A Strategy for Integrating the Inborn Errors of Metabolism Screening with the Congenital Adrenal Hyperplasia Screening in a Single FIA/LC-MS/MS Measurement

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Introduction

The Inborn Errors of Metabolism (IEM) screening protocol, using LC/MS/MS for the analysis of amino acids (AA) and acylcarnitines (AC), is now widely used. Despite its broad acceptance, there is an ongoing debate regarding the need for the derivatization step described in the original sample preparation procedure[1]. The derivatization by butylation hampers the simplicity of the assay and some users have opted to settle for a reduction in sensitivity, resulting from an "underivatized" approach, in exchange for a simplified process.

There is a desire to further expand the IEM screening protocol to include the measurement of certain steroids related to Congenital Adrenal Hyperplasia (CAH), using a simplistic sample preparation approach combined with a fully automated workflow. In this technical note we present a strategy for performing both IEM and CAH assays within a single LC/MS/MS analysis.

Background

- The Inborn Errors of Metabolism (IEM) screening protocol, using tandem mass spectrometry, measures amino acids (AA) and acyl-carnitines (AC) by flow-injection analysis (FIA) on dried blood spot (DBS) samples. A typical measurement takes 1-2 minutes and has been cited to reveal up to 40 different metabolic disorders. The high level of throughput is a key factor in the success of this screening method.
- Congenital Adrenal Hyperplasia (CAH) screening requirements are becoming more stringent in screening programs. Traditionally, 17-hydroxyprogesterone (17OHP) is measured by immunoassays, however LC/MS/MS is an attractive analytical technique for CAH screening because it can also screen for related steroids[2]. The result is a reduction in false positives and a more accurate quantitative result.
- The CAH screening assay using LC/MS/MS achieves a significantly lower throughput than the IEM assay, due to the requirement of a longer chromatographic (LC) runtime.
- Sensitivity challenges in steroid analysis (nM levels) require a larger sample volume or a labor-intensive sample preparation step (Liquid-Liquid, or Solid-Phase Extraction).

- Upon combining the newborn screening and CAH assays, the expected IEM throughput must be maintained. In addition, the sensitivity of the 17OHP measurement must satisfy the requirements of a "screening task" by delegating special measurements requiring higher sensitivity and higher precision to a second-tier test.
- Steroids concentrations are typically at the nM level, whereas concentrations of amino acids and acylcarnitines are typically at the μM level.

Experimental Method

The experimental setup employs special plumbing to separate the injected sample plug into two portions. One portion is used for the FIA-IEM measurement, and the remainder is diverted for a fast LC-separation to measure the steroids associated with CAH, including cortisol, 21-desoxy-cortisol, 11-desoxy-cortisol, androstenedione and 17-hydroxyprogesterone.

Figure 1 shows a schematic of the plumbing. Figure 2 illustrates the valve-switching process used to split the injected sample plug into two portions. The design of the experiment ensures that the FIA measurement for IEM is completed before the first LC peak (cortisol) elutes off the analytical column. The runtime for the entire experiment is 2.5 to 3 minutes, depending on the number of steroids being investigated (Figure 3).

Figure 1. Schematic of the plumbing for combining the FIA-IEM and LC-CAH measurements.

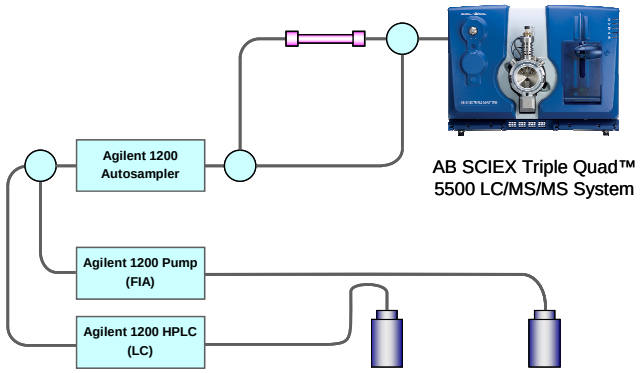


Figure 2. The injection plug must be split to combine the FIA-IEM and LC-CAH measurements.

