

Chromatographic Resolution and Tandem MS Measurement of the Leucine Isomers Associated with the Monitoring of MSUD Patients

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Scott Freeto¹, Donald Mason¹, Jie Chen², Robert Scott², Srinivas Narayan², and Michael Bennett²

¹Waters Corporation - Beverly, Massachusetts, ²Children's Hospital of Philadelphia - Philadelphia, Pennsylvania

INTRODUCTION

Maple Syrup Urine Disease (MSUD) is the result of a genetic defect of the branched-chain α -keto acid dehydrogenase enzyme system. This severe metabolic defect is characterized by an accumulation of branched-chain α -keto acids and their respective branched-chain amino acids leucine, isoleucine and valine. The presence of alloisoleucine is considered pathognomonic for MSUD. Monitoring of therapeutic dietary intervention of patients with MSUD requires accurate measurement of these amino acids, particularly leucine in blood. The ideal methodology for measurement should be sufficiently rapid to provide immediate response while the patient is still in the clinic/ emergency room. The 3 isomeric forms of leucine have identical molecular weights and product ions, making direct injection into a tandem mass spectrometer unsuitable. We present here the results of a study of amino acid measurement in MSUD and phenylketonuria (PKU) patients with a rapid (6 minute) separation using the ACQUITY UPLC™ and tandem mass spectrometry (MSMS) in positive MRM mode. Concentrations of the individual amino acids are calculated using peak area ratios: (area under AA peak/area under internal standard peak) x concentration of internal standard—a process similar to that used by neonatal screening laboratories. For isoleucine and alloisoleucine a relative response factor is generated to accurately quantify these AAs in the absence of isotopically labeled internal standards. An analysis time of six (6) minutes and an injection-to-injection cycle time of nine (9) minutes is used for the analysis—significantly shorter than conventional methods. Split samples were also analyzed on a Beckman 6300 and the results compared by correlation analysis. For all AAs analyzed (Phe, Tyr, Met, Val, aIle, Ile and Leu) the correlation coefficient (r) between methods is > 0.96 . We demonstrate that UPLC-MS/MS technology provides a rapid means of measuring plasma amino acids and may be beneficial in the differential diagnosis and long-term clinical management of patients with MSUD.

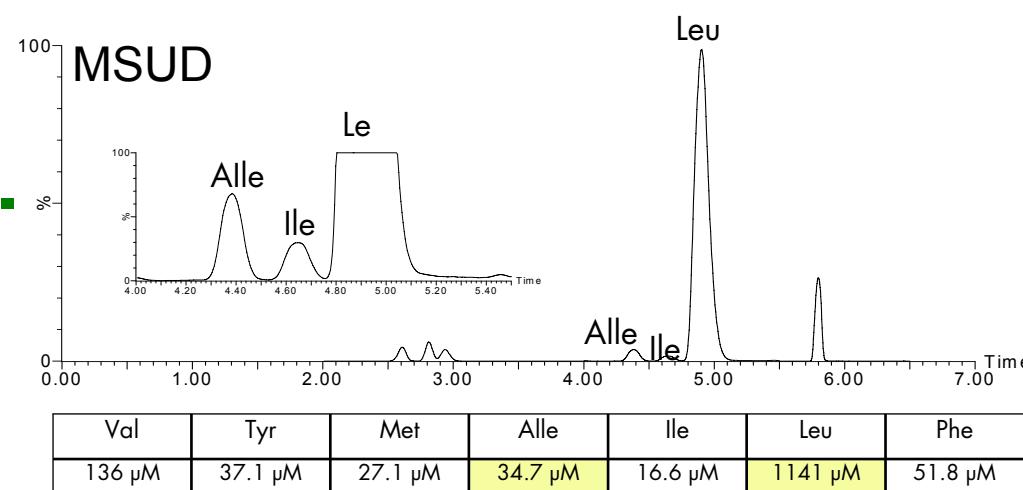
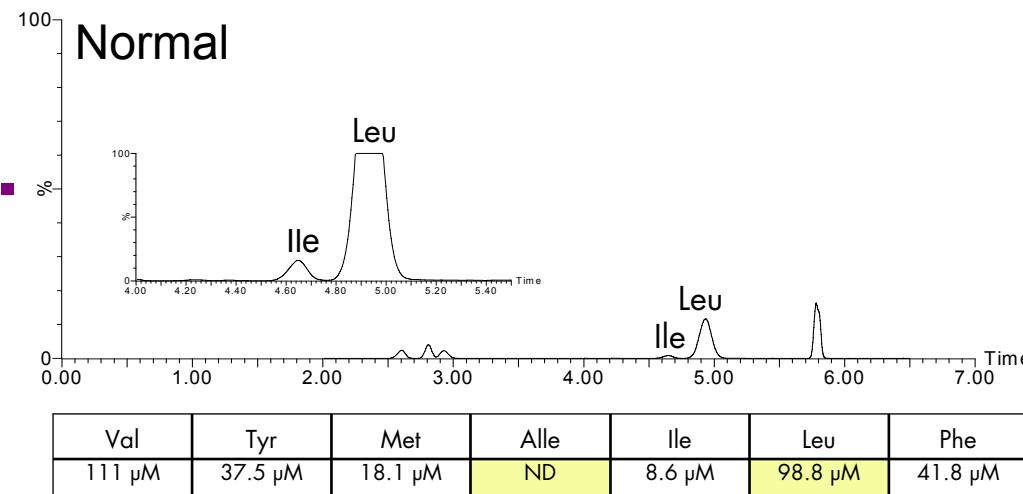


Figure 1 Comparison of a normal plasma specimen to one from a patient diagnosed with MSUD

METHODS

Patient Samples

Seventy-five plasma (sodium heparin) samples consisting of 41 patients with either MSUD or PKU, and 34 patients with neither disease were analyzed using the LC-MS/MS technique described here and on a Beckman 6300 amino acid analysis system. The plasma was stored at -20°C prior to instrument-specific sample preparation.

