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Improved tandem mass spectrometry (MS/MS) derivatized method for the detection of tyrosinemia type I, amino acids and acylcarnitine disorders using a single extraction process

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ABSTRACT

Background: Succinylacetone (SUAC), a specific marker for tyrosinemia type I (Tyr I) cannot be detected by the routine LC-MS/MS screening of amino acids (AA) and acylcarnitines (AC) in newborns. The current derivatized methods require double extraction of newborn dried blood spots (DBS); one for AA and AC and the second for SUAC from the blood spot left after the first extraction. We have developed a method in which AA, AC and SUAC are extracted in a single extraction resulting in significant reduction in labor and assay time.
Methods: The 3.2 mm DBS were extracted by incubating at 45 °C for 45 min with 100 µl of acetonitrile (ACN)-water-formic acid mixture containing hydrazine and stable-isotope labeled internal standards of AA, AC and SUAC. The extract was derivatized with n-butanolic-HCl and analyzed by LC-MS/MS.
Results: The average inter-assay CVs for, AA, AC and SUAC were 10.1, 10.8 and 7.1% respectively. The extraction of analytes with ACN-water mixture showed no significant difference in their recovery compared to commonly used solvent MeOH. The concentration of hydrazine had considerable impact on SUAC extraction.
Conclusion: We developed a new MS/MS derivatized method to detect AA/AC/ SUAC in a single extraction process for screening Tyr I along with disorders of AA and AC.

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1. Introduction

The introduction of tandem mass spectrometry (MS/MS) in newborn screening has tremendously augmented the capability of identifying metabolic disorders involving amino acid, organic acid and fatty acid oxidation from DBS samples [1]. Measurement of tyrosine by MS/MS screening involves the identification of inherent disorders of tyrosinemias that are characterized as type I, II and III, and a temporary physiological state called transient neonatal tyrosinemia (TNT) [2]. TNT is the most common tyrosinemia in neonates caused by the delay in maturing of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). This causes transient increase of tyrosine level in blood and 4-hydroxyphenyllactate and 4-hydroxyphenylpyruvate in urine [3]. On the other hand, tyrosinemia type III is caused by deficiency of HPPD due to genetic defect. Tyrosinemia type II occurs due to the deficiency of cytoplasmic tyrosine transaminase which inhibits the first step of tyrosine metabolism. The inborn deficient activity of the enzyme fumarylacetoacetate hydrolase (FAH, EC. 3.7.1.2) causes Tyrosinemia types I (Tyr I) or hepatorenal tyrosinemia (OMIM 276700), an autosomal recessive disorder. The deficiency of FAH in the tyrosine catabolism pathway results in progressive liver diseases, neurological disorders and hypophosphatemic rickets [4]. This poses significant health problem in the regions with high incidence of the disease such as Canada [5], Tunisia [6] and India [7].

Tyrosinemia II and III show significant elevation of tyrosine while in Tyrosinemia type I normal levels of tyrosine are observed in almost thirty percent [8] of diagnosed Tyr I patients culminating in false negative results. In contrast, lowering of cutoff levels of tyrosine substantially increases the false positive results and increases the burden of repeat analysis. Thus the screening based on analysis and quantitation of tyrosine for the detection of Tyr I is neither specific nor sensitive.

New methods developed for the detection of Tyr I include screening for SUAC, a specific marker produced from metabolism of accumulated fumarylacetoacetate due to the deficiency of FAH. However, established methods for detecting SUAC can be laborious because they require additional extraction due to its insolubility in the first extraction solution and expensive due to increase in operator and instrument usage time. Allard et al. [9] extracted SUAC from residual blood spot left after extraction of AA and AC and analyzed in separate runs which increased both operator and instrument usage time. Turgeon et al. [8] have simultaneously extracted the blood spot for AA/AC and SUAC and pooled both the extracts prior to MS/MS analysis to conserve instrument time. In another report La Marca et al. [10] has used methanol as a solvent for the extraction of AA, AC and SUAC in a single extraction. We have developed a method in which AA, AC and

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SUAC were extracted simultaneously with ACN-water mixture from DBS, derivatized SUAC using hydrazine to SUAC hydrazone followed by derivatization of AA, AC and SUAC hydrazone using butanolic-HCl and analyzed in a single run. This single extraction method is user friendly and cost effective because it reduces the sample processing and instrument use time.

2. Material and methods

2.1. Materials

Amino acids, acylcarnitines and succinylacetone were purchased from Sigma-Aldrich (St. Louis, MO); isotopically labeled internal standards of succinylacetone ($^{13}C_5$-SUAC), amino acids and acylcarnitines from Cambridge Isotope Laboratories, Inc; hydrazine monohydrate (85% solution w/w) and LC/MS grade acetonitrile from Fisher Scientific; formic acid from Acros Organics and 3 N HCl in n-butanol from Regis Technology, Inc. The filter paper 903 Protein Saver used for blood spot samples were purchased from Whatman. The 96-microwell NUNC brand polypropylene v-bottom and truncated bottom plates, aluminum adhesive seal and foil covers, single and multi-channel micropipettes and pipette tips were obtained from VWR International. Low, medium and high level blood spot controls and calibrators were prepared in-house by the quality control pool production section at Genetic Disease Laboratory Branch.

