

A novel liquid chromatography tandem mass spectrometry method for simultaneous determination of branched-chain amino acids and branched-chain α -keto acids in human plasma

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Received: 13 December 2015 / Accepted: 4 March 2016 / Published online: 16 March 2016
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Abstract Branched-chain amino acids (BCAAs) and branched-chain α -keto acids (BCKAs) play significant biological roles as they are involved in protein and neurotransmitter synthesis as well as energy metabolism pathways. To routinely and accurately study the dynamics of BCAAs and BCKAs in human diseases, e.g. cerebral infarction, a novel liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated. The plasma samples were deproteinized with acetonitrile, and then separated on a reversed phase C18 column with a mobile phase of 0.1 % formic acid (solvent A)–methanol (solvent B) using gradient elution. The detection of BCAAs and BCKAs was conducted in multiple reaction monitoring with positive/negative electrospray ionization switching mode. Biologically relevant isomers such as leucine and isoleucine were individually quantified by combining chromatographic separation and fragmentation. Good linearity

($R^2 > 0.99$) was obtained for all six analytes with the limits of detection from 0.1 to 0.2 $\mu\text{g/mL}$. The intra-day and inter-day accuracy ranged from 93.7 to 108.4 % and the relative standard deviation (RSD) did not exceed 15.0 %. The recovery was more than 80 % with RSD less than 14.0 %. The main improvements compared to related, state-of-the-art methods included enhanced sensitivity, enhanced separation of isomers, and reduced complexity of sample processing. Finally, the validated method was applied to analyze the plasma samples of healthy volunteers and patients suffering cerebral infarction, and significant differences in the concentration levels of BCAAs and BCKAs were observed.

Keywords Branched-chain amino acids · Branched-chain α -keto acids · Isomer · LC–MS/MS · Cerebral infarction · Human plasma

Handling editor: D. Tsikas.

Electronic supplementary material The online version of this article (doi:10.1007/s00726-016-2212-5) contains supplementary material, which is available to authorized users.

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Introduction

Branched-chain amino acids (BCAAs) including leucine (Leu), isoleucine (Ile), and valine (Val) are three of nine essential amino acids that are not synthesized *de novo* and must be obtained from diet. Approximately 35 % of indispensable muscle proteins and 40 % of total amino acids required by mammals are comprised of BCAAs (Harper et al. 1984). Unlike other amino acids, BCAAs are primarily catabolised by extrahepatic tissues, e.g. brain, muscle. Catabolism of BCAAs is initiated by transamination reaction with α -ketoglutarate to form glutamate and branched-chain α -keto acids (BCKAs), consisting of α -ketoisocaproic acid (KICA), α -ketomethylvaleric acid (KMVA) and α -ketoisovaleric acid (KIVA). Then, the BCKAs are decarboxylated by the branched-chain α -keto acid dehydrogenase

complex and eventually degraded into acetyl-CoA or succinyl-CoA to fuel the Krebs cycle (Harris et al. 2005; Almeida et al. 2016). Therefore, both BCAAs and BCKAs are essential for normal growth and function at cellular and organism levels. Alterations in the metabolism of them often associate with various diseases. Maple syrup urine disease, methylmalonic acidemia, and propionic acidemia are known genetic disorders resulting from defects in BCAAs catabolic pathway (Henriquez et al. 1994; Ribeiro et al. 2008). In addition, there is ample evidence that patients with chronic diseases, such as diabetes mellitus, have an excess amount of free BCAAs or their catabolic products BCKAs in blood (Shimomura et al. 2006; Giesbertz and Daniel 2016). Thus the accurate detection and quantitation of BCAAs and BCKAs simultaneously could lay a solid foundation for the diagnosis and therapy of these diseases.

Currently, a number of techniques for analysis of amino acids or keto acids have been developed, including nuclear magnetic resonance (NMR) (Ghosh et al. 2015; Del Campo et al. 2016), capillary electrophoresis (CE) (Bouri et al. 2013), high performance liquid chromatography (HPLC) (Qureshi 1987; Kand'ár et al. 2009), gas chromatography–mass spectrometry (GC–MS) (Dettmer et al. 2012; Nguyen et al. 2013), and liquid chromatography–mass spectrometry (LC–MS) (Noguchi et al. 2014; Takach et al. 2014; Domingues et al. 2015; Qi et al. 2015; Ehling and Reddy 2015). However, all of these techniques suffer from various drawbacks, and at our best knowledge, no analytical method has been developed and validated for simultaneous determination of BCAAs and BCKAs in human plasma.

