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Article

A Rapid and Sensitive UPLC-MS/MS-Method for the Separation and Quantification of Branched-Chain Amino Acids from Dried Blood Samples of Patients with Maple Syrup Urine Disease (MSUD)

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Abstract: Newborn screening for MSUD is a special challenge since patients with MSUD can metabolically decompensate rapidly without adequate treatment within the first two weeks of life. However, the screening method does not detect the actual marker metabolite (alloisoleucine) specifically, but only as part of the group of the other isobaric amino acids leucine, isoleucine and hydroxyproline. We describe a sensitive and rapid second-tier UPLC-MS/MS method to determine branched-chain amino acids from the initial extraction of the screening sample. Quantification is based on a seven-point calibration curve. Reference ranges (mean ± SD in µmol/L) were determined from 179 normal, not pre-selected samples from the newborn screening: leucine: 72 ± 27; isoleucine: 37 ± 19; valine: 98 ± 46; hydroxyproline: 23 ± 13. The concentration of alloisoleucine was below the detection limit in about 55% of the cases, and the highest concentration was 1.9 µmol/L. In all 30 retrospectively studied screening samples from patients with confirmed MSUD the concentration of alloisoleucine was significantly increased. In 238 samples with false-positive newborn screening due to a significant increase in the combined concentration of leucine + isoleucine + alloisoleucine + hydroxyproline (400 to >4000 µmol/L), alloisoleucine was below 6.5 µmol/L (n = 57) or not detectable (n = 181). The application of this assay markedly reduces the false-positive rate and the associated anxiety and costs. It is also suitable for routinely monitoring blood spots of patients with MSUD.

Keywords: newborn screening; neonatal screening; maple syrup urine disease; MSUD; dried blood spots; patient follow up; inborn errors of metabolism; IEM

1. Introduction

Maple syrup urine disease (MSUD, OMIM 248600) is caused by a defect of the branched-chain 2-oxo acid dehydrogenase complex. It is autosomal recessively inherited with a worldwide incidence of approximately one in 185,000 [1], with higher incidences in populations with low gene shifting [2], or a high rate of consanguineous marriages [3]. Data from 1.6 million newborns in Bavaria/Germany revealed an incidence of one in 135,000 (95% C.I.; 1:76,000–1:260,000) [4]. For Switzerland we would estimate the same incidence, which is supported from data collected between 1965 to 1989, with an

incidence of one in 143,000 (95% C.I.; 1:80,000–1:286,000). MSUD is a heterogeneous disorder with a wide range of clinical severity, ranging from severe neonatal onset forms to milder variant forms. Newborn screening (NBS) for MSUD is a special challenge since patients with MSUD can metabolically decompensate rapidly without adequate treatment within the first two weeks of life. However, the screening method does not detect the actual marker metabolite (alloisoleucine) [5,6] specifically, but only as part of the group of the other isobaric amino acids leucine, isoleucine and hydroxyproline. The positive effect of early detection, especially of the severe cases, is well documented [7]; however, NBS results cannot discriminate between severe and milder forms [8]. There are also rare cases of hyperhydroxyprolinemia resulting in a false-positive MSUD screening result [9,10], but this seems to be a minor problem in general [4]. Several methods have been published for second-tier measurement of alloisoleucine [11–13], which all use a second extraction from the initial NBS blood spot and chromatographic separation of the isobaric amino acids leucine, isoleucine, and alloisoleucine. We describe a sensitive and rapid second-tier UPLC method by which the branched-chain amino acids leucine, isoleucine, alloisoleucine, valine, and also hydroxyproline can be determined already within the initial extraction of the screening sample. Quantification is based on a seven-point calibration curve. The method can also be used for therapy monitoring in patients with MSUD.

2. Materials and Methods

For routine NBS a Waters 2777C Sample Manager and a Waters Acquity TQD (tandem mass spectrometer) is used. For the separation of branched chain amino acids a Waters Acquity H-Class UPLC system (Quarternary Solvent Manager, Sample Manager FTN, Column Manager) with a Waters Acquity BEH C8 1.7 µm, 2.1 × 100 mm column, and a Waters Acquity TQD is used. Amino acids and Acylcarnitines are extracted from a 3.2 mm dried blood sample using the Neobase test-kit from PerkinElmer (Turku, Finland). After measurement within the routine newborn screening procedure in MRM (multiple reaction monitoring) mode, the residual sample is transferred into a total recovery vial (Waters) and branched chain amino acids are measured from 0.3 µL after separation on the UPLC system using a linear gradient (Figure 1). Flow rate was 0.4 mL/min, flow solvent "A" was water, flow solvent "B" acetonitrile. Both flow solvents contained 0.1% formic acid, and 0.01% heptafluorobutyric acid. Gradient conditions were: 95% "A" and 5% "B" for 0.1 min, then a linear gradient to 100% "B" over 1.4 min, 100% "B" was held for 0.3 min, and then step back to 95% "A" and 5% "B", which was held for 1.2 min. Column temperature was 50 °C. MS parameters are shown in Table 1. Alloisoleucine, isoleucine, leucine, valine, hydroxyproline, heptafluorobutyric acid, acetonitrile (Chromasolve, gradient grade for HPLC) were from Sigma-Aldrich, Buchs, Switzerland. Formic acid was from Merck, Darmstadt, Germany.