2.2. Preparation of controls and calibrators

The DBS controls were prepared by adding AA, AC and SUAC standards to pooled whole blood to achieve different concentrations of analytes, spotting directly on filter papers and drying overnight at room temperature. The prepared cards were placed in zip-lock bags with silica desiccants and stored at −20 ºC until used. The concentration range for SUAC used for the studies was 0–240 μmol/l.

2.3. Sample preparation

The barcode of each dried blood spot calibrator, control and patient specimens were scanned using Datalogic barcode scanner. A 3.2 mm diameter DBS punch was placed in each well of a 96-well truncated v-bottomed polystyrene using DBS puncher (Wallac). The daily working solution (100 μl) of acetonitrile: water (8:2 by volume) containing 0.05% formic acid, hydrazine hydrate (3.0 mmol/l) and internal standards of AA, AC and SUAC ($^{13}C_5$-SUAC, 3.45 μmol/l) was added to each well. The derivatization scheme for SUAC is shown in Fig. 1. The plate was sealed using aluminum foil adhesive cover and gently shaken for 45 min at 45 º C in iEMS incubator /shaker unit (Thermo Electron Corpn). The extracts (75 μl) were transferred to NUNC V-bottomed polypropylene 96-microwell plate using automated Apricot Personal Pipettor (Apricot Designs Inc.) and then dried by placing the trays on heat blocks (VWR International LLC) at 60 º C and blowing of hot air on them for approximately 15–18 min. To the dried residues 3 N HCl in n-butanol (50 μl) was added, sealed with heat sealing film and incubated for 30 min at 60 º C. Excess butanol and HCl was evaporated by placing the trays on the heat block (40 º C) and blowing hot air. The derivatized samples were reconstituted in mobile phase (75 μl; acetonitrile: water — 8:2 containing 0.05% formic acid), covered with aluminum foil and gently shaken for 10 min at 27 º C and placed on autosampler tray for LC-MS/MS analysis.

2.4. LC-MS/MS analysis

A triple quadrupole tandem mass spectrometer, Micromass Quattro Micro (Waters Corporation), was used for analysis and operated in positive-ion mode. The source was operated with a capillary voltage of 3.2 kV and source and desolvation temperature of 120 ºC and 350 ºC respectively. Laboratory generated high purity nitrogen was used at a pressure of 100 psi for the nebulizer and cone gas with a flow rate of 600 and 50 L/h respectively. High purity argon was used as collision gas at a pressure of 10 psi to achieve soft fragmentation. Mass calibration and resolution of both the resolving quadrupoles, Q1 and Q3, were optimized by continuous infusion of setup solution provided by Waters Corporation with the flow rate of 10μL/min into ion source using an infusion pump. Ion spray mass spectra were acquired at unit resolution by scanning the mass range from *m/z* 175.1 to 2034.6. The fragment transitions, cone voltage, and collision energies were optimized by manual infusion of pure standards and their isotope-labeled standards. For optimal multiple reaction monitoring (MRM) transitions, ramping of collision energy

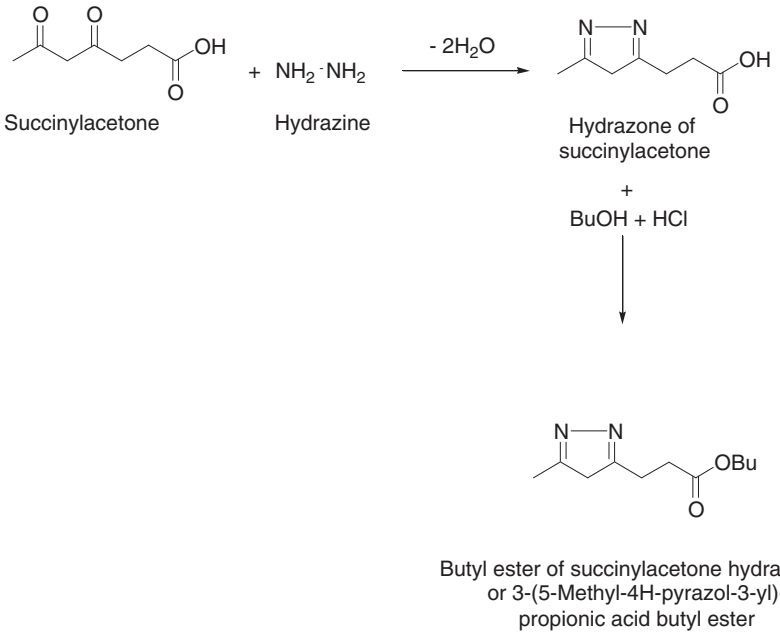

Fig. 1. Derivatization scheme for the formation of butyl ester of succinylacetone hydrazone from SUAC.