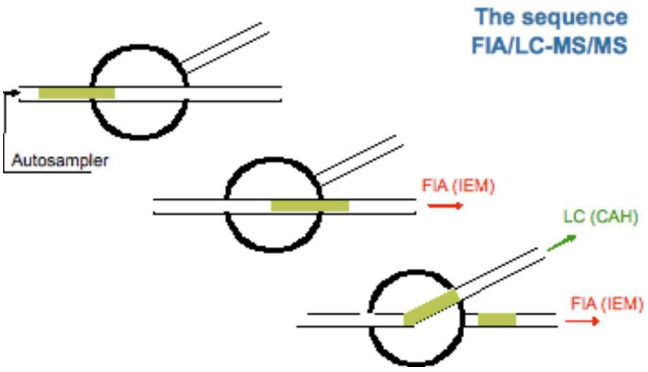
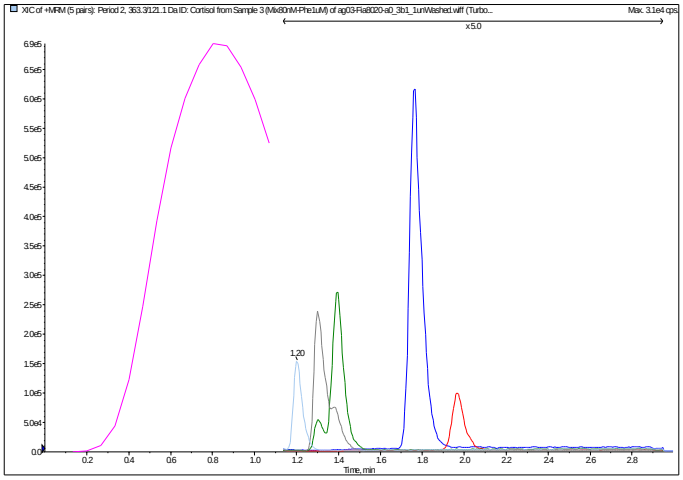


Figure 3. Typical trace obtained from combining the FIA-IEM and LC-CAH measurements. The first part shows the TIC trace from the FIA measurement. The second part shows the MRM traces for the 5 steroids explored.



The sample preparation protocol for the "underivatized" approach is as follows:

1. A 5-mm Dried Blood Spot (DBS) disc is extracted for 20 minutes with an aqueous solution of 90% methanol containing deuterated internal standards for both the classical IEM screening, and for the steroids measurement.
2. If the analysis of succinylacetone (SUAC) is requested, the exhausted paper disc is extracted with an aqueous solution containing acetonitrile, hydrazine and an internal standard[3].
3. After a double-drying step and the addition of MeOH, the supernatant of the first extraction is added to the residue.
4. Two volumes of the resulting solution are added to one volume of a 0.1% formic acid aqueous solution.
5. 100 µL are injected

The hardware configuration for the analysis consists of the following components:

- API 4000™ LC/MS/MS System, API 5000™ LC/MS/MS system or AB SCIEX Triple Quad™ 5500 LC/MS/MS system equipped with ESI probe
- 2 - Isocratic Pumps
- 2 - Switching Valves
- Autosampler
- Column Oven
- Zorbax Eclipse XDB C18, 4.6 x 50 mm column

The plumbing schematic and acquisition methods created for this new application are available on request.

Results

Figure 4 demonstrates how data is captured — the IEM results are returned first (AA + AC + SUAC), followed by the fast LC separation of the steroids.

To demonstrate the sensitivity of the method, only 1 µL of the original specimen was injected. This is significantly (up to 100-200 times) less than is typically required by other steroid quantitation assays. To fulfill the requirement for a screening test, an accepted cut-off level of 70nM is assumed for 17OHP. As shown in Figure 5, the method described here easily achieves the required sensitivity when either the API 4000™ or AB SCIEX Triple Quad™ 5500 LC/MS/MS system is used. Note that the data acquired on the AB SCIEX Triple Quad™ 5500 system shows a 12x improvement in sensitivity.

Figure 4. Representative data from the combined FIA-IEM and LC-CAH measurements.

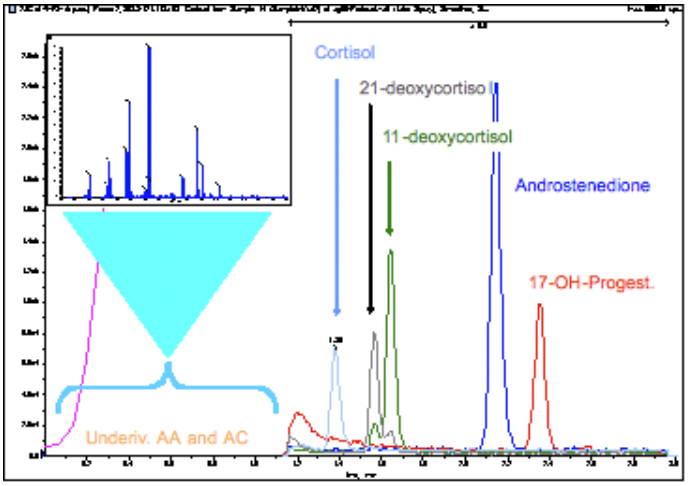


Figure 5. Sensitivity comparison of the LC-CAH measurement from the API 4000™ system (left side) and the AB SCIEX Triple Quad™ 5500 system (right side) The sample is 'blank' blood spiked with 100 nM of each steroid.

