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Improved MS/MS analysis of succinylacetone extracted from dried blood spots when combined with amino acids and acylcarnitine butyl esters

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ABSTRACT

Background: The utilization of succinylacetone (SUAC) as the primary metabolic marker for tyrosinemia Type I is now well known, thus new methods have been developed to analyze SUAC as a first tier test in newborn screening. One approach is to prepare a SUAC hydrazine derivative from the dried blood spots (DBS) previously utilized in the extraction of acylcarnitine (AC) and amino acids (AA). The final derivatized products of SUAC, AA and AC are combined in a single tandem mass spectrometric (MS/MS) analysis. However, butyl esterification techniques may result in contamination of underivatized acylcarnitines by as much as 20%. We have developed a simple wash step to improve the combined analysis of SUAC, AA and AC in DBS by MS/MS.

Methods: AA and AC were extracted with methanol containing labeled internal standard from 3.2 mm punches taken from the DBS specimen. The previously extracted blood spot that remains after removal of the methanol extraction solvent was used in the preparation of SUAC with and without additional washing of the blood spot. The butyl ester eluates of AA and AC, and SUAC hydrazine derivatives were recombined and measured by MS/MS.

Results: Three additional methanol wash steps of the remaining DBS punches prior to SUAC derivatization reduced the presence of underivatized acylcarnitines, resulting in a 4-fold reduction of underivatized palmitoylcarnitine. Palmitoylcarnitine butyl ester is detected at m/z 456 while the underivatized species is detected at m/z 400, which is also the mass of dodecanoylcarnitine butyl ester. The linearity of the SUAC assay was unchanged by the additional wash steps. For butyl esterification methods, the preferred analytic procedure, the presence of AC can compromise the results of a newborn screen for the actual concentrations of acylcarnitines. It is essential to remove any underivatized acylcarnitines prior to SUAC analysis.

Conclusion: The additional methanol wash steps did not alter SUAC assay results but did remove underivatized acylcarnitines which could result in the incorrect quantification of acylcarnitines.

1. Introduction

Before the introduction of tandem mass spectrometry (MS/MS) analysis of amino acids, newborn screening of tyrosinemias was performed in relatively few states with techniques that measure the concentration of tyrosine by such methods as bacterial inhibition assay (BIA) [1], high performance liquid chromatography (HPLC) [2] or fluorometry [3]. These methods have mostly been replaced by amino acid analysis by (MS/MS) [4] as laboratories adopted the technique. When more laboratories began to screen with MS/MS, measuring tyrosine expanded among the newborn screening community.

The tyrosinemias are characterized as Types I, II, or III and as a condition known as transient neonatal tyrosinemia (TNT) [5]. Each of these disorders is characterized by the presence of elevated concentrations of tyrosine in affected patients at some point during the disease state. From a newborn screening perspective, however, the concentration of tyrosine found in a DBS is highly variable depending upon the type of tyrosinemia. One contributor, tyrosinemia Type II, is a defect in tyrosine hydroxylase, the first step for metabolism of tyrosine. The most common cause of tyrosine elevation is a condition known as transient neonatal tyrosinemia (TNT) which is often the result of a deficiency of Vitamin C [4]. However, this elevated value is often identified as a false-positive result. In order to reduce this source of false positives and the impact on follow-up activities, screening laboratories set relatively high decision levels for what is considered an abnormal result. This increases the potential rate for false-negative results especially for one of the important disorders, tyrosinemia type I. It is well known that tyrosine may be normal or only mildly elevated in the newborn period (days 1–7) and a poorly established decision level will lead to a substantial number of false-negative results.

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The tyrosine metabolite, succinylacetone (SUAC) has been proven to be specific marker for tyrosinemia Type I [6]. It was established as the primary marker for detection of tyrosinemia Type I by studies conducted in Quebec, Canada where its frequency is very high in the French Canadian population [3]. The methods used require a separate assay for SUAC using classical biochemical methods. Recently, however, several methods to measure SUAC by MS/MS with minimal alterations in the laboratory preparation have been reported [7–13]. Allard et al. detail the use of hydrazine to achieve SUAC derivatization in dried blood spots (DBS). In order to minimize the use of additional punches from patient specimens, the residual blood spot punches from the extraction of amino acids and acylcarnitines were used in sample preparation. These derivatized analytes may be recombined with the acylcarnitine and amino acid eluate in the final sample preparations, thus allowing for the simultaneous analysis of amino acids, acylcarnitines and SUAC in a single MS/MS analysis [12,13].

