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S. J. Bruce, C. Roux, P. A. Binz, A. Ferrari Gyger and O. Braissant

Clinical Chemistry Laboratory, University Hospital of Lausanne (CHUV), Lausanne, Switzerland

Comprehensive Clinical Acylcarnitines by LC-MS/MS

Introduction

Our hospital has been performing quantitative acylcarnitine profiling using tandem-MS for inherited disorders of β-oxidation and organic acid metabolism for well over a decade. The method (direct infusion of butyl-ester derivatives and MS detection in the MRM mode) has remained unchanged throughout this time. Additionally, we employ a separate targeted method for the quantification of free and total carnitine in plasma by LC-MS/MS. Technological advancements of LC-MS systems has made it feasible to develop a different, more efficient approach that avoids cumbersome sample derivatisation, arguably improves on analytical quality and for us, the possibility to replace two existing routine methods with one. We have developed a single LC-MS/MS method for quantitative acylcarnitine profiling within which free carntine (C0) and total carnitine can also be accurately quantified. This new methodolgy is in the process of being implemented for routine clinical work within our hospital.

Methods (¹ New method, ² Established methods)

¹ Sample preparation: Plasma samples (10 µl) undergo a rapid protein precipitation (isotopic dilution), the resulting supernatants are further diluted by MeCN + 0.2% formic acid and then transferred to LC-MS vials ready for analysis.

¹ LC-MS/MS analysis: Samples are injected onto a UPLC Acquity HILIC Column (2.1 x 100 mm, 1.8 µm, Waters) using a Waters Acquity I-Class LC module coupled to a triple-quad MS system (TQ-S, Waters). LC-MS run time per sample = 12 min.

² Targeted C0 and total carnitine by LC-MS/MS: Samples are split into non-hydrolysed (for C0) and hydrolysed groups (for total carnitine), the latter group undergo hydrolysis with HCl. Following on, all samples undergo a SPE (Oasis MCX) and an additional dilution before injection onto a HPLC Atlantis HILIC column (2.1 x 50 mm, Waters) and MS detection in the MRM mode.

² "Classical" acylcarnitine profiling: Samples are derivatised (butylation), dried under N_2 and reconstituted in mobile phase. Samples are directly infused to the MS via the LC system without separation, analytes are detected and quantified in the MRM mode.

Results

Patient samples were compared between methods (targeted C0 and total carnitine by LC-MS/MS Vs the new acylcarnitine profiling approach by LC-MS/MS) for C0 and total carnitine (n = 57). The C0 comparison between the new approach and the classical acylcarnitine profiling approach is also shown here (n = 83). The corresponding Passing-Bablok regression plots are presented in Figure 2.