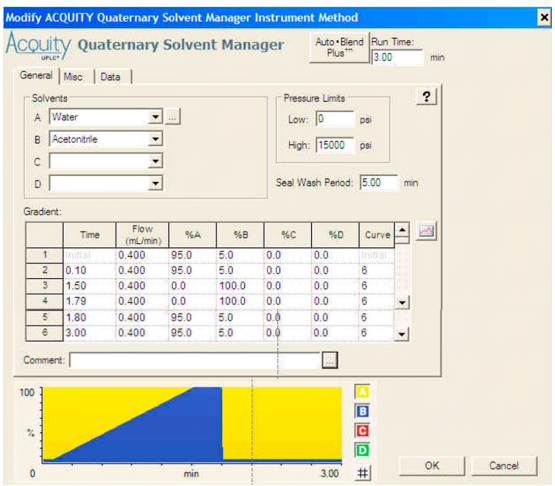

Figure 1. Mobile phase A: water with 0.1% formic acid and 0.01% heptafluorobutyric acid; Mobile phase B: acetonitrile with 0.1% formic acid and 0.01% heptafluorobutyric acid.

Table 1. Mass spectrometry parameters.

Compound	Parent [Da]	Daughter [Da]	Dwell Time [s]	Cone Voltage [V]	Collision Energy [eV]
Leucine *	132.1	86.1	0.05	22	10
$^{2}H_{3}$-leucine #	135.1	89.1	0.05	22	10
valine	118.1	72.1	0.05	22	10
$^{2}H_{8}$-valine #	126.1	80.1	0.05	22	10

* Leucine, isoleucine, alloisoleucine, and hydroxyproline are measured with the same transition and settings; # stable isotope labeled internal standard; Da = Dalton (or ***u*** for unified atomic mass unit).

3. Results

Reference ranges (mean ± SD in µmol/L) for the four isobaric amino acids were determined from 179 normal, not pre-selected samples from the NBS: leucine: 72 ± 27; isoleucine: 37 ± 19; valine: 98 ± 46; hydroxyproline: 23 ± 13. The concentration of alloisoleucine was below the detection limit in about 55% of the cases, and the highest concentration was 1.9 µmol/L. In all 30 retrospectively studied screening samples from patients with confirmed MSUD, the concentration of alloisoleucine was significantly increased. An example of the separation is shown in Figure 2. In 238 samples with false-positive NBS due to significantly increased concentrations of leucine + isoleucine + alloisoleucine + hydroxyproline (300 to >4000 µmol/L), alloisoleucine was below 6.5 µmol/L (Figure 3). The coefficient of variation was determined from 29 consecutive measurements of two control samples. Control samples were prepared from whole blood spiked with different amounts of the four isobaric amino acids and dried on filter paper. The final concentrations were 546 and 846 µmol/L for leucine, 310 and 910 µmol/L for isoleucine, 103 and 303 µmol/L for alloisoleucine, and 839 and 1339 µmol/L for valine, respectively (see Table 2). Since the inclusion of MSUD in the NBS panel in Switzerland in November 2014, approximately 124,000 newborns have been screened for MSUD. Alloisoleucine was measured in a second-tier test in approximately 0.15% of all newborns. All reanalyzed samples have been normal to date, going along with a recall rate of 0.0%.

