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Discussion

# Comments on quantitation of carnitine esters by high-performance liquid chromatography

Eberhard Schmidt-Sommerfeld*, Duna Penn

*Department of Pediatrics, Louisiana State University, New Orleans, LA 70118, USA*

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The analysis of specific acylcarnitines in body fluids has become an important clinical tool for the diagnosis of inborn errors of amino acid and fatty acid metabolism. It is particularly useful for elusive diseases like medium-chain acyl-CoA dehydrogenase (MCAD) deficiency for which the routine analysis of organic acids in urine is unreliable [1]. Recent data on the influence of nutrition and drugs on carnitine ester profiles suggest that quantitation of acylcarnitines in urine or plasma may be required for accurate diagnosis [2].

Two different high-performance liquid chromatography (HPLC) methods have been suggested: (a) pre-column enzymatic exchange of radiolabeled carnitine into the acylcarnitine pool first described by Kerner and Bieber [3,4] and adapted by us for clinical use [2,5]; and (b) pre-column derivatization of carnitine esters with a phenacyl reagent. The latter method has been subject of two recent publications in this journal [6,7].

In both papers, the radioisotopic exchange method [3,4,8] is criticized on the basis of theoretical problems that might arise with the use of commercial carnitine acetyl transferase (CAT) in the exchange reaction. The following addresses the 3 major concerns:

(1) *Limited substrate specificity of the enzyme*: We have demonstrated that the following carnitine esters exchange with commercial CAT: acetyl-, propionyl-, (iso)butyryl-, $C_5$-acyl- (3 isomers), hexanoyl-, octanoyl- and decanoylcarnitine [2]. Fortunately, most metabolic diseases that affect carnitine metabolism will be detected by abnormal concentrations of these metabolites. Dicarboxylic acylcarnitines and valproylcarnitine are not substrates for the enzyme and therefore cannot be detected. The achievement of isotopic equilibrium for individual carnitine esters, a prerequisite for reliable quantitation, depends on the conditions of the exchange reaction. For example, incubation with 4 U CAT (Boehringer, Mannheim, Germany) at room temperature for 1–2 h is optimal for complete radioisotopic exchange of all of the above listed carnitine esters except $C_5$-acylcarnitines. The latter require longer incubation times.

(2) *Possible inhibition of the enzyme by unusual carnitine esters in patient samples*: In our experience, this does not occur in plasma. It may occur in urine, but can easily be overcome by diluting the specimen to *ca.* 25 μmol total carnitine/l without loss of sensitivity.

(3) *Presence of acylcarnitine hydrolase activity in commercial CAT* [9]: We found no significant release of free carnitine after incubating a mixture of authentic acylcarnitine standards with desalted CAT for up to 2 h at room temperature.

* Corresponding author.

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Measurement after addition of different concentrations of authentic standards to urine and plasma showed excellent recovery of added acylcarnitines [2]. Moreover, there was good agreement between the measurements of acetylcarnitine (the substrate found to be most sensitive to hydrolysis, ref. 9) by the radioisotopic exchange/HPLC and an independent spectrophotometric method [8]. These data do not suggest that hydrolysis of acylcarnitines under our incubation conditions significantly affects the results.

In summary, although the criticism put forward in refs. 6 and 7 may theoretically be valid, in practice, problems with the enzymatic exchange can be overcome if incubation conditions are carefully controlled. The major advantage of the method is its high sensitivity achieved by specific labeling of the acylcarnitines at the carnitine moiety. This contrasts with other published HPLC procedures that use non-specific derivatization of carboxyl groups for detection and require extensive sample clean-up. These procedures may not be sensitive enough for the reliable diagnosis of inborn errors of metabolism in all cases. For example, urinary concentrations of octanoylcarnitine, the most important carnitine metabolite for the detection of MCAD deficiency, were reported to be in the order of 10 mmol/mol creatinine using these methods [7,10]. In contrast, in a multicenter blinded analysis of 79 urine specimens from 47 patients with MCAD deficiency using the radioisotopic exchange/HPLC method, we found concentrations of octanoylcarnitine in urine as low as 0.1 mmol/mol creatinine [2]. In a similar study, we have also recently established the reliability of this method for plasma and blood spots [5].

We suggest that any method of acylcarnitine analysis should be tested in a blinded fashion to establish its sensitivity and specificity for the detection of metabolic diseases before its clinical applicability is proposed.

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