

Waters

A RAPID UPLC™ - MS/MS METHOD FOR DETERMINING SPECIFIC AMINO ACIDS ASSOCIATED WITH MAPLE SYRUP URINE DISEASE AND PHENYLKETONURIA MANAGEMENT

Scott Freeto<sup>1</sup>, Donald Mason<sup>1</sup>, Jie Chen<sup>2</sup>, Robert Scott<sup>2</sup>, Srinivas Narayan<sup>2</sup>, and Michael Bennett<sup>2</sup>  
<sup>1</sup>Waters Corporation - Beverly, Massachusetts, <sup>2</sup>Children’s Hospital of Philadelphia - Philadelphia, Pennsylvania

ABSTRACT

Maple Syrup Urine Disease (MSUD) and Phenylketonuria (PKU) are severe inborn errors of amino acid (AA) metabolism which, if left untreated, can have catastrophic consequences for the child. Maple Syrup Urine Disease results from a genetic defect of the branched-chain  $\alpha$ -keto acid dehydrogenase enzyme system. This metabolic defect is characterized by an accumulation of branched-chain  $\alpha$ -keto acids and their respective branched-chain amino acids, leucine, isoleucine and valine. The presence of alloisoleucine is considered pathognomic for MSUD. PKU is the result of a deficiency in phenylalanine hydroxylase, the enzyme responsible for converting phenylalanine to tyrosine. PKU is therefore characterized by an abnormal accumulation of phenylalanine, reduced levels of tyrosine or by an increased relative phenylalanine / tyrosine ratio. Monitoring of therapeutic dietary intervention of patients with MSUD requires specific measurement of these amino acids, particularly leucine, in blood. The ideal analytical method 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 PKU patients with a rapid (6 minute) separation using the ACQUITY UPLC™ and tandem mass spectrometry. Deproteinized plasma samples are esterified with 3N HCl in butanol and the amino acid butyl esters are separated on an ACQUITY UPLC BEH C18 column. Isocratic elution is used to resolve valine, tyrosine, methionine and the leucine isomers, followed by a rapid gradient to elute phenylalanine and to wash the column prior to the next injection. The relevant AAs are detected in the positive ion MRM mode on a tandem quadrupole mass spectrometer. Concentrations of the individual AAs 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. Seventy-five plasma 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 results were compared by correlation analysis and each of the 75 samples fell into the same PKU, MSUD or normal cohort by both methods. For each AA the correlation coefficient (r) between methods is > 0.96. We demonstrate that UPLC-MS/MS technology provides a rapid means of measuring plasma AAs and may be beneficial in the differential diagnosis and long-term clinical management of patients with MSUD and PKU.

METHODS

Patient Samples

Seventy-five plasma 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. Venous blood was collected in sodium heparin-containing collection tubes and centrifuged at 900g for 5 min at 4°C. The plasma was stored at -20°C prior to instrument-specific sample preparation.

Sample Preparation for Amino Acid Analyzer

Plasma samples, calibrators or controls (500µl) were added to a 1.7ml polypropylene microcentrifuge tube followed by the addition of 50µl 35% sulfosalicylic acid. The mixture was vortex mixed and centrifuged at 13,000g for 5 min. After centrifugation 100µl of supernatant was transferred to a 0.5ml microcentrifuge tube to which 100µl of Li220 diluting buffer was added. The tubes were mixed thoroughly at which time the samples were ready for analysis¹.

Sample Preparation for LC-MS/MS

Plasma samples, calibrators or controls (50µl) were added to a 1.7ml polypropylene microcentrifuge tube along with 950µl 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 (300µl) was transferred to a clean microcentrifuge tube and evaporated to dryness in a Savant centrifugal evaporator. To the dried residue, 50µl 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 200µl 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 (2.5µM) with d10-alloisoleucine (d10-alle) and 13C6-isoleucine (13C6-Ile) also obtained from Cambridge Isotope Laboratories. Concentrations of alle and lle 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 (alle) 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.

Chromatography

Chromatographic separation was achieved using an ACQUITY® BEH C18 2.1x100mm, 1.7µm analytical column maintained at 30°C and ACQUITY UPLC system using a binary high pressure gradient. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The gradient profile used is shown in Table 1.

Time (min.)	%A	%B	Flow (mL/min.)	Gradient Curve
0.00	90	10	0.60	Init
1.50	83	17	0.60	6
4.50	83	17	0.60	6
7.00	50	50	0.60	6
7.50	90	10	0.60	6
8.00	90	10	0.60	11

Table 1. Liquid chromatography gradient program used for the separation of the targeted amino acids in this study.

