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ORIGINAL ARTICLE

Use of HPLC-UV method for the analysis of maple syrup urine disease in plasma sample first time in Saudi Arabia

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ABSTRACT

Background: Measurement of branched-chain amino acids (BCAAs) [valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu)] in plasma and dry blood spot samples is important for the diagnosis and monitoring of maple syrup urine disease (MSUD), which is a metabolic disorder that affects metabolism of BCAAs. We used a temporally short program on an High performance liquid chromatography (HPLC) (Biochrom 30) for the first time in Saudi Arabia to analyze plasma samples for the diagnosis of MSUD.

Methodology: 500 µl of plasma were mixed with 500 µl of 10% sulphosalicylic acid, followed by shaking for 5–10 seconds on a vortexer. The mixture was incubated at 4°C for 10 minutes followed by centrifugation at 10,000 rpm for 5 minutes. Supernatants were transferred into HPLC vials for injection.

Results: For all amino acids, the limit of detection and limit of quantification were 5.0 and 10.0 µmol/l, respectively. The method was linear in the range of 10–2,500 µmol/l. The method was specific and selective for the detection of BCAAs, including alloisoleucine. The correlation coefficient (CV) of method comparison was greater than 0.980 and percent recovery of all amino acids in plasma was 80%–100%, which was evaluated at two amino acid concentrations. Precision was assessed by repeat analysis of spiked plasma samples and percent CV was found to be <8% for all amino acids tested at two concentrations.

Conclusion: The short program of amino acid analysis was helpful for the early diagnosis of MSUD.

Keywords: MSUD analysis by HPLC.

Introduction

Maple syrup urine disease (MSUD) is a recessive inherited genetic disorder characterized by the inability to metabolize branched-chain amino acids (BCAAs) including leucine, isoleucine, and valine, as well as the corresponding α-ketoacids. MSUD patients are deficient in the ketoacid multienzyme dehydrogenase complex that catalyzes the first step of BCAA metabolism (1,2). As a consequence, BCAAs and the corresponding α-keto acids accumulate in bodily fluids, which is responsible central nervous system (CNS) toxicity and the sweet odor of affected urine (3). MSUD is a rare metabolic disease with an estimated incidence of 1 in 185,000 births worldwide (4) and 1 in 22,000 births in Saudi Arabia (5). It is the most common and severe form of branched-chain ketoaciduria that manifests between the fourth and seventh days following birth life. MSUD symptoms include lethargy, poor appetite, weight loss, and neurological problems. Other symptoms include convulsions, hypothermia, coma, unwillingness to eat, and death if not treated in time. Early diagnosis is important for treatment and prognosis as it may prevent neurological complications and deterioration.

Testing for MSUD is part of the newborn screening program and is conducted by tandem mass spectrophotometric analysis of dry blood spots. Elevated concentration of leucine, isoleucine, and valine in dry blood spots are indicators of MSUD. All commercially available MS/MS reagent kits cannot differentiate between isoleucine, leucine, and alloisoleucine, later is the typical pathognomonic marker of MSUD (6). Newborn screening sample positive for MSUD are typically confirmed by

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plasma amino acid analysis using a Biochrom 30 HPLC analyzer, which requires approximately 130 minutes per sample. In this work (and for the first time in Saudi Arabia), we utilized a temporally shorter HPLC program in which different buffers and temperatures were used on the same HPLC analyzer for the quantification of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) in patient plasma samples, which facilitates earlier and faster diagnosis of MSUD (within 60 minutes). Separation of amino acids in ion-exchange chromatography is dependent on pH, concentration, and flow rates of the different buffers at the different temperatures (relative to the standard HPLC program) (7). By changing buffers concentrations and column temperatures, all BCAAs were separated and analyzed within 60 minutes, which shortened sample analysis by approximately 70 minutes. After confirmation and determination of these amino acids, the shorter HPLC method was used to monitor the effectiveness of patient treatment and follow-up visits. The method was fully validated according to Food & Drug Administration Authority USA (FDA) bioanalytical method validation guidelines.

Materials and Methods

Reagent and chemicals

European Pharmacopeia isoleucine (I0460000), leucine (L0375000), and valine (V0030000) reference standards were purchased from Sigma-Aldrich. High-purity (97%) alloisoleucine (860719) and suphosalicylic acid were also purchased from Sigma-Aldrich. HPLC grade lithium buffers, loading buffers, ninhydrin, and ultrasolve were obtained from Biochrom, UK.

