiency and an 11β-hydroxylase deficiency, which together account for >99% of all cases [1]. This requires separation of 21-deoxycortisol (21-Deox) and 11-deoxycortisol (11-Deox), specific for these two forms of CAH [1]. Most previous assays for 21-Deox and 11-Deox did not allow analysis of DBSs [4, 5]; and those which did, achieved acceptable but not baseline resolution of these isobaric analytes [6].

Accordingly, we aimed to develop a single LC-MS/MS assay for the concurrent confirmation and differential diagnosis of CAH through the analysis of 21-Deox, 11-Deox, cortisol (Cort), 4-androstenedione (4-AD) and 17-OHP in DBS. We also set out to apply this method for

newborn screening in the frame of the Hungary-Romania Cross-Border Cooperation Project (HU-RO) 0802/008 SCREENGEN. Cross-border cooperation may be expected to improve the effectiveness of newborn CAH screening and eliminate possible issues (high initial costs and the need for trained analysts) concerning the implementation of the LC-MS/MS technique [7, 8, 9].

Experimental

For sample preparation, two spots 4.7 mm in diameter (corresponding to 13.6 µl blood) were punched out from filter cards and placed into 96-well round-bottom microtiter plates. For steroid extraction, 200 µl of the deuterated internal standard working solution was added to each vial. The plate was sealed and shaken for 50 min at ambient temperature. After centrifugation, the supernatant (150 µl) was transferred to a second plate and dried for 45 min. The underivatized residues were reconstituted in 45 µl methanol/water 20/80 (v/v), sealed and shaken for 20 min at room temperature.

The analysis was performed on a PerkinElmer Flexar UHPLC system (two FX-10 binary pumps, solvent manager with a degasser, autosampler and thermostatic oven; all *PerkinElmer Inc., Waltham, MA, USA*), and an AB SCIEX QTRAP 5500 MS/MS triple quadrupole mass spectrometer, controlled by Analyst 1.6.1 software (both *AB SCIEX, Framingham, MA, USA*). Following optimization of the MS/MS settings, 15 µl of sample was analyzed using multi-step gradient elution (total assay time: 13 min) using a Phenomenex Kinetex XB-C18 100x3.0 mm, 2.6 µm core-shell analytical column and a SecurityGuard Ultra Cartridge guard column (both *Phenomenex, Torrance, CA, USA*). Eluents A and B consisted of ultrapure water plus 0.1% formic acid, and methanol plus 0.1% formic acid, respectively.

In the frame of the Hungary-Romania Cross-Border Cooperation Project, DBS samples from newborns screened in county Timis, Romania, and suspected of having CAH on the basis of the primary screening (17-OHP DELFIA® assay, *Wallac Oy, PerkinElmer Inc., Turku, Finland*), were sent to our laboratory. The reported LC-MS/MS assay was applied as a second-tier test, using the same specimen as in the primary screening. After prompt determination of the steroid levels in Szeged, the results were immediately reported to the primary screening centre to facilitate early intervention. The Cross-Border Cooperation Project was approved by the Ethical Committees of all participating institutions (the University of Szeged, the Louis Turcan Emergency Hospital for Children and the Vasile Goldis Western University of Arad).

Results and discussion

Traditionally, the confirmation of primary CAH screening results was based on LC-MS/MS findings of elevated levels of 4-AD and 17-OHP, a decreased level of Cort and an elevation in the ratio (4-AD+17-OHP)/Cort [3]. In contrast with 4-AD and 17-OHP, 21-Deox and 11-Deox are specific markers for 21-OH and 11β-OH deficiencies, respectively [1]. Thus, their concurrent determination can improve the specificity of the assay and allow the differential diagnosis of the two most frequent forms of CAH [1].

Previous assays for 21-Deox and 11-Deox have almost exclusively used serum samples [4] or urine [5]. However, serum and urine can be obtained only with a delay which can hamper early recognition and treatment. Instead, the analysis of the same DBS specimen as in the primary screening eliminates the need for repeated blood sampling, can shorten time to diagnosis and improve the cost- and time-effectiveness of screening [2]. The avoidance of repeated blood sampling in the large number of false-positive cases can eliminate unnecessary family anxiety and the accompanying increase in the frequency of hospitalization [9].

