Evaluation of selected short chain acylcarnitines in blood spots, plasma and urine by UPLC-MS/MS for the differential diagnosis of branched-chain organic acidurias

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Abstract:
Acylcarnitines analysis by tandem mass spectrometry (MS/MS) is now routinely used to detect organic acidurias and defects of fatty acid oxidation in blood spots, plasma, and urine. The direct flow injection method is useful, especially when high throughput is desired. This method, however, cannot separate isomeric or isobaric species, requiring additional tests to identify the specific metabolic defect. The biggest impact is observed with the short-chain acylcarnitines, a chain length of 4 and 5 carbon atoms, hydroxylated and non-hydroxylated. These species derive primarily from the intermediate metabolism of branched-chain amino acids, valine and the two isoforms, leucine and isoleucine. Several defects have been identified in these pathways, all characterized by the accumulation of specific acylcarnitine species. In isovaleric acidemia, an inborn error of leucine metabolism, the specific marker is isovaleryl carnitine (IVC); in 2-methylbutyrylglycinuria, an inborn error of isoleucine metabolism, the marker is 2-methylbutyrylcarnitine (2MBC), which is isomeric to isovaleryl carnitine. In the valine pathway, isobutyryl-CoA dehydrogenase deficiency is characterized by accumulation of isobutyrylcarnitine (IBC), a 4-carbon atom species with the same mass/charge ratio of its isomer isovaleryl carnitine (IVC). This is the specific marker for SCAD (Short-Chain Acyl-CoA Dehydrogenase) deficiency, a disorder of fatty acid oxidation. Finally, the hydroxylated species with 5-carbon atoms, 3-hydroxyisovaleryl carnitine (3HIVC), a marker for 3-methylcrotonylglycinuria, a disorder of leucine metabolism, as well as 3-methyl-3-hydroxybutyrylcarnitine (3MHCBC), the marker for beta-ketothiolase deficiency, a disorder of isoleucine and ketone body metabolism. We developed a fast and reliable chromatographic method to separate all of these species and evaluate whether this method could be used, independently from specimen type, to identify the specific metabolic defect in these patients, limiting the number of additional tests/specimen.

Introduction:
The analysis of acylcarnitines by tandem mass spectrometry (MS/MS) is now routinely used to detect organic acidurias and defects of fatty acid oxidation in blood spots, plasma, and urine. The direct flow injection method is useful, especially when high throughput is desired. This method, however, cannot separate isomeric or isobaric species, requiring additional tests to identify the specific metabolic defect. The biggest impact is observed with the short-chain acylcarnitines, a chain length of 4 and 5 carbon atoms, hydroxylated and non-hydroxylated. These species derive primarily from the intermediate metabolism of branched-chain amino acids, valine and the two isoforms, leucine and isoleucine. Several defects have been identified in these pathways, all characterized by the accumulation of specific acylcarnitine species. In isovaleric acidemia, an inborn error of leucine metabolism, the specific marker is isovaleryl carnitine (IVC); in 2-methylbutyrylglycinuria, an inborn error of isoleucine metabolism, the marker is 2-methylbutyrylcarnitine (2MBC), which is isomeric to isovaleryl carnitine. In the valine pathway, isobutyryl-CoA dehydrogenase deficiency is characterized by accumulation of isobutyrylcarnitine (IBC), a 4-carbon atom species with the same mass/charge ratio of its isomer isovaleryl carnitine (IVC). This is the specific marker for SCAD (Short-Chain Acyl-CoA Dehydrogenase) deficiency, a disorder of fatty acid oxidation. Finally, the hydroxylated species with 5-carbon atoms, 3-hydroxyisovaleryl carnitine (3HIVC), a marker for 3-methylcrotonylglycinuria, a disorder of leucine metabolism, as well as 3-methyl-3-hydroxybutyrylcarnitine (3MHCBC), the marker for beta-ketothiolase deficiency, a disorder of isoleucine and ketone body metabolism. We developed a fast and reliable chromatographic method to separate all of these species and evaluate whether this method could be used, independently from specimen type, to identify the specific metabolic defect in these patients, limiting the number of additional tests/specimen.

Method:
Reagent standards: BC, IBC, IVC, 2MBC, PVC, VC, d3-BC, and d9-IVC were purchased. 3OHIVC and 3OH2MBC were synthesized
Sample preparation: Plasma, 20 μL, extracted with acetonitrile containing 0.4% formic acid. Blood spots, 4.76 mm punch, extracted using methanol. Urine, 0.01 μ mole creatine equivalent, extracted with acetonitrile containing 0.4% formic acid. Acylcarnitines were derivatized as butyl esters for detection.
UPLC-MS/MS: Waters Acquity UPLC / Acquity BEH C18 column, 100x2.1 mm, 1.7 um. Mobile phase A: Water / 0.05% formic acid. Mobile phase B: AcCN / 0.05% formic acid. Isocratic elution with 18% B for 9 min, then 20% B for 9.5 min.
Ion transitions: 288.85 for BC and IBC; 291.85 for d3-BC; 300.85 for TG and 3MCC; 302.85 for IVC, 2MBC, PVC, and VC; 311.85 for d9-IVC; 318.85 for 3OHIVC and 3OH2MBC.

Results:
The upper limit of linearity was 50 μmol/L for BC and IBC, and 100 μmol/L for PVC and 2-MBC, in plasma. The concentrations of TC, PVC, and VC were estimated using the calibration curve of PVC. The limit of quantitation was 0.05 μmol/L, for all acylcarnitine species studied. The analytical characteristics of this assay were the same in all specimen types (blood spots, plasma, urine). The acyl group of the two species, 2MBC and 3OH2MBC, contains chiral centers, resulting in, respectively, 2 and 4 chromatographic peaks. For these two species, the sum of all the isomers was used to calculate the concentration.

Figures 2-4 show the ion traces for the individual acylcarnitine species obtained from plasma samples in normal controls and in affected patients. There is a significant difference between the two groups. This difference is observed also in urine and blood spots (data not shown).

Conclusions:
1. The chromatographic separation of short-chain acylcarnitines by UPLC prior to MS/MS detection is a fast and reliable method to differentiate the isomeric forms. 2. Analysis of samples obtained from controls and patients with different organic acidurias indicated that specific markers were significantly increased in all affected patients, independently from specimen type. 3. This method can be used as a second tier test when acylcarnitine analysis is performed by direct flow injection, without the need for additional specimen.

Abbreviations
BC – Butyrylcarnitine
IBC – Isobutyrylcarnitine
IVC – Ivaleryl carnitine
2MBC – 2-Methylbutyrylcarnitine
PVC – Propionylcarnitine
VC – Valencarnitine
TC – Tiglylcarnitine
3MCC – 3-Methylcrotonylcarnitine
3HIVC – 3-Hydroxyisovaleryl carnitine
3OH2MBC – 3-Hydroxy-2-Methylbutyrylcarnitine
BCO – Butyryl Co-A dehydrogenase deficiency
SCAD – Short chain acyl-CoA dehydrogenase deficiency
2MBC – 2-Methylbutyrylglycinuria
3MCC – 3-Methylcrotonylglycinuria
BKTD – Beta-Ketothiolase deficiency