However, the current methods that utilize butyl esterification of amino acids and acylcarnitines do not account for the 10–20% residual methanol eluate containing underivatized acylcarnitines and amino acids in the sample plates. Although, Turgeon et al. noted that C12 was elevated in the combined assay of SUAC and acylcarnitines but concluded it had no impact on 10,000 newborn screens [13]. We have examined the extent of interferences caused by these underivatized acylcarnitines on the acylcarnitine butyl ester profiles and have determined that they are substantial. Further, we have improved the method by addition of three wash steps prior to hydrazine derivatization of the residual DBS punches.

2. Materials and methods

Succinylacetone (4,6-dioxoheptanoic acid) was purchased from Sigma-Aldrich (St. Louis, MO). Sets of isotope-labeled internal standards NSK-A and NSK-B, $^{2}H_{3}$-glutarylcarnitine, and $^{13}C_{5}$-SUAC were purchased from Cambridge Isotope Laboratories, Inc. (CIL, Andover, MA). All unlabeled acylcarnitines were purchased from Life Science Resources (Milwaukee, WI) except for the C14:1, which was purchased from the Universidad Autónoma (Madrid, Spain). Hydrazine monohydrate and formic acid were both reagent grade and purchased from Sigma-Aldrich (St. Louis, MO). All solvents were purchased from Fisher Scientific (Fairlawn, NJ). Butanolic-HCl was from Regis Technologies (Morton Grove, IL).

All blood used to prepare the DBS materials for this study was purchased from Tennessee Blood Services (Memphis, TN). A single unit of whole blood collected in CPDA-I was adjusted to 55% hematocrit by plasma removal before it was spotted onto Whatman® Grade 903 filter paper to prepare the four different sets of DBS materials for the study. All blood pools were spotted onto filter paper in 75 µl aliquots and dried overnight at ambient temperature and then stored in zip-closure, low-gas permeable plastic bags with desiccant packs to minimize moisture levels [15]. Sets one through three contained DBS enriched acylcarnitines: tetradecenoylcarnitine (C14:1, 2.62 µmol/l) and palmitoylcarnitine (C16, 25.0 µmol/l) were added to set one, octanoylcarnitine (C8, 10 µmol/l) was added to set 2, and an unenriched quality control set was prepared for set 3. The fourth set contained enriched SUAC (20.0 µmol/l).

Samples were prepared for MS/MS analysis as follows. Using DBS enriched with selected acylcarnitines, tyrosine and/or SUAC, samples were prepared in triplicate using the following preparation scheme: a single 1/8″ (3.2 mm) diameter filter paper punch was individually placed into 96-well polypropylene microtiter plates, and extracted subsequently with 100 µl of methanolic solution containing stable isotope-labeled acylcarnitines internal standards for 30 min at ambient temperature with gentle agitation according to methods previously described [4,14]. The methanolic eluates were transferred to new 96-well plates for derivatizing into butyl esters while the residual filter paper punch plates were utilized for subsequent SUAC extraction. Plates containing methanolic eluates were dried under a gentle flow of heated (50 °C) nitrogen for 15 min. Then, 50 µl of butanolic-HCl (3 N) was added to each well, covered, and incubated for 15 min at 65 °C. After incubation, the plate was dried under heated (50 °C) nitrogen for 15 min. The plates containing butyl esters of amino acids and acylcarnitines were reconstituted with 100 µl of mobile phase (acetonitrile:water:formic acid; 50:50:0.02% by volume) as previously described [4,14] before combining with SUAC sample plates.

To the plate containing residual DBS punches, 100 µl 80:20% acetonitrile:water containing 0.1% formic acid, 15 mmol/l hydrazine hydrate (0.1% by volume), and 100 nmol/l of the internal standard, $^{13}C_{5}$-SUACwere added to each well. The plate was covered with aluminum foil to prevent evaporation and incubated for 45 min at 37 °C with gentle agitation. After incubation, the extract was transferred to another 96-well plate and dried under gentle flow of nitrogen (50 °C, 15 min). In order to remove any residual hydrazine following drying, methanol (50 µl) was added to each well, agitated, and evaporated under nitrogen flow (50 °C, 10 min) [13].

