Application Note # CA-271518

Using Gas Chromatography – Tandem Mass Spectrometry to Improve the Reliability and Accuracy of Organic Acid Analyses in Urine

Organic acids are water-soluble compounds containing one or more carboxyl groups, as well as other functional groups (-keto, -hydroxy). They occur as physiologic intermediates in a variety of metabolic pathways. The organic acidurias are a group of disorders in which one or more of these pathways are blocked, resulting in a deficiency of normal products and an abnormal accumulation of intermediate metabolites (organic acids) in the body. These excess metabolites are excreted in the urine and can be analyzed by capillary gas chromatography-mass spectrometry (GC-MS). Herein we describe a tandem GC/MS approach for analysis of fifteen organic acids in newborn urine.

Introduction

Screening of pediatric populations for small (~ C3 to C8) organic acids in the urine is commonly used as a diagnostic for genetic metabolic disorders and in the treatment of autism. Typically, positive results warrant further genetic or metabolic screening. Oftentimes, these screens suffer from endogenous interferences in the urine matrix (even in selected ion monitoring: SIM mode), which impair accurate compound identification and quantitation. Here tandem mass spectrometry (MS/MS) is presented as a means to overcome this limitation.

Experimentals

Instrumentation:

- Bruker 320-MS
- CTC Combi-PAL Liquid Autosampler

Materials & reagents:

All standards and reagents were purchased from Sigma-Aldrich (St. Louis, MO); including hydrochloric acid and ethyl acetate. Chem Elut cartridges were purchased from Varian Inc. Test tubes were purchased from VWR. Regisil RC-2 was purchased from Regis Technologies (Morton Grove, IL).

Sample preparation:

1. Determine the creatinine levels of all samples to be analyzed.
2. Add 60 µL of internal standard mix to a 12 x 75 mm disposable glass test tube labeled with the sample ID.
3. Add 2 mL control sample, patient urine, or blank. Cap the tubes and vortex.
4. Add approximately 5 drops of 6N HCl to each tube to acidify. Cap the tubes and vortex.
5. Place the Chem Elut columns in the upper rack and the 16 x 100 mm disposable glass tubes labeled with sample ID in the lower rack.
6. Apply the sample to the column and wait for the sample to flow-though (takes about 5-10 minutes).
7. Add 10 mL of ethyl acetate and allow it to flow through the column completely.
8. Transfer 2 mL aliquot of each sample eluent to a 10 mL conical tube labeled with corresponding sample ID. Save the left over 8 mL sample in the refrigerator. Place the conical tubes under N_2 flow and evaporate to total dryness.
9. For samples with urine creatinine less than 50 mg/dL, add 50 µL of Regisil RC- 2(BSTFA) for derivatization. For samples with urine creatinine greater than 50 mg/dL, add 100 µL of Regisil for derivatization. Cap and vortex for 5 seconds. Incubate the tubes at 96 °C for 20 minutes.
10. Remove the tubes and cool to room temperature.
11. For samples with urine creatinine less than 50 mg/dL, add 1 mL of ethyl acetate. For samples with urine creatinine greater than 50 mg/dL, add 2 mL of ethyl acetate. Cap and vortex.
12. Transfer aliquots into labeled autosampler vials.
13. Place the autosampler vials in the carousel of the liquid autosampler and analyze by GC/MS/MS.

GC Conditions

Column	5% phenyl 95% dimethylpolysiloxane 30 m, 250 µm ID, 0.25 µm df
Carrier	Helium; 1 mL/min constant-flow
Injector Temperature	250 °C; 50:1 split
Injection Volume	1 µL

GC Oven Program

Temperature (°C)	Rate (°C/min)	Hold (min)
90	0	1.5
130	10	1.0
250	10	1.0
280	15	10