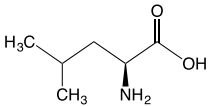
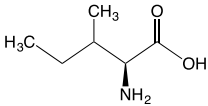
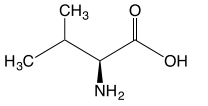
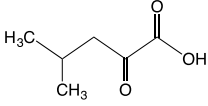
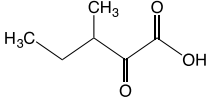
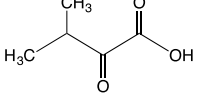
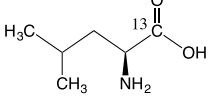
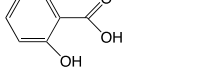
NMR enjoys the attractive advantages of ease-of sample preparation, unbiased approach towards analytes, and easy for quantification of the spectral peaks. However, it requires a large volume of plasma sample (>300 μ L) because of its relatively low sensitivity and selectivity (Ghosh et al. 2015). CE exhibits excellent performance for quantitation of organic acids based on its advantages of high resolution, low sample loading and economical equipment, but lacks robustness for routine analysis of biological extracts. HPLC is a reproducible technique in terms of high sample throughput analysis, and has been commonly applied to determine BCAAs and BCKAs separately. For example, Ziegler et al. (2014) used a combination of derivatization and reverse phase HPLC to selectively and sensitively detect and quantify amino acids from plant samples. Takach et al. (2014) also reported combining ultra-performance liquid chromatography (UPLC) separation with stable isotope labeling resulting in the elution and quantitation of 44 amino acids within 5 min. Unfortunately, these methods often require a pre-column derivatization procedure which is time-consuming and might lead to some problems, such as reagent interferences, side reactions and derivative instability (Önal et al. 2013). GC–MS is indeed

a durable and sensitive method with commercial spectral libraries and unchallenged chromatographic resolution, but the high temperature in GC will cause the isomerization of amino acids and thus may lead to inaccurate results (Christou et al. 2014; Liu et al. 2014).

On the basis of selectivity, sensitivity, ease-of-use, robustness to matrix, and robustness in routine operation, LC–MS is identified as the optimal platform (Buescher et al. 2010; Krumpochova et al. 2015). Since BCAAs and BCKAs are polar metabolites including two pairs of isomers (Leu and Ile, KICA and KMVA), the simultaneous separation and quantitation of them by LC–MS has also historically suffered from some drawbacks. In traditional reserved phase LC–MS, it is difficult to obtain a good retention as well as a good separation of polar analytes in a single run. For instance, Ehling et al. (2015) separately determined the concentrations of Leu and KICA in human breast milk by UPLC–MS/MS using one C18 column with two different gradient programs. Hydrophilic interaction liquid chromatography (HILIC) is able to retain polar analytes and suitable for MS detection, but resolves only few bioactive isomers (Tang et al. 2014; Qi et al. 2015). Ion pair chromatography (IPC) is widely used to selectively analyze acids and bases. How et al. (2014) analyzed 18 free amino acids in natural waters by LC–MS/MS with trifluoroacetic acid as ion-pairing reagent, and Buescher et al. (2010) presented an ion-pairing UPLC–MS/MS method using tributylamine for determination of 138 compounds in primary metabolism including BCKAs. However, no ion-pairing reagent could satisfy BCAAs and BCKAs at the same time due to the opposite charged states of amine and carboxylic acid. It should be noted that separate analysis of BCAAs and BCKAs often consumes more time, labor, sample and organic solvent, especially in long/large clinical studies. Therefore, the development of a LC–MS method to achieve simultaneous determination of BCAAs and BCKAs is in demand and remains the superior advantageous of simplicity and flexibility.

We hence worked toward a simple and effective LC–MS-based method for targeted and quantitative analysis of BCAAs and BCKAs. Building on the excellent capacity of ion suppression reverse phase LC to separate the isomers, we established a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI^{+/−}-MS/MS) method, which was the first analytical method developed for simultaneous investigating BCAAs and BCKAs in human plasma. Compared to related, state-of-the-art methods, the main improvements were enhanced sensitivity, enhanced separation of isomers, and reduced complexity of sample processing. The key parameters of separation and detection were systematically investigated and optimized. The method was fully validated and successfully applied to analyze the plasma samples from 32 healthy volunteers and 33 cerebral infarction patients.