A second plate containing residual DBS punches was prepared in the same manner as the first with the following exceptions. The small volume of methanol extract and blood spot punches following the transfer of amino acids and acylcarnitines to a new plate was subjected to washing steps (3×) by addition of 100 µl of fresh methanol, agitation and removal of wash fluid. The washing steps took less than 5 min for one full plate. The washed plate was then subjected to derivatization for SUAC analysis as described previously for the first plate.

In both cases, the combined amino acids, acylcarnitines and derivatized extracts were analyzed by MS/MS using methods previously described [4,13,14]. Samples were prepared in triplicate for 3 separate analytical runs ($n=9$). A Sciex API 3200, Thermo Ultra and Quattro Ultima MS/MS systems with electrospray ionization were utilized for the studies and optimized for the analyses of acylcarnitines, amino acids and SUAC as described previously [4]. All three systems yielded comparable results. Precursor ion scans (Pre 85) and neutral loss scans (NL 102) for the acylcarnitines and amino acids and SRM scans of the precursor/product ions (m/z 155/137) of SUAC and its internal standard $^{13}C_{5}$-SUAC (m/z 160/142) were obtained. Quantification of acylcarnitines was achieved by calculating the ion abundance ratios of the pure unlabeled compound relative to their respective deuterated internal standards for all analytes.

3. Results

The MS/MS profile of acylcarnitine butyl esters before and after additional wash steps, that add <5 min additional preparation time per plate, is shown in Fig. 1A and B. Fig. 1A is an acylcarnitine profile from a sample preparation step that included SUAC without the wash step. In a typical acylcarnitine profile that did not include SUAC sample preparation, the metabolites at m/z 400, 426, 428, 456, 480, 482 and 484 would be expected to be the butyl esters of C12, C14:1, C14, C16, C18:2, C18:1, and C18 respectively. The mass values at m/z 370, 400, 424, 426, 428 could also be underivatized C14, C16, C18:2, C18:1 and C18, respectively. Therefore m/z 400 could be the butyl ester of C12 (C12BE) or underivatized C16 (C16und), m/z 426 could be C14:1BE or C18:1und, and m/z 428 could be C14BE or C18und. The presence of an important marker of underivatized species is observed at m/z 403, which can only be the mass of underivatized $^{2}H_{3}$ palmitoylcarnitine internal standard since no $^{2}H_{3}$-C12 was present in the internal standard mixture utilized. Measurement of m/z 403 estimates the degree of contamination of underivatized acylcarnitines or incomplete derivatization. Fig. 1B shows the same profile but with the wash procedure incorporated into the method. Note the absence of m/z 403 and other acylcarnitines. Calculation of the relative ratios of 403 and 459 was determined to estimate the degree of underivatized acylcarnitines. Analysis of samples in triplicate showed that the ratio of 403/459 for unwashed samples was 0.097 ± 0.05 versus 0.022 ± 0.29 for washed specimens with standard deviations of 5% for each analysis.

With regard to the impact of the wash step on the SUAC analysis, DBS prepared with different enrichment of SUAC at 0, 1.5, 3, 5, 10, 20, 50 and 100 µmol/l were evaluated. SUAC linearity was similar for both methods, with r^2 values of 0.9976 and 0.9944, respectively. The recovery of SUAC at different enriched (spiked) concentrations, based on liquid standard response, was comparable with both linear regressions resulting in a slope of 0.41.

4. Discussion

The addition of a methanol washing step to the method reported by Turgeon et al. [13] has been shown to remove the underivatized acylcarnitine from the analysis of acylcarnitine butyl esters by MS/MS. The SUAC assay cannot be considered completely separate from the acylcarnitine analysis since the two final extracts are combined prior to introduction into the MS/MS system. A significant portion of underivatized "key" acylcarnitines are detected in an unwashed analysis. Three methanol rinses significantly reduce underivatized acylcarnitines as measured by quantification of the ion signal at m/z 403 relative to 459. There is an approximate four-fold reduction of underivatized acylcarnitines based on the measurements of the internal standard $^{2}H_{3}$ palmitoylcarnitine. The presence of a measurable quantity of underivatized acylcarnitines (>5%) can be estimated by measuring m/z 403, which is the mass of underivatized $^{2}H_{3}$ palmitoylcarnitine internal