Optimum peak shape and dynamic range were achieved using 5µl partial loop injections with a 50µl loop. The weak needle wash was 10% methanol in water and the strong needle wash was 32% acetonitrile, 32% isopropanol, 30.8% methanol, 5% water and 0.2% formic acid. The column eluent was connected directly to the electrospray probe of the mass spectrometer without the use of flow splitting or solvent diversion.

Mass Spectrometry

Both Quattro micro™ API and Quattro Premier™ XE tandem quadrupole mass spectrometers were used in the positive ion electrospray mode with similar results for linearity and precision. (Between instrument sensitivity was not evaluated in this study.) All analytes were monitored in MRM mode. Complete system control and data processing was accomplished with MassLynx™ version 4.1 software. Chromatographic peaks were integrated and quantified via least squares linear regression with 1/x weighting using the supplied QuanLynx™ application manager. The mass spectrometer was tuned by infusing a mixture of all seven amino acids (and their corresponding internal standards) at a concentration of 10µg/mL via syringe pump (10µL/min.) into a 50:50 mixture of mobile phases A and B flowing at 0.6mL/min. Under these conditions, the mass spectrometer was optimized to provide the greatest abundance of the leucine butyl ester precursor (m/z 188.0). In MS/MS mode the collision energy for the most abundant leucine product ion (m/z 86.0) was optimized. These parameters were found to provide adequate sensitivity for all other amino acids. Optimized MRM transitions were grouped temporally to optimize the mass spectrometer duty cycle for each amino acid and internal standard as shown in Table 2.

Time	Analyte	MRM Transition (m/z)	Dwell Time (sec.)
1—3.75 min.	Valine	174.0 > 72.0	0.040
	d3-Valine	182.0 > 80.0	0.040
	Tyrosine	238.0 > 136.0	0.040
	13C6-Tyrosine	244.0 > 142.0	0.040
	Methionine	206.0 > 104.0	0.040
3.75—5.6 min	d3-Methionine	209.0 > 107.0	0.040
	Alloisoleucine	188.0 > 86.0	0.050
	d10-alloisoleucine	198.0 > 96.1	0.050
	Isoleucine	188.0 > 86.0	0.050
	13C6 Isoleucine	194.0 > 91.0	0.050
	Leucine	188.0 > 86.0	0.050
5.6—7.5 min.	d3-Leucine	191.0 > 89.0	0.050
	Phenylalanine	222.0 > 120.0	0.050
	d3-Phenylalanine	227.0 > 125.0	0.050

Table 2. MRM transitions and dwell times for the seven amino acids and corresponding internal standards monitored in this study.

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 . Figure 2 shows the method comparison of three representative analytes. Each of the 75 specimens was classified into the same PKU, MSUD or normal cohort by both methods when independently evaluated. Figure 3 compares a normal specimen to one drawn from a patient with MSUD and one from a patient with PKU (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 r² values in excess of 0.99 as shown in Figure 2.

Precision

Within day and day-to-day precision for all control levels and all seven analytes ranged from 1.5 to 7.0% and are summarized in Table 3. Correlation analysis of alle concentrations in controls using d10-alle vs. d3-Leu yielded y=1.02x—0.079, r²=0.998 and lle concentrations using 13C6-Ile vs. d3-Leu yielded y=1.02x—0.39, r²=0.997.

