Hydrolysis Monitoring for Accurate Clinical Measurement of Total Carnitine

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Objectives

- The objective was to develop an automated flag to detect samples with incomplete hydrolysis in our free/total carnitine assay
- First: Free carnitine (C0) was measured
- Not all carnitine molecules are free. Many are conjugated to acyl-CoA to form acylcarnitine
- Then: All acylcarnitine species were hydrolyzed, and free carnitine species were measured again. This value becomes total carnitine
- Total carnitine – Free carnitine = Acylcarnitine
- Ammonium hydroxide was used for hydrolysis
- How can we know that hydrolysis is complete? Can we use acetylcarnitine (C2) as a marker for incomplete hydrolysis?
- If C2 is detectable in total carnitine samples, it is possible that hydrolysis is not complete

Introduction

Carnitine plays a crucial role in transporting fatty acids into the mitochondrial matrix, and conjugation of free carnitine to an acyl group to form an acylcarnitine is a necessary step in this process. Free and total carnitine are routinely measured in order to diagnose carnitine deficiency as well as inherent mitochondrial defects. Free carnitine is measured by tandem mass spectrometry after a simple protein crash, and it represents the fraction of carnitine unconjugated to fatty acid. Similarly, total carnitine is measured by flow injection-MS/MS after alkaline hydrolysis to liberate carnitine from associated acyl groups. Acylcarnitine is calculated as the difference between total and free carnitine. Because of the dependence on efficient alkaline hydrolysis, both the total and acylcarnitine values may be artifactually low if carnitine is not fully liberated from bound acyl groups. Importantly, the ERNDIM quality assurance program only tests free carnitine, and thus it is possible to miss systematic problems in the hydrolysis step if total carnitine is also measured.

Methods and Materials

Our underivatized free/total carnitine method monitors free carnitine (C0) by flow injection-MS/MS (quantifier: 162.25 -> 85.17; qualifier: 162.25 -> 103.19) and a d3-carnitine IS. We added an additional MRM transition to monitor acetylcarnitine (C2) (204.25 -> 85.17), which was also normalized using the C0 IS. We monitored the decrease in normalized peak area of C2 in free vs. total carnitine samples in order to detect appropriate hydrolysis. This calculation was also added to Indigo Ascent, and a rule was constructed to flag samples with insufficient % decreases in C2.

Results

Although many acylcarnitines are present only in low abundance in healthy individuals, acetylcarnitine (C2) is normally present at levels of approximately 4-21 µmol/L, with notably high values present with ketosis. As such, measuring its decrease as a result of alkaline hydrolysis may provide a convenient way to detect the efficiency of this step. We added an MRM transition for C2 to our flow injection free/total carnitine assay, and because of co-elution during flow injection we used the C0 IS to provide overall normalization of the C2 peak (designated nC2).
During assay validation, we identified outlier measurements of a high control sample. Interestingly, these outliers showed a substantially higher (total nC2) / (free nC2) ratio (0.62-0.67) compared with other samples (<0.2), consistent with suboptimal hydrolysis. This corresponded with lower measured total carnitine in these results compared with the other values in the precision study, as would also be expected for incomplete hydrolysis. Free carnitine values were comparable across the precision replicates, showing that this did not reflect a global change in the ability to measure carnitine. This data provided evidence that total / free nC2 is a useful measure to identify samples in which incomplete hydrolysis has occurred. Subsequently, this measurement has been implemented as a rule in Indigo Ascent, and we show a clinical example where a sample would have been reported with an artifactually low total carnitine if the nC2 ratio had not flagged incomplete hydrolysis.

