University of Applied Sciences and Arts Northwestern Switzerland
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Determination of Marker Metabolites for CAH from DBS by MS/MS

Bader Pascal
Master thesis, Molecular Technologies

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INTRODUCTION

Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive disorder which can appear at birth. It is diagnosed by the newborn screening program at the University Children's Hospital Zurich (Kinderspital Zürich). In the case of CAH, the biosynthesis of hormones such as cortisol, aldosterone and testosterone are disturbed by a deficiency of enzymes. The whole steroidogenesis involving several metabolites is unbalanced. The current newborn screening method for congenital adrenal hyperplasia, which quantifies 17-hydroxyprogesterone (17-OHP), has a positive predictive value of 2% due to its poor specificity. There could be a possibility to substitute the currently used immuno-assay method which has a lot of cross-reactivity issues, with a tandem mass spectrometry method for dried blood spots (DBS) sample which would allow for a much higher positive predictive value. In order to allow the measurements of the high number of daily samples, the idea is to analyze the DBS by tandem mass spectrometry (TMS) without any preliminary chromatographic step. The goal of the Master's Thesis was to investigate if there is a possibility to quantify metabolites marker for CAH by TMS without chromatography. The cutoff values for newborns are between 20 and 300 nmol/l in blood. Therefore, the targeted quantification concentration was between 0.6 and 10 nmol/l, since the current extraction method has a 32x dilution factor.

MS/MS instrumentation:
Waters Xevo TQD (ESI)

UPLC-MS/MS instrumentation:
Waters Acquity H-class UPLC
(Acquity BEH C18 2.1x50mm, 1.7um)
Waters Xevo TQD (ESI)

Fig. 1: Sampling of the blood on filter paper by heel pick

CONCEPT

Two methods have been developed; a TMS method without chromatography and another TMS method with a preliminary UPLC (Ultra Performance Liquid Chromatography) separation, both to quantify 8 steroids including 17-OHP, cortisol, androstenedione, 11-deoxycortisol (11-DC), 21-deoxycortisol (21-DC), corticosterone, deoxycorticosterone and progesterone. The UPLC-MS/MS method has been created in order to observe the interferences and could also be useful for a 2nd tier method.

The first step was to find the optimal MS transitions and the best conditions such as collision energy and cone voltage. It has been discovered that 17-hydroxyprogesterone, which is the most important marker metabolite for CAH, shares the same MS transitions than deoxycorticosterone. This was problematic for the quantification in the MS/MS method without chromatographic separation. 11-DC, 21-DC and corticosterone had also identical precursor and product ions. Progesterone, androstenedione and cortisol have specific monoisotopic masses and their differentiation by MS/MS was not problematic.

Four UPLC methods for steroids found in publications [1]-[4] have been repeated in order to find a good method to separate the 5 challenging compounds. Leading to bad resolution (<1), a new UPLC method has finally been developed using the systematic KαN approach, which is based on the enhancement of the gradient retention factor, the median selectivity and the median theoretical plates number, in that order.

It has been quickly found out that the signal intensity would be too low to quantify at the aimed levels. Different strategies have been done in order to optimize the signals. A fine tuning of each MS parameters has been done, an investigation of alternative ionization mode (ESI-) and also other mobile phase additives than formic acid, such as ammonium acetate and ammonium fluoride have been examined. A new quantification algorithm has been conceived in order to discern compounds having the same monoisotopic mass and inducing the same product ions, using two non-specific transitions. Some tests on an Agilent instrument has also been performed. All tests were made in solvent, without matrix.

With the best possible conditions of the system, the quantification with internal standards for both MS method with and without UPLC have been characterized by determining their LOD, LOQ, linearity, recovery, intra- and inter-assay precision. Ion suppression has been determined for the method without chromatography.

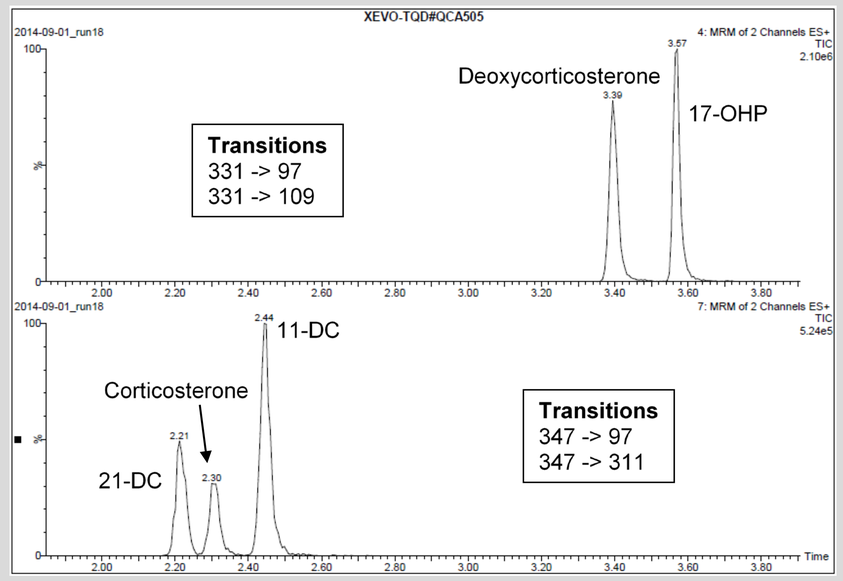

Fig. 2: Successful separation of deoxycorticosterone and 17-OHP on one hand, and 21-DC, corticosterone and 11-DC on the other hand.

RESULTS & CONCLUSION

The UPLC method allows the separation of the compounds with success (R>1) with a run time of 6.6 minutes. During the signal optimisation, the negative ESI mode trials were unsuccessful and the best results have been found with 1 nmol/l of NH_4F as additive in ESI+. The new quantification algorithm allowed to use more intense peaks for the method with no chromatography. All those strategies meant to increase the intensity, led to a remarkable enhancement of 30 times higher signals. It was possible to quantify 17-OHP by MS/MS as low as 4 nmol/l using the TMS method without UPLC, and at 20 nmol/l if it is necessary to discern it from deoxycorticosterone also without chromatography. Even though some repeatability issues were observed, the characterization of the quantification method of 17-OHP, cortisol, androstenedione and progesterone led to encouraging results for both methods. The tests on the alternative TQD showed acceptable indicative recovery and intra-assay precision at 0.6 nmol/l.

OUTLOOK

It has been concluded that the sensitivity is yet too low to quantify the steroids at the aimed level. However, tests on the minimal injection volume has been made and led to the conclusion that the dilution factor of the extraction method could be decreased from 32x to 9x. It could be possible to work at 7x higher concentration by using a new extraction method and by taking two 3.2mm DBS instead of one as it is in the current method, and even at 16x higher levels if taking a 6mm DBS.

Another possibility would be to perform further investigation on an alternative instrument. Indeed, the trials on an Agilent TQD 6460 showed very promising results. Finally, a third option would be to measure serum sample instead of dried blood spots. This would allow to take higher sample amount and concentrate it to have higher levels to measure. However, this would induce a huge change in the whole newborn sampling process and sample preparation.

TMS techniques will surely replace the non-specific immuno-assay method for the screening for CAH. Perspectives have been proposed which will certainly lead to a new screening method for CAH using MS technique with a much higher positive predictive value than the current method.

REFERENCES

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