Instrumentation

The Biochrom 30 HPLC system (Biochrom Company, UK) was used in this work, which consisted of a binary pump and a MIDAS sample injector. All four amino acids were detected at 570 nm at 65 minutes. HPLC columns were packed with Ultropac C8 (Octasillyl) cation-exchange resin, which was used as the stationary phase. HPLC-grade lithium buffers 1, 2, 3, 4, 5, and 6 were used as the mobile phase. An injection volume of 40 µl was loaded into the HPLC system. Elite EZ Chrom software was used to process the data.

Calibration and quality control preparation

Isoleucine, leucine, valine, and alloisoleucine were dissolved in 0.1 N HCL at a stock concentration of 5,000 µmol/l. The solution was further diluted with 0.1 N HCl to 500 µmol/l that was used as a working standard solution. A single-point calibration standard was prepared from the working standard solution for analysis.

Amino acid quality controls were prepared by drawing whole blood from 30 healthy donors in lithium heparin tubes. Blood samples were centrifuged to separate the plasma. All plasma samples were combined, mixed, and analyzed in duplicate to obtain baseline concentrations. Known amounts of amino acid standard solution were spiked into pooled plasma to prepare two concentrations of quality control samples corresponding to the normal and abnormal reference ranges. Aliquots of the prepared quality controls (500 µl) were labeled as AA-QC1 (normal control) and AA-QC2 (abnormal QC) and were stored at −70°C until use.

Preparation of solutions

Needle wash solution consisted of 20% isopropanol that was prepared by mixing 200 ml of pure isopropanol with 800 ml of HPLC-grade water. This solution was stable until consumed. Ninhydrin solution was prepared by mixing ultrasolve plus solution with Ultra Ninhydrin solution in a clean analyzer reagent bottle. The solution was purged with nitrogen gas for 30 minutes. This ninhydrin reagent was stable for 15 days and was sufficient for 120 hours of continuous HPLC analyzer operation. Precipitation reagent was prepared by dissolving 1.5 g of sulfosalicylic acid crystals in 15 ml of HPLC-grade water. The solution was stable for 10 days.

HPLC procedure

Five hundred microliter of working standard solution, 500 µl of quality controls, and 500 µl of patient sample were separately transferred into 1.5 ml microcentrifuge tubes. Next, 500 µl of sulfosalicylic acid solution was added to each tube that was then mixed for 30 seconds followed by incubation at 4°C for 10 minutes. The microcentrifuge tubes were centrifuged at 15,000 rpm for 5 minutes and supernatants were transferred into HPLC vials. The HPLC system was equilibrated with buffer 6 for 30 minutes before injection. The total run time was 60 minutes per sample.

Results and Discussion

Method optimization

In the HPLC chromatogram, the valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) peaks were well-resolved in calibration samples, quality control samples, and patient samples. The HPLC system was equilibrated with buffer 6 before starting the analysis. Buffers other than buffer 6 disturbed the separation of peaks in the HPLC column. A small methionine peak appeared before alloisoleucine in all plasma sample chromatograms. In older HPLC columns (i.e., after 1,000 samples), the methionine peak was merged with the alloisoleucine peak. During mobile phase gradient flow, buffer 6 and the column temperature were increased to solve this problem. When analyzing non-MSUD patient samples, no peaks were detected in the retention time of alloisoleucine, which confirmed the specificity of the method.

Specificity and selectivity

Separate standard solutions of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine