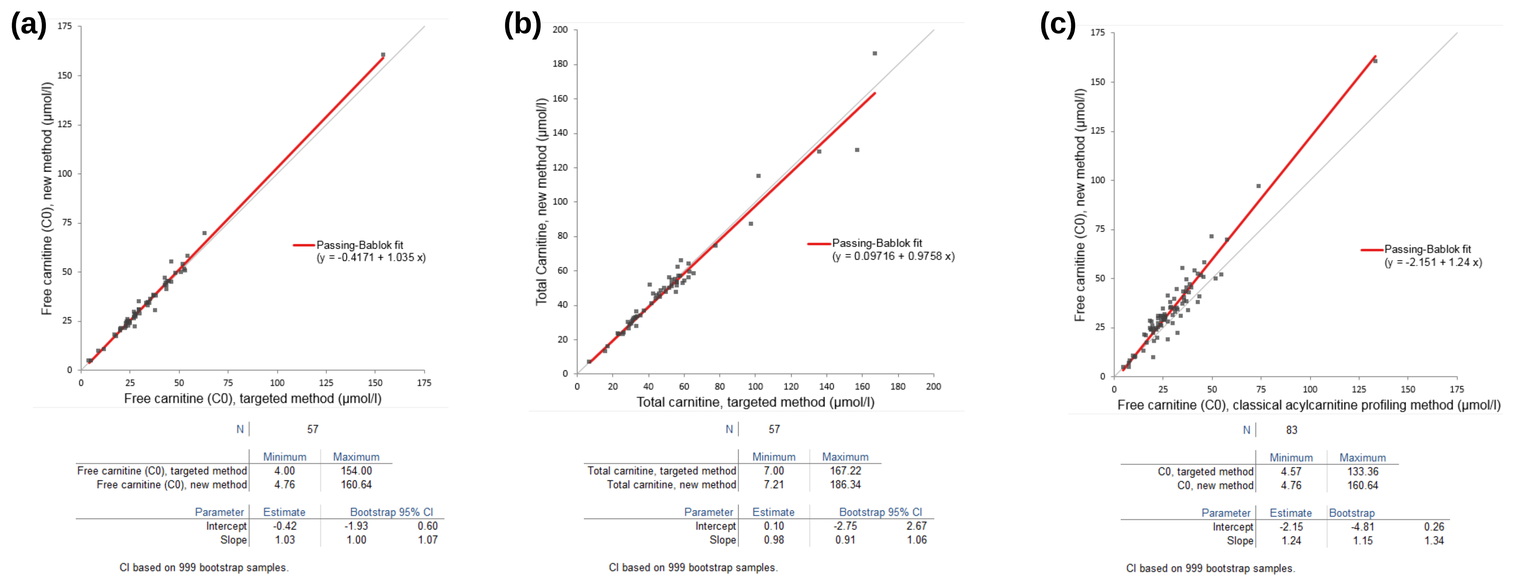
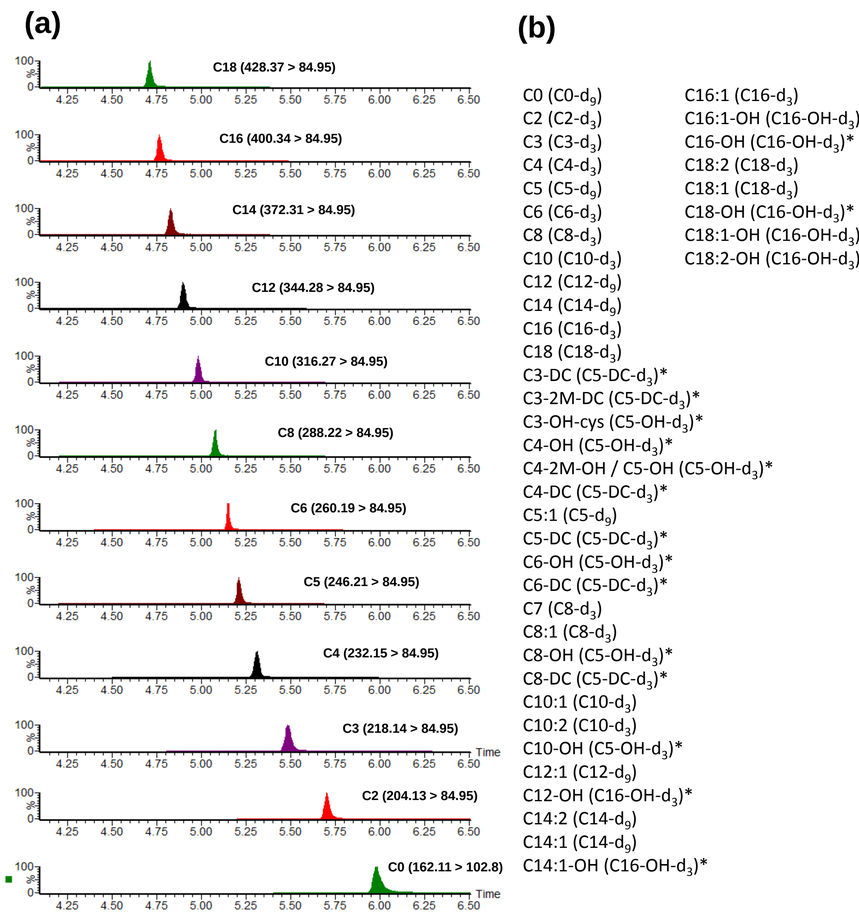


Figure 2. Passing-bablok regression plots: **(a)** C0 targeted Vs C0 new method, n = 57. **(b)** Total carnitine targeted method Vs total carnitine new method, n = 57. **(c)** C0 classical acylcarnitine profiling method Vs C0 new method n = 83.

Individual acylcarnitine comparisons were made across all samples with good agreement for all straight-chain and unsaturated straight-chain species. For the hydroxy (OH) and dicarboxylic (DC) acylcarnitine species, all elevated levels linked with known diagnostic samples were detected, but increases were not always in good agreement, notably for C5-DC with which the comparison was a linear one but with a 2.8 fold increase Vs classical profiling approach (Figure 3). However a small inter-lab test and external control results (data not shown) revealed that for C5-DC many other laboratories are in agreement with higher levels. This difference could be due to the fact that the classical method quantifies the C5-DC by extrapolating onto a 'C5 calibration curve'.