MS Conditions

Ionization Mode	EI Positive, 70 eV
Collision Gas	2.0 mTorr Argon
Source Temperature	200 °C
Manifold Temperature	40 °C
Transfer Line Temperature	280 °C
Q1 Peak Width	0.7 Da
Q3 Peak Width	3.0 Da
Dwell Time	100 ms
Detector	Electron Multiplier with Extended Dynamic Range (EDR)

Results

Example MRM chromatograms of 2-hydroxy butyric acid, 3-hydroxy butyric acid, 3-methyl-2-oxovaleric acid, and α-hydroxyisocaproic acids are provided above (clockwise from upper left). These chromatograms are typical of the 15 target analytes and the internal standard. The chromatograms were obtained from a sample of normal human urine to which the example acids were added to a concentration of 25 nmol/mL. Unlike full-scan or SIM analyses, matrix effects of the urine have been significantly reduced by the MRM scanning technique, greatly simplifying identification and quality control activities. The elimination of urine matrix interference also helps with the linearity of calibration. Calibration data for the 15 analytes and 1 internal standard demonstrate good linearity, as seen in table one below.

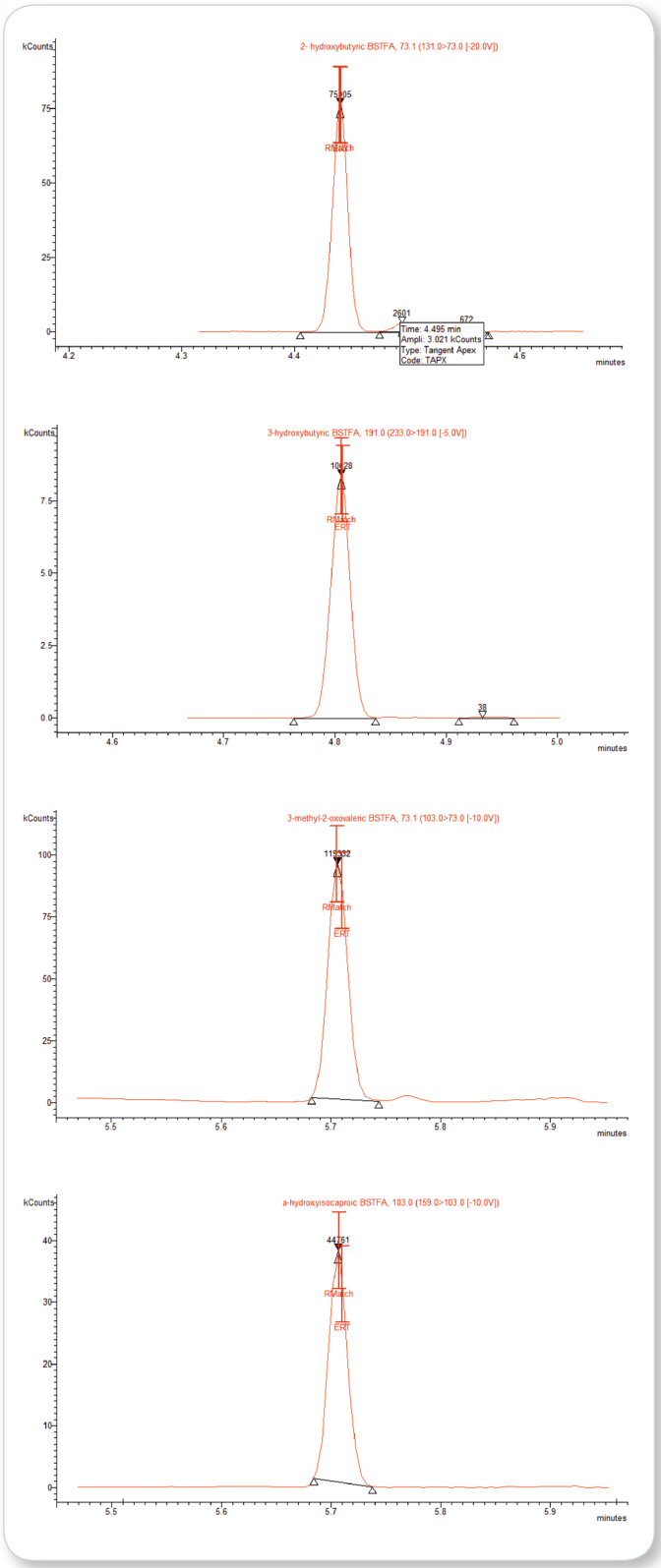

Figure 1: MRM chromatograms of 2-hydroxy butyric acid, 3-hydroxy butyric acid, 3-methyl-2-oxovaleric acid, and α-hydroxyisocaproic acids.

This analysis was originally intended to only be a screen, and as such only a three-point calibration was used and no rigorous determination was made of either reproducibility or of detectability. However, S/N ratios were measured for the 25 nmol /mL spike, and based on those an approximate detection limit can be extrapolated, assuming that a detectable peak must have an area ≥ 3x that of the S/N ratio. Based on that assumption, the approximate detection limits were determined in nmol/mL, and given in Table 2 below. To convert from nmol/mL to ppb, multiply the detection limit by the molecular weight in ng/nmol. For example, 2-hydroxybutyric acid = 104 g/mol = 104 ng/nmol, so the detection limit for this compound is 104 * 0.11 = 11.4 ng/mL = 11.4 ppb. Using this conversion, detection limits in ppb can be found in Table 3.