Table 1 List of the structures, ESI models, selected MRM parameters, collision energy for each analyte measured

Analyte	Chemical structure	Molar mass (g/mol)	ESI model	Precursor ion (m/z)	Collision energy (V)	Product ion (m/z)
Leu		131.17	+	132.2	-27	43.0
Ile		131.17	+	132.2	-19	69.0
Val		117.15	+	118.2	-15	72.0
KICA		130.14	-	129.1	7	129.1
KMVA		130.14	-	129.1	7	129.1
KIVA		116.12	-	115.1	5	115.1
IS-1		132.17	+	133.2	-13	86.0
IS-2		138.12	-	137.0	6	137.0

Materials and methods

Chemicals and reagents

Standard BCAAs and BCKAs, L-leucine (>98 %), L-isoleucine (>98 %), L-valine (>98 %), KICA (>95 %), KMVA (>98 %), KIVA (>98 %), were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-¹³C₁-leucine (IS-1, >99 %) and salicylic acid (IS-2, >99 %) as internal standards were also purchased from Sigma-Aldrich. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Formic acid, ammonium acetate, ammonium formate and ammonia solution were of analytical grade and obtained from Nanjing Chemical Reagent Co. Ltd (Jiangsu, China). Ultrapure deionized water was supplied by a Milli-Q system from Millipore (Watford, UK).

Apparatus and analytical conditions

Separation of analytes was achieved by an ion suppression reverse phase method developed for ultrahigh performance systems and eventually implemented on a Shimadzu

Nexera UHPLC system (Shimadzu, Kyoto, Japan) using an Agilent ZORBAX SB-C18 column with dimensions 100 × 3 mm, 3.5 μm (Agilent, MA, USA) temperature-controlled at 40 °C. The linear gradient program was set as follows with mobile phase (A) 0.1 % formic acid solution and phase (B) methanol: 0 min, 20 % B; 1.5 min, 20 % B; 1.7 min, 40 % B; 3.5 min, 40 % B; 5 min, 65 % B; 8 min, 65 % B. The flow rate was 0.4 mL/min. The injection volume was 2 μL with full loop injection. The column was equilibrated for 4 min (approximately 4.4 column volumes) before each injection.

Selective and sensitive detection of analytes was achieved by coupling the liquid chromatograph to Shimadzu LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) using an electrospray ionization (ESI) source. The mass spectrometer was operated in positive/negative switching mode with multiple reaction monitoring (MRM). Electrospray ionization parameters were optimized for 25 % methanol at a flow rate of 0.4 mL/min and used for the entire gradient: spray voltage 4.5 kV from 0 to 3.5 min and -3.5 kV from 3.51 to 8 min, nebulizer gas 3.0 L/min, drying gas 15.0 L/min, desolvation line

temperature 250 °C, heat block temperature 400 °C, CID gas 230 kPa. Collision energy and fragment ions were optimized individually for all analytes (Table 1). Ion optics were set to 0.5 amu Q1 resolution, 0.5 amu Q3 resolution, 0.01 amu scan width, and 100 ms dwell time.

Standard and working solutions preparation

Stock standard solutions (1 mg/mL) of all analytes were prepared by dissolving each compound in water/methanol (50:50, v/v) and stored at 4 °C. From the stock solution, by serial dilution using water/methanol (50:50, v/v), working solutions (different concentrations of 0.4–200 µg/mL) for calibration and quality control (QC) were prepared. QC samples were prepared by spiking pooled human plasma with low, medium and high concentrations of standards, respectively. All working solutions and QC samples were prepared daily.

Sample preparation

An aliquot of 40 µL human plasma was pipetted into a 1.5 mL Eppendorf tube and spiked with 10 µL internal standard working solution (50 µg/mL IS-1, 5 µg/mL IS-2) and 160 µL acetonitrile. After vortex mixing for 5 min, the mixture was centrifuged at 4 °C for 5 min at 15,000g. Then, an aliquot of 100 µL supernatant was transferred into another 1.5 mL Eppendorf tube, evaporated by vacuum drying at 37 °C, and dissolved in 40 µL of water/methanol (80:20, v/v). Finally, the reconstituted extract was centrifuged (4 °C, 15,000g, 5 min) and the supernatant was transferred to an autosampler vial for analysis.

Method validation

This method was validated according to the US-FDA document and other related guidelines (González et al. 2014; Ruiz-Angel et al. 2014) with respect to linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, recovery, matrix effect and stability.