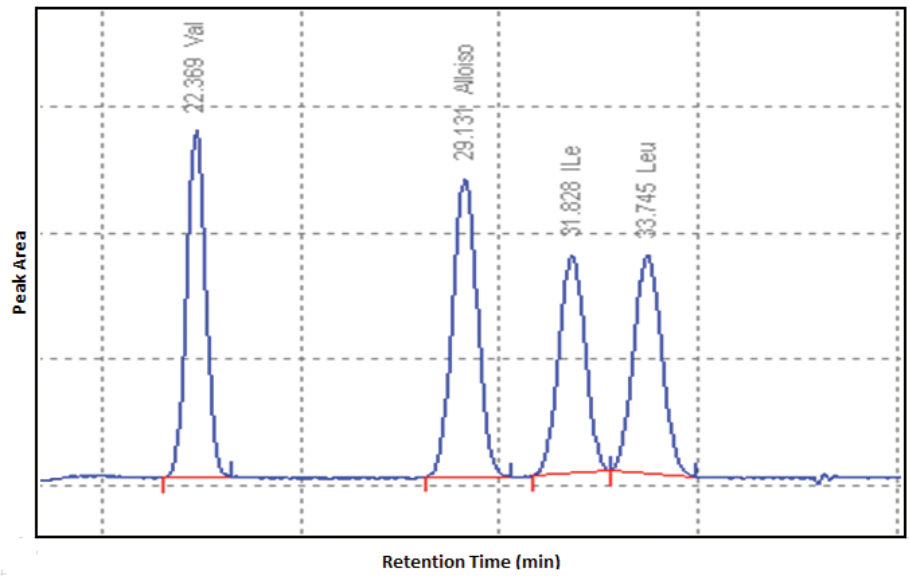

Figure 1. *Chromatogram of MSUD calibration standard solution.*

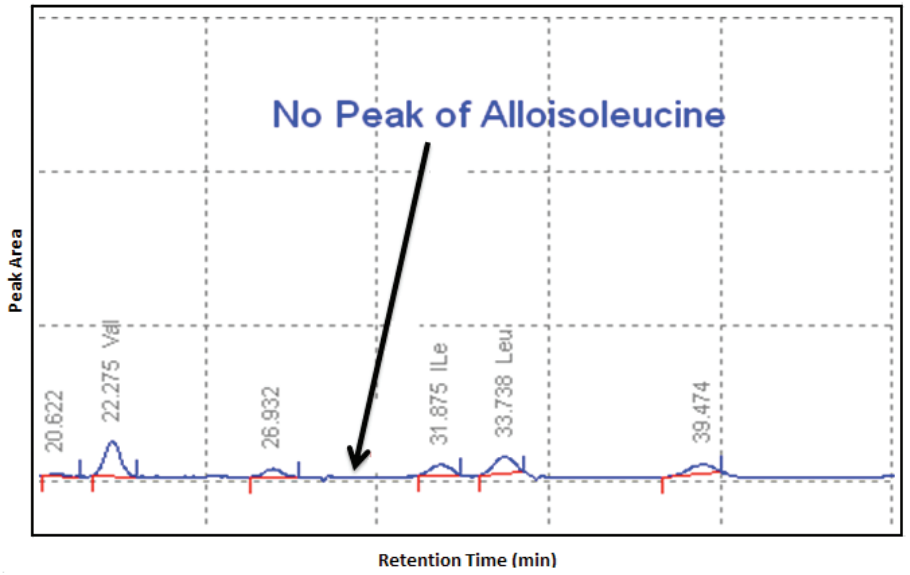

Figure 2. *Chromatogram of a non-MSUD patient.*

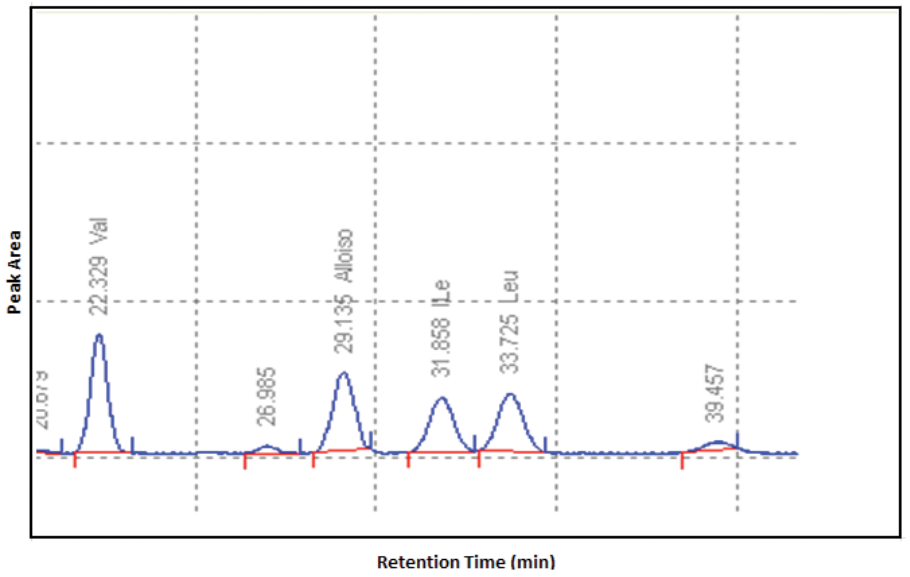

Figure 3. *Chromatogram of a MSUD patient.*

(Leu) were prepared (500 µmol/l) and extracted according to the assay procedure. Each extracted standard solution was injected five times to determine individual retention times. A combined mixture of these amino acids was also analyzed to confirm the validity of their individual retention times. Approximately 20 non-MSUD patient samples were injected; no alloisoleucine peak was detected, which demonstrated the specificity of the method. A pool of patient samples were spiked with increasing concentrations of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) that was then analyzed by the assay procedure. The detected levels of all four amino acids increased, which demonstrated that the method was specific and selective. Chromatograms of standard solution, a non-MSUD patient, and a MSUD patient are shown in Figures 1–3, respectively.