Conclusion

Although the analysis of organic acids in urine matrices is not novel, the use of triple-quadrupole technology is a new approach. Implementation of this technology greatly simplifies identification and reliability by the minimizing the effects of the urine matrix. Additionally, method linearity is superior to that obtainable from the traditional single-quadrupole technique.

Table 1: Calibration Curve Correlation Coefficients.

Acid	R^2	Acid	R^2
2-Hydroxybutyric	0.997	4-Hydroxybutyric	0.992
3-Hydroxybutyric	0.997	Undecanoic Acid (IS)	
α-Hydroxy-isovaleric	0.985	β-Oxoglutaric Acid	0.975
Malonic	0.997	3-Hydroxy-3-methylglutaric	0.982
Methylmalonic	0.998	α-Oxoglutaric	0.999
α-Oxoisovaleric	0.992	Suberic	0.999
3-Methyl-2-oxovaleric Acid	0.995	Arabinose	0.981
Hydroxy-isocaproic	0.995	Homogentisic	0.992

Table 2: Approximate Detection Limits (nmol/mL).

Acid	D/L	Acid	D/L
2-Hydroxybutyric	0.11	4-Hydroxybutyric	0.33
3-Hydroxybutyric	0.07	Undecanoic Acid (IS)	
α-Hydroxy-isovaleric	0.49	β-Oxoglutaric Acid	5.5
Malonic	0.51	3-Hydroxy-3-methylglutaric	2.1
Methylmalonic	0.50	α-Oxoglutaric	0.27
α-Oxoisovaleric	3.3	Suberic	2.1
3-Methyl-2-oxovaleric Acid	0.38	Arabinose	32
Hydroxy-isocaproic	0.18	Homogentisic	0.5

Table 3: Approximate Detection Limits (ppb).

Acid	D/L	Acid	D/L
2-Hydroxybutyric	11.4	4-Hydroxybutyric	34.5
3-Hydroxybutyric	6.2	Undecanoic Acid (IS)	
α–Hydroxy-isovaleric	58	β–Oxoglutaric Acid	804
Malonic	53	3-Hydroxy-3-methylglutaric	347
Methylmalonic	59	α–Oxoglutaric	39.4
α–Oxoisovaleric	380	Suberic	366
3-Methyl-2-oxovaleric Acid	336	Arabinose	4803
Hydroxy-isocaproic	26	Homogentisic	84.1

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Keywords
Organic acidurias
Newborn urine
GC/MS/MS
metabolites

Instrumentation & Software
Bruker 320-MS
CTC Combi-PAL Liquid Autosampler

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