Calibration curves were constructed by spiking standards on top of endogenous levels in pooled plasma samples. Five samples were prepared for each concentration and averages were used to make calibration curves. Each calibration equation was fitted by the linear regression equation $y = mx + b$, where y = (the peak area of analyte in spiked plasma—the peak area of analyte in blank plasma)/the peak area of IS, x = the concentration of the spiked analyte. Since BCAAs and BCKAs were endogenous in human plasma, LOD and LOQ were estimated by calculating the standard error of the intercept (Sb) on the calibration curves, and expressed as 3.3 and 10 times the Sb/m , respectively.

Precision and accuracy were carried out in six replicates at three QC levels on the same day and on three consecutive

validation days. The precision was expressed as the relative standard deviation (RSD), and the accuracy was evaluated by the percentage ratio between the measured and nominal concentrations of QC samples. The accuracy was required to be in the range of 85–115 %, while the precisions not to exceed 15 %.

Recovery for each analyte was studied at three levels of concentration (low, medium and high QC), and calculated as follows: $\text{recovery}(\%) = \frac{A-C}{B-C} \times 100$, where A is the analyte response in a plasma sample spiked before the extraction and processed, B is the response in a plasma sample processed and then spiked with the analyte, C is the background value of analyte in a blank plasma sample processed. Six replicates were run at each concentration.

Matrix effects were evaluated by comparing the response of each analyte dissolved in the supernatant of the processed blank plasma at three QC levels subtracted those of blank samples with that of the standard solution dissolved in mobile phase. If the ratio <85 or >115 %, the matrix effect was implied. Six human plasma samples from different sources were employed to ensure the representation.

Stability was assessed on human plasma QC samples after long-term storage (three months at –80 °C), short-term storage (10 h at room temperature), and three freeze–thaw cycles, by comparison of the results with those obtained from freshly prepared samples. In addition, post-preparative stability was assessed in the final extract by testing reproducibility in autosampler tray over a single batch period (15 °C for 12 h).

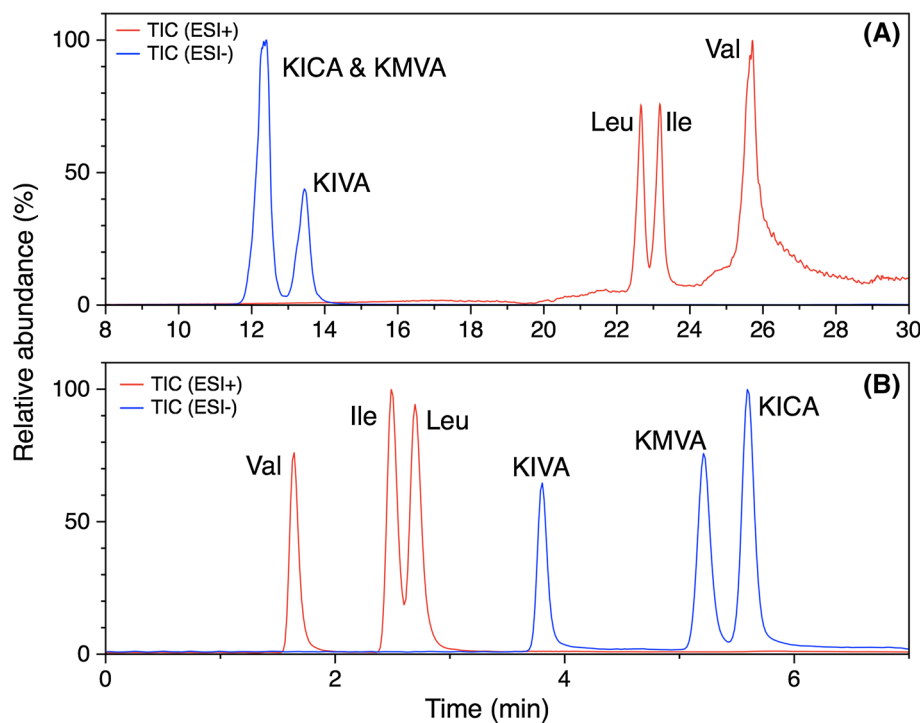
Participants and sample collection

Thirty-three patients diagnosed with cerebral infarction were recruited from the Second Affiliated Hospital of Harbin Medical University. The plasma samples of the case group were collected from them within 24 h after their hospitalization. The clinical diagnosis and pathological reports of all the patients were also obtained from the hospital. The control group consisted of plasma samples from 32 individuals who came to the hospital for routine physical check-up, and their age, gender matched with that of the patients. All plasma samples of both case and control group were immediately frozen and stored at –80 °C until analysis. The participants' clinical information is provided in Supporting Information Table S1. The experimental protocol was reviewed and approved by the Local Committee of Medical Ethics. Written informed consents were obtained from all subjects.