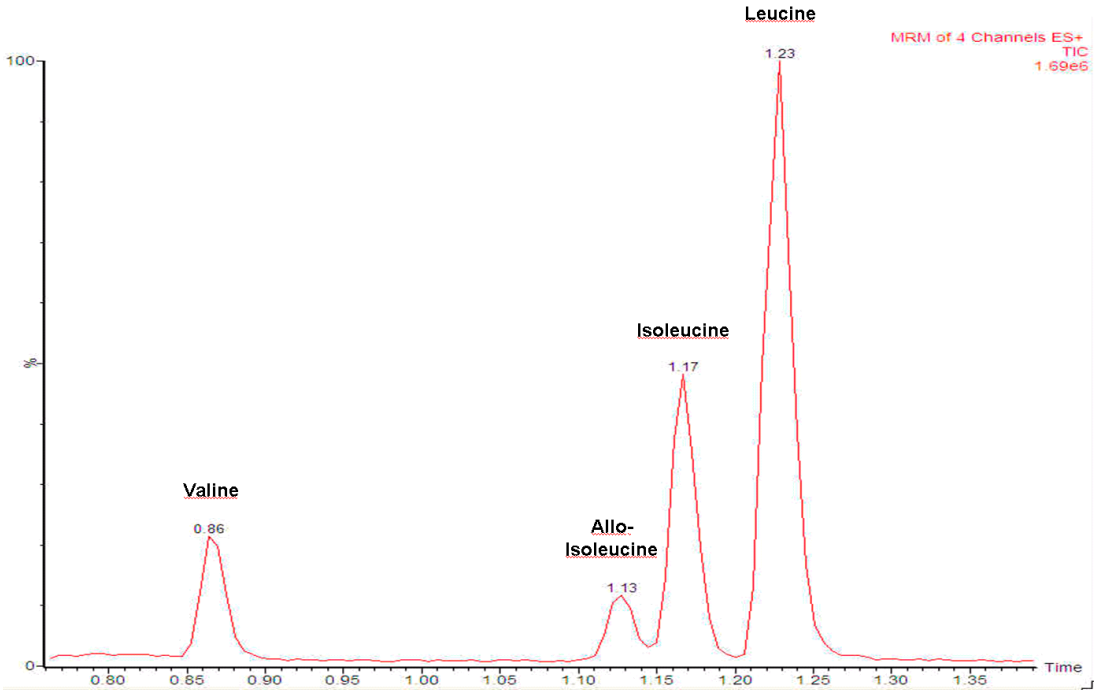

Figure 2. Separation of branched-chain amino acids from a dried blood spot of a patient with MSUD.

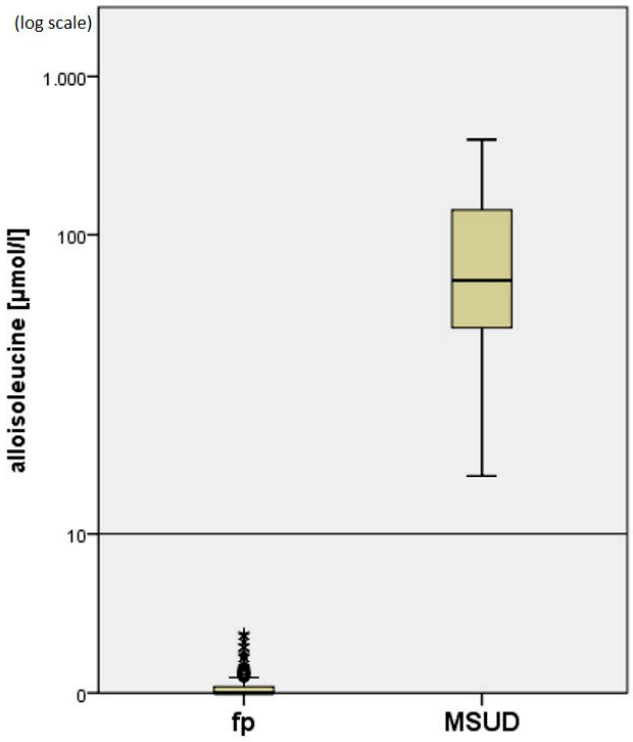

Figure 3. Distribution of alloisoleucine in dried blood spot (DBS) of newborns with false-positive (fp) newborn screening, and newborns with MSUD.

Table 2. Combined concentrations of leucine, isoleucine, alloisoleucine, and hydroxyproline (xle; measured in MRM mode), and concentrations of leucine, isoleucine, alloisoleucine (Allo-Ile), and valine in normal newborn samples (mean ± SD), screening samples of patients with confirmed MSUD, and false positive samples of newborns on total parenteral nutrition (TPN/FP).

Samples	*n*	xle *	Leucine *	Isoleucine *	Allo-Ile *	Valine *
normal	179	137 ± 36	72 ± 27	37 ± 19	n.d.–1.9	98 ± 46
MSUD	30	247–3530	280–4000	79–643	10–400	157–1206
TPN/FP ‡	238	300 – >4000	400 – >4000	107 – >3000	n.d.–7	397 – >3000
CV #	29	-	10.3/10.8	7.1/12.9	12.2/17.5	15.3/15.9

* concentration of amino acids in µmol/L; # CV was determined at two different concentrations (QC_{low}/QC_{high}); ‡ TPN/FP: Total parenteral nutrition/False positive; n.d. = not detectable.