Results

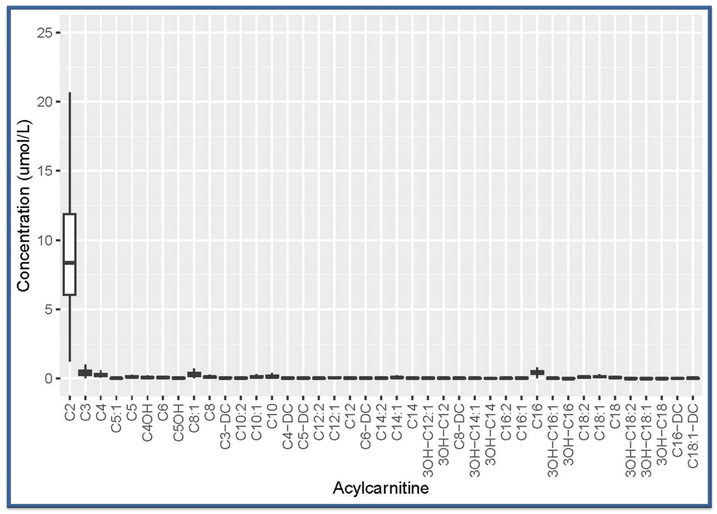

Figure 1: Acylcarnitine results over a year time span showing that C2 is present in both healthy and diseased patients. The normal range of C2 is 4-21 µmol/L, creating the opportunity to utilize C2 as a marker for the hydrolysis process.

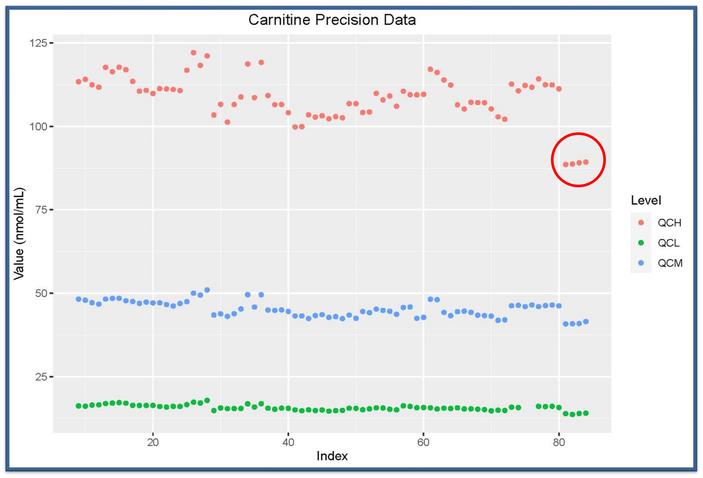

Figure 2: Precision data of the carnitine assay showing the QCs of 38 consecutive batches prepared across 19 different days including two different instruments. Outliers were noted (final 4 data points) and further investigated. Notably, the values are lower and would be consistent with incomplete hydrolysis.

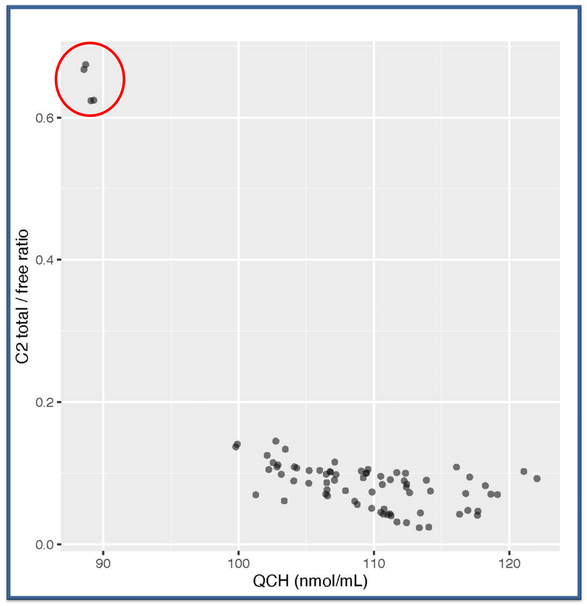

Figure 3: QCH data points were plotted against C2 Total/Free Ratio; demonstrating that the outliers from the precision study (Figure 2) have a high C2 Total/Free Ratio compared to other samples.