Most importantly, all the confirmed diagnostic cases tested were correctly and clearly identified by the new method. Additionally due to the LC separation, specific acylcarnitine constitutional isomer species can be identified, thereby potentially adding important diagnostic information. Some examples of correctly identified diagnostic cases with additional information from the new method are shown in Figure 4.



*****Acylcarnitines quantified by ratio calculation with their internal standards (= semi-quantification), all other acylcarnitines are quantified via calibration curves (= full quantification)*

Figure 1 (above). New LC-MS/MS acyclarnitine method: **(a)** MRM channel extractions for acylcarnitines within a calibrator sample used to construct the calibration curve. **(b)** List of all acylcarnitines targeted within the LC-MS/MS method with their selected internal standards used for quantification.

Figure 4 (below). Diagnostic case results, new method: **(a)** A β-ketothiolase deficiency, new method can separate the constitutional isomers C4-2M-OH and C5-OH. **(b)** A rare HIBSCH or ECHS1 deficiency with elevated C3-OH-cys. **(c)** A 3-methylcrotonyl-CoA carboxylase deficiency with elevated C5-OH. **(d)** Deficiencies (a) and (c) in which the C5:1 isomers of 3-methyl-crotonylcarnitine and tiglylcarnitine can be separated / identified.

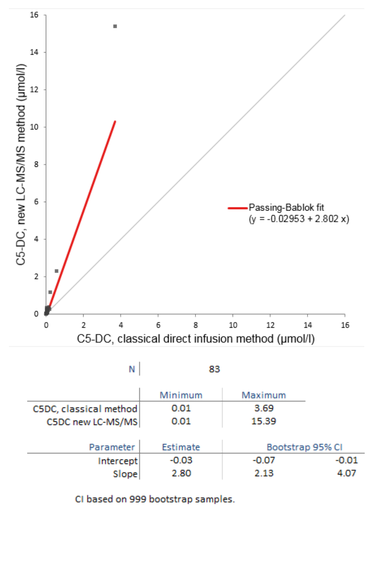
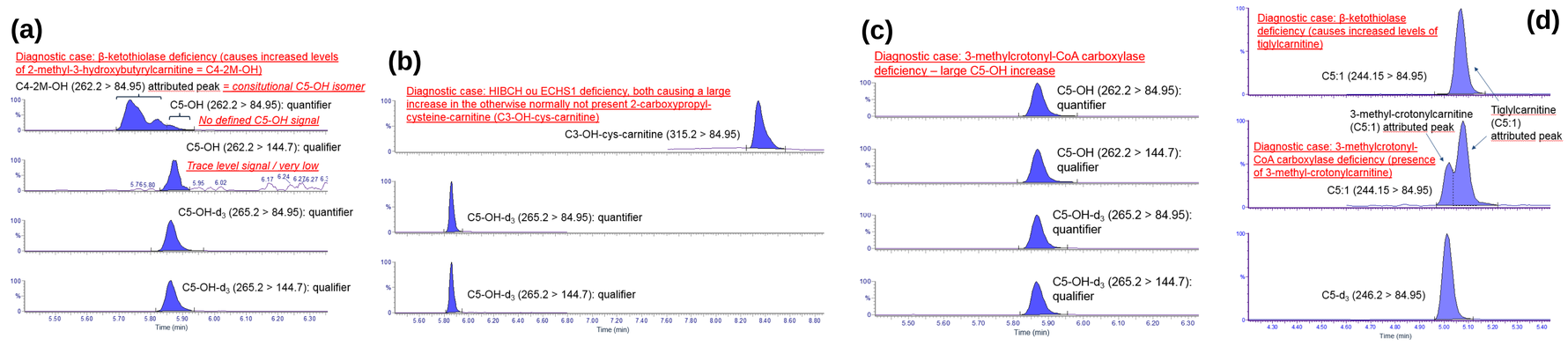


Figure 3. Passing-bablok regression plot of the classical MS acylcarnitine profiling method Vs the new LC-MS/MS method for C5-DC, n = 83.



Conclusions

- A new method for quantitative acylcarnitine screening by LC-MS/MS has been developed for clinical routine analysis, enabling simultaneous quantification of total carnitine in plasma / serum samples. This method, which will replace two existing routine methods in our laboratory is estimated to save ~1-1 ½ days per week of both instrumental and technical labour time.
- The LC separation enables a much easier sample preparation and more detailed information that can help with diagnostic cases with the separation and detection of constitutional isomer species.
- This method can be applied to blood spot samples (as described for plasma / serum) and urine for free carnitine quantification.

For more information: **Steve.Bruce@chuv.ch**