Clinical applications of 3-hydroxy fatty acid analysis by gas chromatography–mass spectrometry

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Samples

Serum and plasma samples from patients who were known to be on a diet high in medium-chain triglycerides (MCT) were utilized for studies. Samples from patients which were LCHAD and M/SCHAD deficient were obtained from patients submitted for testing who were subsequently or previously diagnosed with these disorders. Fibroblast studies were carried out on the fibroblast cultures submitted for other fatty acid oxidation studies. Control samples were derived from the cell lines that had normal fatty acid oxidative flux, while patient sample lines were from patients confirmed both enzymatically and mutationally. All studies were completed under institutional IRB approval.

Standards and internal standards

Natural and stable isotope labelled standards and internal standards were synthesized using the Reformatsky reaction by Dr. Paul Fennessey at the University of Colorado Health Science Center, Denver. We selected to synthesize even-chain length saturated 3-hydroxy fatty acid species from chain lengths C6–C18. The purities of each standard and internal standard were evaluated by gas chromatography–full scan mass spectrometry and found to be greater than 99%. For the purposes of our analysis, unsaturated 3-hydroxy fatty acid species were quantified based on the equivalent chain length saturated standard curve and internal standard.

Method

Briefly, 10 μL of each of seven 500 μM stable isotope internal standards is added to 500 μL of serum or plasma. Essentially, each sample is prepared in duplicate, and one of the duplicates is hydrolysed prior to addition of internal standards with 500 μL of 10 M NaOH for 30 min. This hydrolysed sample provides the total 3-hydroxy fatty acid content while the unhydrolyzed sample represents the free fatty acid content. The samples are then all acidified with 6 M HCl, 125 μL for unhydrolyzed samples and 2 mL for hydrolysed samples. Samples are extracted twice with 3 mL of ethyl acetate each time, dried down under nitrogen at 37 °C and then derivatized with 100 μL of N,O-bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane for an hour at 80 °C. Derivatized samples are placed on an autosampler and 1 μL is injected into the GC-MS which was an Agilent 5890 series II system using a HP-5MS capillary column for analysis. The analytical run conditions an initial oven temperature of 80 °C for 5 min. The oven temperature was then programmed to rise 3.8 °C/min to a temperature of 200 °C and then rise at 15 °C/min to 290 °C where it remained for 6 min. Quantitation was against the characteristic ion of *m/z* 233 for the unlabelled 3-hydroxy fragment and *m/z* 235 for the labelled internal standard. Culture media samples for fibroblast culture medium experiments differed from this method slightly by utilizing 4 mL of medium and acidifying with 250 μL of 6 N HCL.

The GC/MS analysis is performed in the SIM mode, looking for the specific ions shown in **Table 1** for each native fatty acid and stable isotope labelled internal standard. The amount of native 3-hydroxy fatty acid for chain lengths of C6 to C18 is calculated from the relative abundance of native compound to stable isotope internal standard and the internal standard concentration.

Table 1. $[M\text{-}CH_3]^+$ *m/z* ions for the 3-hydroxy fatty acid species.

3-Hydroxy fatty acid	$[M\text{-}CH_3]^+$ m/z ion	
	Native	Stable isotope
3-Hydroxy hexanoic (C6)	261	263
3-Hydroxy octanoic (C8)	289	291
3-Hydroxy decanoic (C10)	317	319

3-Hydroxy fatty acid	$[M-CH_3]^+$ m/z ion	
	Native	Stable isotope
3-Hydroxy dodecanoic (C12)	345	347
3-Hydroxy tetradecanoic (C14)	373	375
3-Hydroxy hexadecanoic (C16)	401	403
3-Hydroxy hexadecenoic (C16:1)	399	403
3-Hydroxy octadecanoic (C18)	429	431
3-Hydroxy octadecenoic (C18:1)	427	431
3-Hydroxy octadecadienoic (C18:2)	425	431

The assay for the quantitative measurement of 3-hydroxy fatty acids was developed and reference intervals were established defining the expected concentration at each chain length from C6 to C18. Imprecision of the assay was determined at multiple levels. Coefficients of variation (CV's) of 1.0–10.5% were obtained at 30 μmol/L and 3.3–13.3% at 0.3 μmol/L. **Fig. 1** demonstrates the reference intervals under normal physiological conditions for free and total 3-hydroxy fatty acids and **Fig. 1B** shows the extension of the upper limit of the range under conditions of ketosis or of medium-chain triglycerides in the diet, when concentrations of 3-hydroxy-C6 to 3-hydroxy-C10 can become extremely elevated.

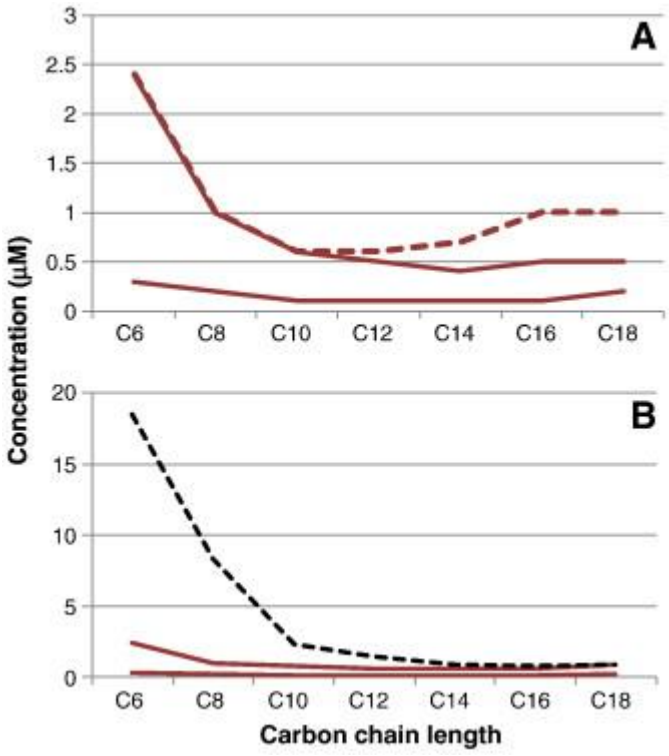

Fig. 1. Reference intervals for 3-OHFA from C6 to C18, showing: A) upper and lower limits for free 3-OHFAs (solid lines) and the upper limit for total 3-OHFAs (dotted line) and B) upper and lower limits for free 3-OHFAs (solid lines) and the upper limit 3-OHFAs seen under conditions of MCT dietary supplementation or fasting ketosis (dotted line).