Development of an LC-MS/MS-based second-tier screening method for Vitamin B12 deficiency and related disorders at Charite Berlin

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Introduction

Starting in May 2026, newbornscreening (NBS) laboratories in Germany had to extend their screening panel to include tests for inborn errors of vitamin B12 deficiency and related disorders as methylmalonic acidemia, propionic acidemia (PA) and homoycsteinemia.[1] While rather rare, these disorders stem from inherited disruptions of cobalmin, propionate or homocysteine (Hcy) metabolism and can cause serious medical problems.[2] This work describes the development of LC-MS/MS-based second tier screening workflow to identify and distinguish these disorders at Charité Berlin.

Methodics

In the clinical setting, first indicators for a diagnosis are recorded during FIA-MS based first-tier screening, recognizing abnormal levels of Phe/ Met or certain carnitines.[3] To confirm and differentiate these results, a second-tier analysis is required, that can identify the underlying disorders, based on the detection of elevated levels of Hcy, methylmalonic acid (MMA), 3-hydroxypropionic acid (3-HP) and methylcitric acid (MCA). A systematic evaluation of extraction (e.g. solvents compositions, additives, temperature) and LC-MS/MS conditions (e.g. column material, solvents, gradients) was utilized for method optimization.

Results

While only focused on four major analytes, the method development exhibited specific challenges. As expected,[4] extraction of the highly polar target analytes increased with the water content of the extraction solution, however likewise increasing the amount of undesired matrix contaminants. Balancing of extraction conditions and chromatographic separation was used to increase signal intensity, while preventing accumulation of dirt on the instrument. The analysis of MMA was impacted by the presence of the naturally occuring isomer succinic acid, requiring prior chromatographic separation. Finally, direct quantification of Hcy was impacted by oxidative dimerization of the analyte forming homocystin and required implementation of a reliable reduction step to allow for quantification.

Conclusion

Optimizing the extraction of the very polar acids MMA and MCA was challenging, as the yield decreased significantly with higher content of organic solvents. Increasing the water content, the addition of acids or concentrating the extracts, increased the analyte signals, yet also the amount of unwanted contaminants. The issue was overcome by adapting the LC-MS method and diverting contaminants to waste instead of the ion source, thus using an "online-cleaning" approach. In summary we compare the impact of diverse extraction protocols and analytical approaches and the impact on method development.

References

[1] G-BA. Beschluss Kinder-Richtlinie: Früherkennung eines Vitamin B12-Mangels und weiterer Zielerkrankungen (Homocystinurie, Propionazidämie und Methylmalonazidurie) im erweiterten Neugeborenen-Screening. Available at: https://www.g-ba.de/beschluesse/7229. Accessed April 08, 2026

[2] Gramer G., Hoffmann G.F. Vitamin B12 Deficiency in Newborns and Their Mothers—Novel Approaches to Early Detection, Treatment and Prevention of a Global Health Issue. *Curr. Med. Sci*. **2020**; 40:801–809.

[3] Gramer, G.; Fang-Hoffmann, J.; Feyh, P.; Klinke, G.; Monostori, P.; Mütze, U.; Posset, R.; Weiss, K.H.; Hoffmann, G.F.; Okun, J.G. Newborn screening for vitamin B12 deficiency in Germany—Strategies, results, and public health implications. *J. Pediatr.* **2020**, *216*, 165–172.

[4] Kilgore, M. B.; Platis, D.; Lim, T.; Isenberg, S.; Pickens, C. A.; Cuthbert, C.; Petritis, K. Development of a universal second-tier newborn screening LC-MS/MS method for amino acids, lysophosphatidylcholines, and organic acids. *Anal. Chem.* **2023**, *95*, 3187– 3194.