Linearity and range

From the stock calibration standard, solutions of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) (5, 10, 25, 50, 500, 1,000, 1,500, and 2,500 µmol/l) of were prepared and were analyzed in duplicate according to the procedure. Linear regression results are shown in Table 1.

Limit of detection (LOD) and lower limit of quantification (LOQ)

A standard solution of 5.0 and 10.0 µmol/l of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) were prepared and analyzed five times by the assay procedure. Recovery and precision were calculated. The LOD was found to be 5.0 µmol/l and the LOQ was 10.0 µmol/l (Table 1).

Accuracy and precision

A plasma sample was analyzed in duplicate to establish baseline concentrations of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu). This plasma sample was spiked with known amounts of stock standard solution (containing all four amino acids) at two different concentrations of 150 and 250 µmol/l. These samples were analyzed five times to assess accuracy and precision and the results are summarized in Table 2.

Method comparison

Approximately 20 patient samples containing various concentrations of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) were analyzed on the

Table 1. *Linearity and sensitivity data.*

Parameter	Valine	Alloisoleucine	Isoleucine	Leucine
Range	5–2,500 µmol/l	5–2,500 µmol/l	5–2,500 µmol/l	5–2,500 µmol/l
Slope	0.970	1.1229	0.7286	0.6975
Intercept	3.765	6.636	6.6330	3.1149
R^2	1.000	0.9952	0.9993	0.9991
LOD	5.0 µmol/l	5.0 µmol/l	5.0 µmol/l	5.0 µmol/l
LOQ	10.0 µmol/l	10.0 µmol/l	10.0 µmol/l	10.0 µmol/l

Table 2. *Accuracy and precision data.*

Parameter	Levels	Valine	Alloisoleucine	Isoleucine	Leucine
% Recovery	Level 1	99	99	99	100
	Level 2	92	87	90	80
Precision (%CV)	Level 1	3.6	4.4	8.1	3.7
	Level 2	4.3	3.8	2.1	1.9

Table 3. *Method comparison: Biochrom 30 and Biochrom 30 plus.*

Parameter	Valine	Alloisoleucine	Isoleucine	Leucine
Slope	1.09	1.14	1.10	0.976
Intercept	−2.4	−12.2	0.73	6.39
R^2	0.981	0.986	0.988	0.992

Biochrom 30 on different days using the short program. The same samples were also analyzed on a different HPLC system, the Biochrom 30 Plus. Statistical analysis was performed by comparing the mean values of both methods and bias. No significant bias nor differences in patient results were found, as indicated by the statistical analysis of the two mean values (Table 3). We analyzed approximately 20 different samples from the External Quality Assurance Program for Amino Acids proficiency testing program and the results were found to be within the acceptable range.

Application

The shorter HPLC program was successfully applied for the determination of valine (Val), alloisoleucine (allo-Ile), isoleucine (Ile), and leucine (Leu) levels in plasma to confirm and follow-up diagnoses of MSUD for the first time in Saudi Arabia.

Conclusion

The use of the shorter HPLC program for the confirmation of MSUD was helpful as it can clearly identify and quantify alloisoleucine in plasma samples. Early diagnosis is important for starting treatment without having to wait for the entire amino acid profile, which takes approximately 120 minutes. This modified method had never been used for Saudi populations. It is highly selective and sensitive for BCAAs s in plasma samples and can detect and quantify up to 5 µmol/l of each analyte. In the routine method, the methionine peak is frequently merged with the alloisoleucine peak but is fully separated by the shorter program, which confirms the selectivity and specificity of the method. The method can also detect and quantitate alloisoleucine in dry blood samples (DBS), which is a pathognomonic marker of MSUD; however, the method was not validated for DBS because plasma s is commonly used for diagnosis and follow-up due to the high concentrations of alloisoleucine in MSUD patients.

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Consent for publication
Not applicable.

Ethical approval
The method is approved by the institutional quality management team responsible for new method development and implementation.

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Declaration of conflicting interests
The authors declare that there is no conflict of interests.

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