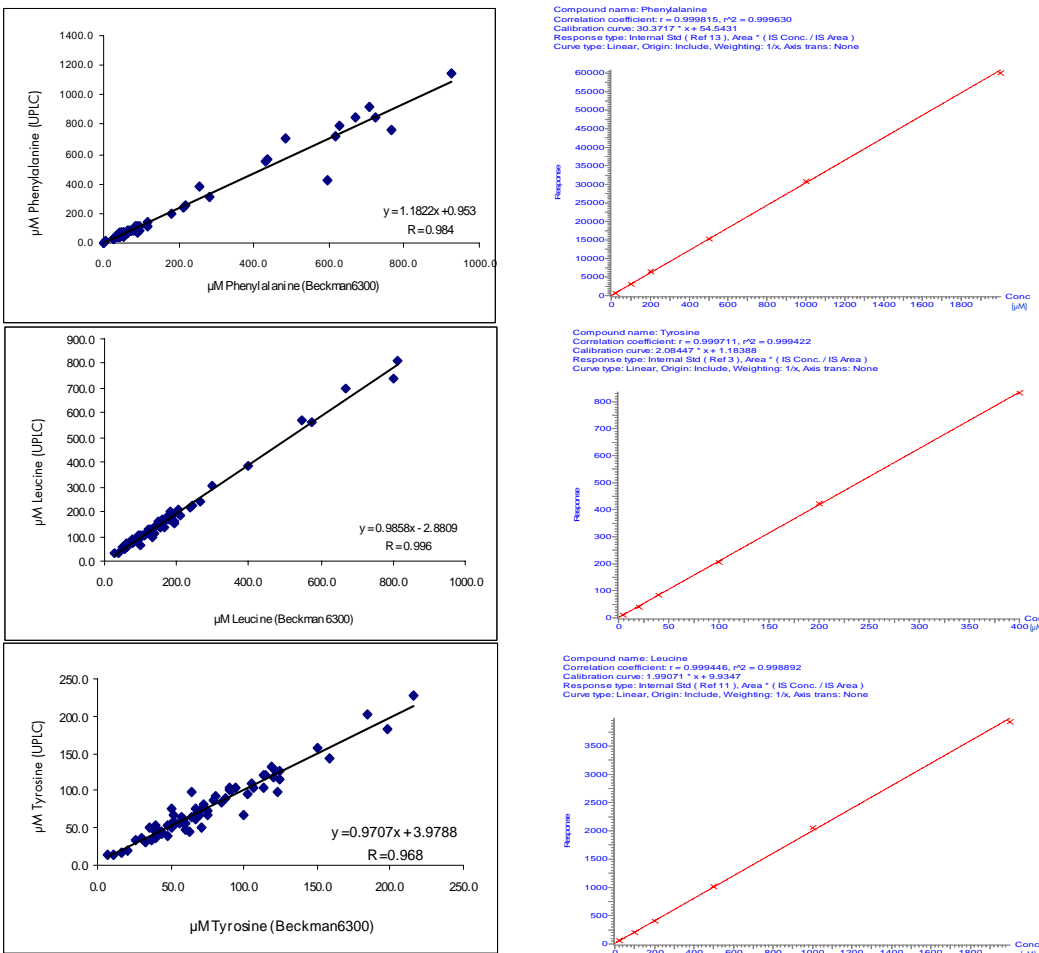


Figure 2. Selected amino acid method correlation and linearity.

	Within Day Precision													
	QC Level 1							QC Level 2						
	Val	Tyr	Met	alle	Ile	Leu	Phe	Val	Tyr	Met	alle	Ile	Leu	Phe
Mean (µM)	73	29	72	7.3	7.6	152	147	148.7	59.3	146	15.1	14.8	297	300
SD	1.4	1	1.2	0.5	0.4	2.82	3.6	3.4	1.8	5.4	0.5	0.8	6.3	10.0
% cv	1.9	3.5	1.6	7.0	4.8	1.9	2.5	2.3	3.1	3.7	3.3	5.5	2.1	3.3
	Day-to-day Precision													
	Val	Tyr	Met	alle	Ile	Leu	Phe	Val	Tyr	Met	alle	Ile	Leu	Phe
Target (µM)	75	30	75	7.5	7.5	150	150	150	60	150	15	15	300	300
Avg. mean (µM)	75	30	74	7.4	8.19	152	153	153.4	60.5	151	15.2	15.7	307	310
SEM	3.0	3.2	4.9	6.6	6.3	1.9	2.2	2.1	2.8	2.5	3.8	6.3	1.6	2.2

Table 3. Summary of within-day and day-to-day precision for each of the 7 amino acids in three levels of control material.