Results

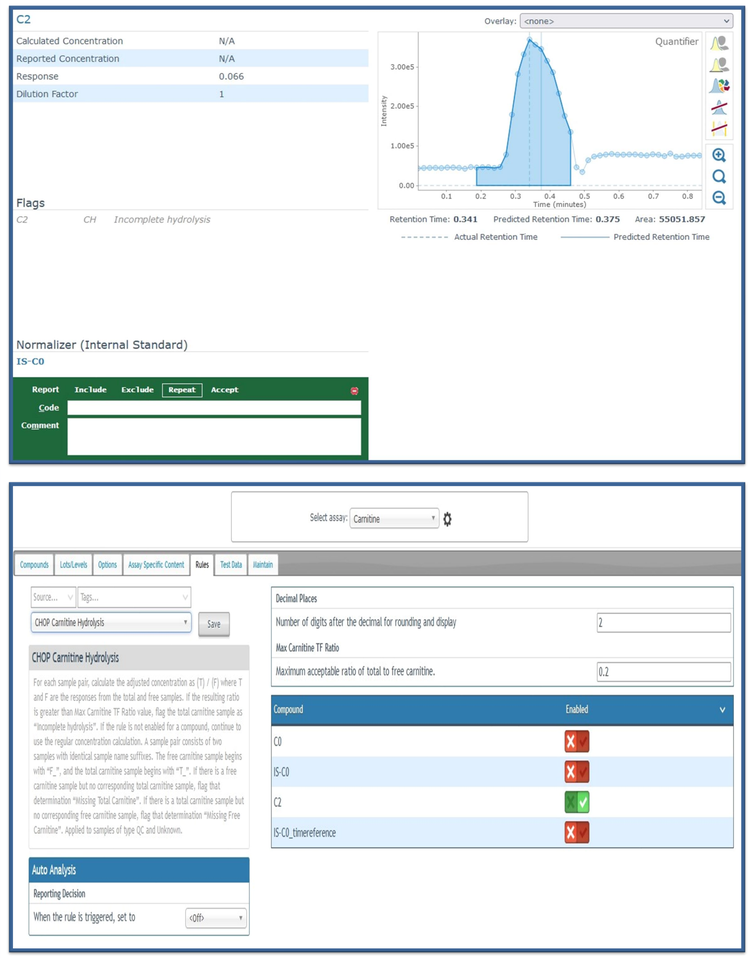

Figure 4: (upper) Demonstrating the integration of a C2 peak from a patient sample. Newly established "incomplete hydrolysis" flag was reported because the C2 Total/Free Ratio was high (>0.2).
Figure 5: (lower) Explanation of the Criteria for the Incomplete hydrolysis rule in Indigo Ascent V4.

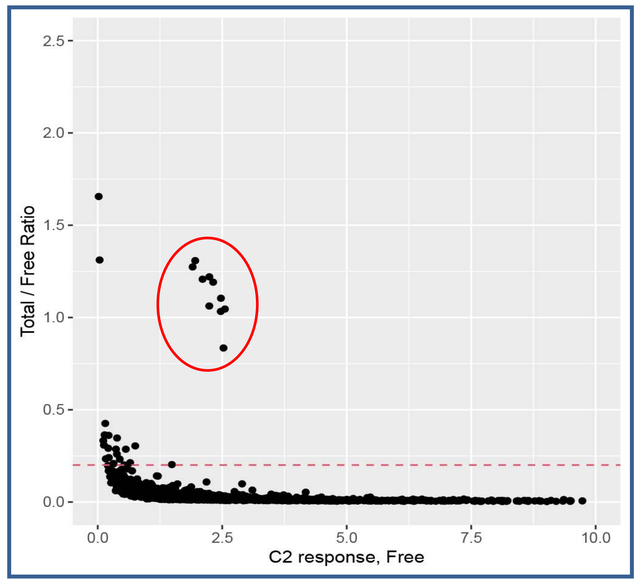

Figure 6: C2 Total/Free Ratio of 2012 samples. The rule cut-off threshold of 0.2 (Total/free < 20%) is shown (dotted red line). 11 out of 2012 samples failed hydrolysis (circled), yielding a "true" failure rate of 0.55%. Note that low endogenous C2 levels can lead to a false positive rule failure.

Conclusions

Utilizing the C2 Total/Free Ratio has shown to be effective in monitoring the hydrolysis process. An automated method was established in Ascent to using a rule-based flag for incomplete hydrolysis.