4. Discussion

As already outlined in the introduction, NBS for MSUD is extraordinary due to the necessity of a rapid screening test with a high positive predictive value. The merely combined concentrations of leucine, isoleucine, alloisoleucine, and hydoxyproline (xle) are not helpful, as demonstrated in Table 2. Since the concentration of alanine is frequently decreased or in the low normal range, the inclusion of alanine (ala) and/or the ratios xle/ala and val/ala have already improved the interpretation of MSUD-NBS [2,4]. The biochemical and metabolic basis for this phenomenon has been described already in 1978 [14]. A schematic overview of alanine metabolism in healthy humans and in patients with MSUD is shown in Figure 4. Normally branched-chain amino acids are metabolized to their respective 2-oxo acids. This reaction needs 2-oxo glutarate as a cofactor which is converted to glutamate. Then 2-Oxo glutarate is "recycled" from glutamate and pyruvate, generating alanine at the same time. If there is a block in the 2-oxo acid dehydrogenase, such as in MSUD, these reactions are reversible, leading to low normal or decreased concentrations of alanine.

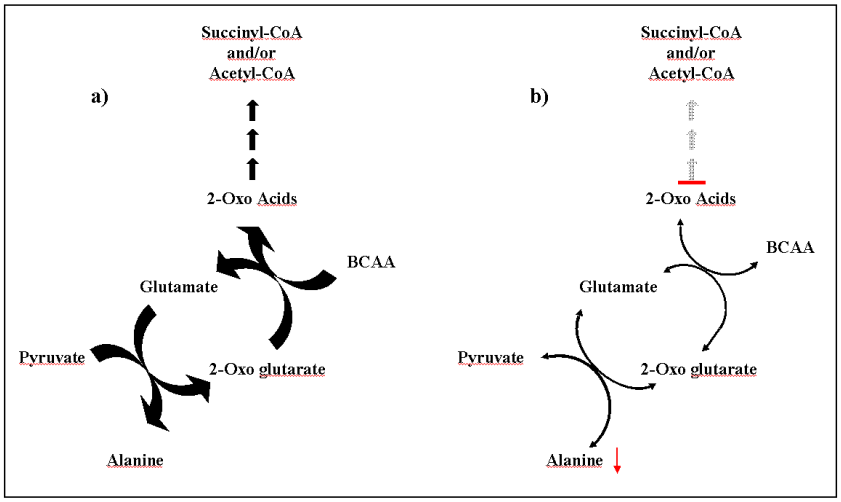

Figure 4. Alanine metabolism in healthy humans (**a**) and in patients with MSUD (**b**). (BCAA: branched-chain amino acids)

The application of a second-tier test, however, allows a clear identification of affected individuals. All previous described methods are more time-consuming, and they all need significantly more dried blood spot (DBS) material: either 2.5 times more material and 16 min separation time [11], four times more material and 10 min separation time [12], or two times more material and 3 min separation time [13], compared to our approach with no additional material, no additional extraction and direct injection of the leftover extract. By this the confirmation of a positive NBS result for MSUD can be accomplished already 1 h after the initial measurement of elevated branched-chain amino acids including all steps of conditioning and preparing the UPLC-MS/MS system.

5. Conclusions

The described method is rapid, sensitive and reliable. It can be used either as a second-tier method for MSUD newborn screening, or for therapy monitoring in patients with MSUD. Samples can be taken by heel-prick or finger-prick, either by the parents or the patients themselves. The integration within the routine newborn screening requires only minimal additional work. For MSUD newborn screening, the results of the branched-chain amino acids determined by the second-tier method are available within one hour after the positive screening result. The approach markedly reduces the false-positive rate and improves the positive predictive value of NBS for this potentially fatal condition. In addition, both a clinical follow-up can be avoided and the newborn's family can be spared the trouble connected with a false-positive screening result.

Author Contributions: Ralph Fingerhut and Markus Heck conceived and designed the experiments; Ralph Fingerhut performed the experiments; Ralph Fingerhut, Wulf Röschinger and Markus Heck analyzed the data; Wulf Röschinger contributed materials; Ralph Fingerhut wrote the paper; Wulf Röschinger and Markus Heck reviewed the manuscript and made considerable contributions to the submitted manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MSUD:	Maple Syrup Urine Disease
UPLC:	Ultra Performance Liquid Chromatography
MS/MS:	Tandem Mass Spectrometry
NBS:	Newborn Screening
CV:	Coefficient of Variation
TPN:	Total Parenteral Nutrition
FP:	False Positive

MRM: Multiple Reaction Monitoring
C.I.: Confidence Interval

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