We have successfully developed a single LC-MS/MS assay for the concurrent confirmation and differential diagnosis of CAH through the analysis of 21-Deox and 11-Deox in DBS

(together with Cort, 4-AD and 17-OHP). This method was validated in terms of linearity, lower limit of detection (LLOD; 1.0-5.0 nM), lower limit of quantitation (LLOQ; 2.5-12.5 nM), intra-assay precision (coefficient of variation, CV: <7%), inter-assay precision (CV: <10%), variation between injections (CV: <5%), retention time stability (CV: <1%) and extraction efficiency (CV: 69.7-85.2% for DBS calibrator *vs* methanolic standard; and 75.3-90.4% for DBS calibrator *vs*. spiked whole blood). Excellent baseline resolution (R_s>1.5) was achieved for all five metabolites in DBSs: the R_s was 4.28 for the pair Cort and 21-Deox; 3.46 for the pair 21-Deox and 11-Deox; 5.68 for the pair 11-Deox and 4-AD; and 6.51 for the pair 4-AD and 17-OHP. A representative LC-MS/MS chromatogram of a DBS calibrator is presented in Figure 1.

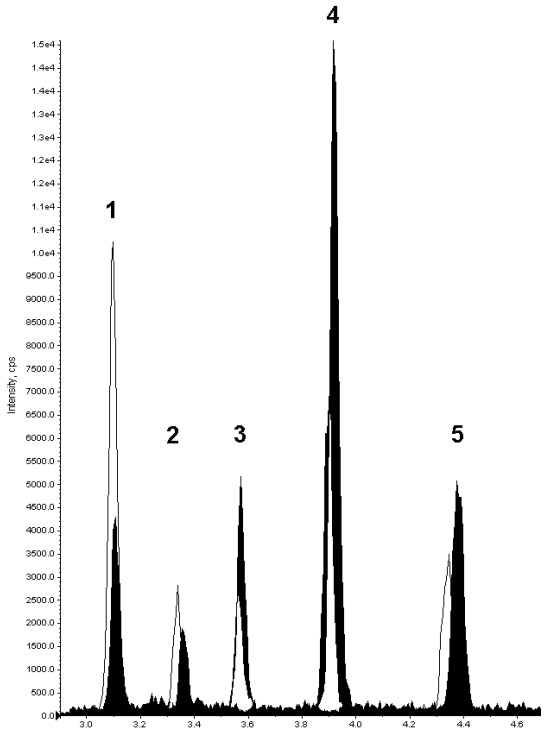

Figure 1. Representative LC-MS/MS chromatogram of a DBS calibrator.
Peak numbers: 1: Cort; 2: 21-Deox; 3: 11-Deox; 4: 4-AD; 5: 17-OHP (black peaks: unlabelled analytes; unfilled peaks: deuterated internal standards).

In addition, our LC-MS/MS assay proved applicable for the confirmation and differential diagnosis of CAH in cross-border cooperation for newborn screening. A total of 163 newborn DBS samples (including 16 prematures) submitted for second-tier testing were tested with the present assay. The results were compared with reference values of healthy mature newborns and prematures (related to gestational age). The classic 21-hydroxylase deficient form of CAH could be confirmed in one sample on the basis of the markedly elevated levels of 21-Deox, 4-AD and 17-OHP, the increased ratio (21-Deox+17-OHP)/Cort and the decreased Cort level (11-Deox concentration and ratio 11-Deox/Cort were normal). All other samples proved to be false-positives; an 11β-hydroxylase deficiency was not detected.

Conclusion

An LC-MS/MS method with excellent resolution of 21-Deox and 11-Deox in DBS has been developed, allowing the simultaneous confirmation of CAH and reliable differentiation between its two main forms, the 21-hydroxylase and 11β-hydroxylase deficiencies. The assay can use the same DBS as in the primary screening (second-tier approach), which can facilitate

early therapeutic intervention, improve the cost- and time-effectiveness of screening, and eliminate unnecessary family anxiety by decreasing the high recall rates caused by false-positives. Our method was successfully applied to clinical DBS samples in cross-border cooperation for newborn screening for CAH. The specificity and sensitivity of LC-MS/MS and the use of analyte ratios are expected to facilitate the diagnosis of mild, non-classic CAH.

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