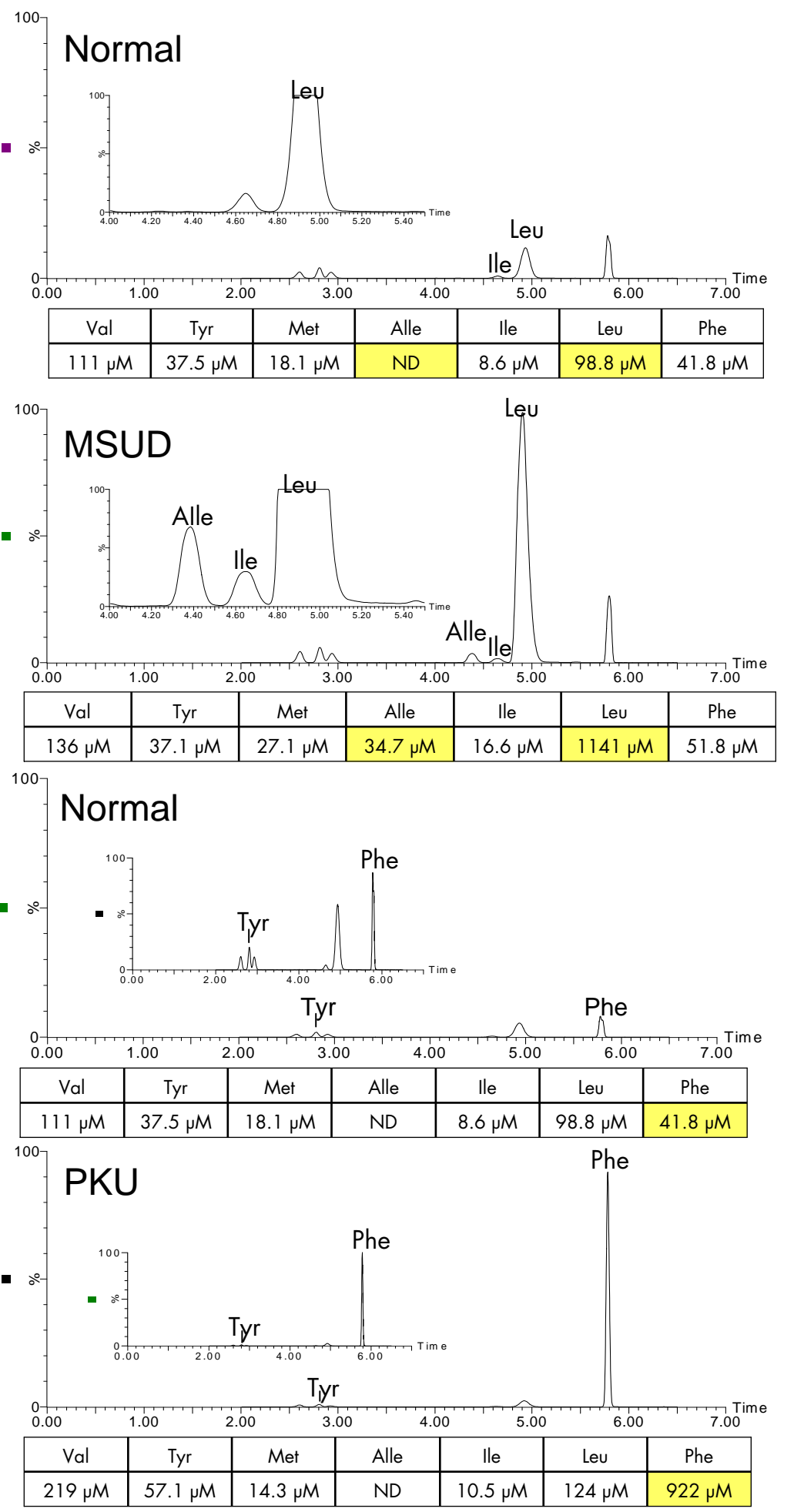


Figure 3. Comparison of a normal plasma specimen to one from a patient diagnosed with MSUD and one with PKU (note highlighted values).

DISCUSSION

The measurement of amino acids from whole blood collected on filter paper has been dramatically improved in terms of sensitivity and turn-around time with the introduction of electrospray-tandem mass spectrometry. It is now possible to accurately quantitate almost all physiological amino acids using only a few microliters of blood within a period of approximately two (2) minutes. This technological advance has allowed for population-based screening of newborns for inherited disorders of amino acid metabolism and is now considered 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.

One such disorder, Maple Syrup Urine Disease presents an unusual challenge in that three of the critical amino acids, leucine, isoleucine and alloisoleucine are isobaric and cannot be differentiated without chromatographic separation. Traditional ion-exchange chromatography can provide this separation but takes up to an hour per sample for analysis and the detection system lacks the sensitivity of mass spectrometric approaches. We have developed a rapid (less than 6-minute) separation protocol utilizing UPLC with mass spectrometric analysis to monitor the individual isobaric branched-chain amino acids in patients with Maple Syrup Urine Disease. In addition, this method also measures phenylalanine, tyrosine and methionine, which are critical amino acids for monitoring patients with phenylketonuria and homocystinuria, respectively. We have also demonstrated that that all three 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. 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.

CONCLUSIONS

- UPLC technology provides the ability to chromatographically resolve the leucine isomers in less than 6 minutes with mass spectrometer friendly mobile phases.
- The method described here 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 alle and lle when internally standardized using d3-Leu or their individual stable isotopes d10-alle and 13C6-Ile

Reference

1. Slocum RH, Cummings JG. Amino acid analysis of physiological samples. In: Hommes FA, ed. *Techniques in Diagnostic Human Biochemical Genetics—A Laboratory Manual*. New York: Wiley-Liss; 1995:87-126.