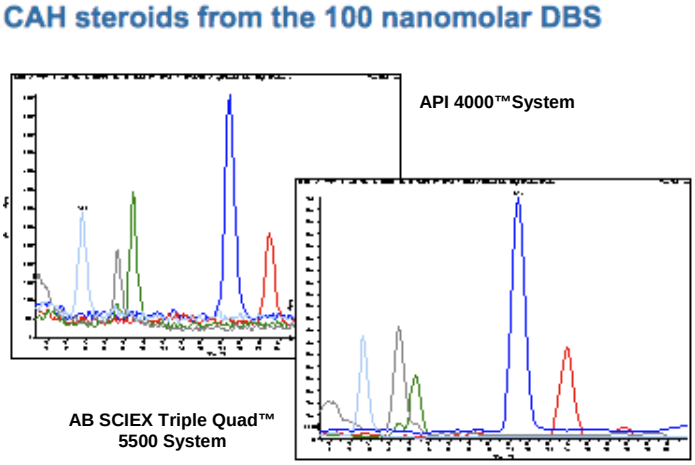


Figure 6 demonstrates the sensitivity of the androstenedione measurement when run on the API 4000™ and AB SCIEX Triple Quad™ 5500 systems.

In order to assess the robustness of this approach, several spiked samples were assayed without the addition of an internal standard. Figure 7 demonstrates the linearity obtained for the analysis of 17OHP.

Figure 6. Sensitivity of the androstenedione measurement on the API 4000™ (top) and the AB SCIEX Triple Quad™ 5500 (bottom) systems. The sample is a 'blank' blood spiked with 25nM of each steroid.

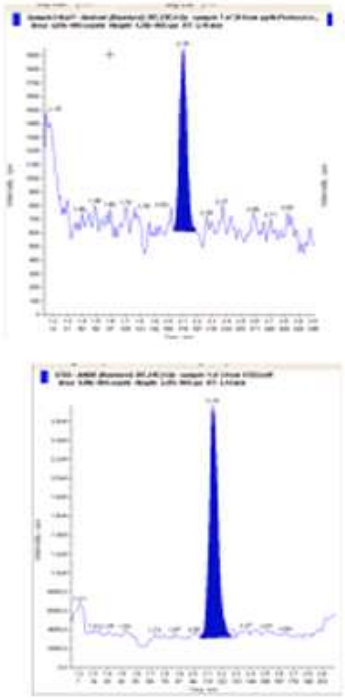
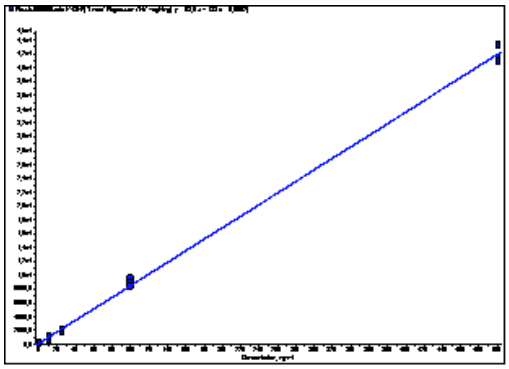


Figure 7. Linearity obtained for the analysis of 17OHP. Sample concentrations range from 1 to 500 nM, and no internal standard was used.



Conclusions

The method described here for Inborn Errors of Metabolism (IEM) and Congenital Adrenal Hyperplasia (CAH) screening uses valve switching to split the injected sample plug in two, thus enabling the concurrent measurements. Different flow-rate regimes are utilized during the method: less than 100µL/min for the FIA-IEM analysis, and 800 µL/min for the LC-CAH analysis. The Turbo V™ ion source is capable of tolerating the continuous swapping between different flow-rate regimes, and this method has proven to be both viable and robust.

This approach is intended for an initial screening, and uses a small volume of injected sample, only 1 µL. The use of a high-performance LC/MS/MS system is recommended in order to achieve the appropriate level of sensitivity needed to measure the steroids. Using this method, the observed limit of quantitation (LOQ) for the analysis of 17OHP is approximately 6 nM.

The overall run time of this combined screening method, for both IEM and CAH, is comparable to that of the standard IEM-only screening method. Note, however, that the run time is highly dependent on the number of steroids investigated. By targeting only 17OHP for a first-tier test and omitting other related steroids, the runtime may be shortened by approximately 1 minute.

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References

1. Casetta, B., edited by Vekey, K., Telekes, A., Vertes, A. 2008. *Neonatal Research.* Medical Applications of Mass Spectrometry, Elsevier. ISBN 978-0-444-51980-1.
2. Lacey, J.M., Minutti, C.Z., Magera, M.J., Tauscher, A.L., Casetta, B., McCann, M., Lymp, J., Hahn, S.H., Rinaldo, P., Matern, D., (2004). *Improved Specificity of Newborn Screening for Congenital Adrenal Hyperplasia by Second-tier Steroid Profiling using Tandem Mass Spectrometry*. Clinical Chemistry 50:3 621-625.
3. Turgeon, C., Magera, M.J., Allard, P., Tortorelli, S., Gavrilov, D., Oglesbee, D., Raymond, K., Rinaldo, P., Matern, D. 2008. *Combined Newborn Screening for Succinylacetone, Amino Acids, and Acylcarnitines in Dried Blood Spots*. Clinical Chemistry 54:4 657–664.

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