Sample Preparation for Amino Acid Analyzer

Plasma samples, calibrators or controls (500 μ L) were added to a 1.7 mL microcentrifuge tube to which 50 μ L 35% sulfosalicylic acid was added. The mixture was vortex mixed and centrifuged at 13,000g for 5 min. After centrifugation, 100 μ L of supernatant was transferred to a 0.5 mL microcentrifuge tube to which 100 μ L of Li220 diluting buffer was added. The tubes were mixed thoroughly prior to analysis.

Sample Preparation for LC-MS/MS

Plasma samples, calibrators or controls (50 μ L) were added to a 1.7mL polypropylene microcentrifuge tube along with 950 μ L of a methanolic working internal standard. The tubes were capped, vortex mixed for 30 seconds, and centrifuged at 13,000 RPM for 3 minutes. The clear supernatant (300 μ L) was transferred to a clean microcentrifuge tube and evaporated to dryness in a Savant centrifugal evaporator. To the dried residue, 50 μ L of butanolic HCl was added, the tubes were capped, vortex mixed for 30 seconds and placed in a 60°C dry heat block for 20 minutes to facilitate derivatization. After derivitization the tubes were uncapped and again evaporated to dryness in the centrifugal evaporator. The residue was resuspended in 200 μ L 50:50 mobile phases A and B and vortex mixed for 30 seconds. The resuspended samples were diluted 1:50 in MilliQ® water in an autosampler vial prior to analysis.

Internal Standards and Calibrators

Isotopically labeled amino acid internal standards were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). The majority of the internal standards were included in a labeled amino acid standards mix that was reconstituted and diluted per the manufacturer's instructions. The working internal standard was supplemented (2.5 μ M) with d₁₀-alloisoleucine (d₁₀-aIle) and ¹³C₆-isoleucine (¹³C₆-Ile) also obtained from Cambridge Isotope Laboratories. Concentrations of aIle and Ile in the control samples were evaluated using their individual, stable isotope internal standards as well as using d₃-Leu as the internal standard for all three leucine isomers.

Precision and Linearity

Calibration and linearity curves and controls were made by fortifying analyte - stripped human serum obtained from Biocell Laboratories (Rancho Dominguez, CA) with authentic amino acid standards. Powders of valine (Val), tyrosine (Tyr), phenylalanine (Phe), methionine (Met) and alloisoleucine (aIle) were obtained from Sigma (St. Louis, MO). Leucine (Leu) and isoleucine (Ile) were obtained from Fluka. Within-run precision was evaluated by analyzing 10 aliquots of each of the three prepared control samples within a single analytical run. Run-to-run precision was evaluated by analyzing these same control samples in triplicate on 6 separate days.

DISCUSSION

The introduction of electrospray-MSMS has allowed for facile quantitation of most physiologic amino acids using just a few microliters of blood in less than 2 minutes. This technique has made possible population based screening of newborns for inherited disorders of amino acid metabolism which is now the standard of care in the United States and much of the developed world. One consequence of this expansion in newborn screening is the detection of a greater number of disorders and a greater number of affected individuals – putting increased pressure on metabolic diagnostic laboratories to provide management services.

MSUD, however, presents an unusual challenge in that three of the critical amino acids, leucine, isoleucine and alloisoleucine are isobaric and can not be differentiated without chromatographic separation. We have developed a rapid 6-minute, assay using UPLC with MS detection that previously took as much as 1 hour by ion exchange HPLC. In addition, this method also measures phenylalanine, tyrosine and methionine for monitoring patients with phenylketonuria and homocysteineuria. We have also demonstrated that the leucine isomers may be accurately quantified using d₃-Leu as the internal standard eliminating the additional expense of including specific stable isotopes for each analyte. Additionally, the method exhibits excellent linearity over the clinical reporting range of concentrations, is precise and compares favorably with existing, time-consuming ion exchange chromatographic techniques.

RESULTS

Method Comparison

The results of the two methods (AAA vs LC/MS/MS) were compared by correlation analysis where, for each amino acid, the correlation coefficient (R) was greater than 0.96. Each of the 75 specimens was classified into the same PKU, MSUD or normal cohort by both methods when independently evaluated. Figure 1 compares a normal specimen to one drawn from a patient with MSUD (note the presence of alloisoleucine in the MSUD specimen and its absence in the normal).

Linearity

All amino acids yielded linear calibration curves in the LC-MS/MS analysis over two orders of magnitude, a range which included all patient specimens, having r^2 values in excess of 0.99.

Precision

Within day and day-to-day precision for all control levels and all seven analytes ranged from 1.5 to 7.0%. Correlation analysis of aIle concentrations in controls using d₁₀-aIle vs. d₃-Leu yielded $y=1.02x-0.079$, $r^2=0.998$ and Ile concentrations using ¹³C₆-Ile vs. d₃-Leu yielded $y=1.02x-0.39$, $r^2=0.997$.

CONCLUSIONS

- UPLC technology provides chromatographic resolution of the leucine isomers in less than 6 minutes with mass spectrometer friendly mobile phases.
- The described method correlates well with a "gold-standard" amino acid analyzer utilizing an ion-exchange methodology that takes nearly five times longer.
- This method yields excellent linearity, precision and sensitivity.
- Similar results are obtained for aIle and Ile when internally standardized using d₃-Leu or their individual stable isotopes d₁₀-aIle and ¹³C₆-Ile