Statistical analysis

Statistical analysis was performed with SPSS statistics 20.0 software (IBM SPSS Inc., USA) and statistical significance was set at $p < 0.05$. Differences in study variables were

Fig. 1 Total ion chromatograms (TIC) of standard (1 µg/mL) BCAAs and BCKAs using **a** ZIC-HILIC column and **b** ZORBAX SB-C18 column



compared using Student's t test for continuous measures and χ^2 test for categorical variables.

Results and discussion

Optimization of chromatographic conditions

To separate all analytes with good efficiency in a short time, we tested many combinations of stationary phases, solvents and modifiers, pH, and gradients. Particular attention was paid to the separation of isobaric species such as Leu and Ile. The resolution (R_s) is calculated using the half-height method: $R_s = \frac{1.18(t_2 - t_1)}{w_{0.5,1} + w_{0.5,2}}$, where t_1 and t_2 are the retention times of two consecutive peaks, and $w_{0.5,1}$ and $w_{0.5,2}$ are the peak widths measured at half height. Poor separation ($R_s = 0$) for Leu-Ile was obtained using ZORBAX 300SB-C18 (100 × 2.1 mm, 1.8 µm), SB-CN (150 × 2.1 mm, 5 µm) and SB-AQ (150 × 2.1 mm, 3.5 µm), a better separation ($R_s = 0.99$) was obtained using ZORBAX SB-C18 (100 × 3 mm, 3.5 µm), and a complete separation ($R_s = 1.53$) was obtained with ZIC-HILIC (150 × 2.1 mm, 3.5 µm). Although HILIC method was useful for the separation of highly polar substances such as un-derivatized amino acids (Miller et al. 2012; Domingues et al. 2015), unlike the ZORBAX SB-C18 column, the HILIC column failed to separate the other pair of isomers KICA-KMVA ($R_s = 0$) (Fig. 1).

Since the interaction with the end-capped C18 phase depends on inherent and ion suppression mediated hydrophobic properties, the retention of all analytes is sensitive

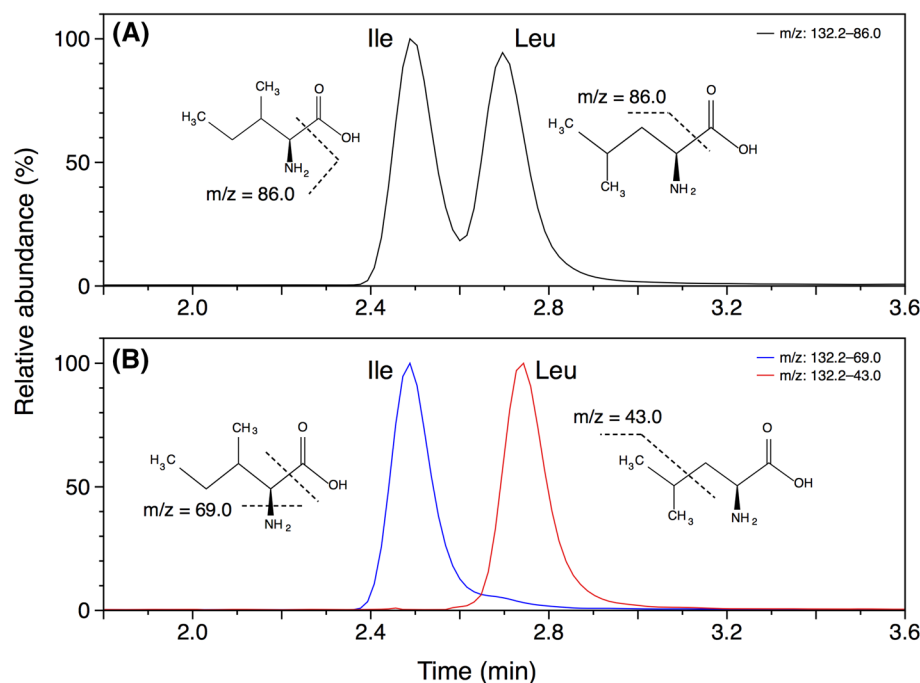
to the composition of mobile phase. Five aqueous solvents and two organic solvents were investigated, including 0.1 and 0.2 % formic acid, 0.1 % acetic acid, 10 mM ammonium formate, 10 mM ammonium acetate, methanol and acetonitrile. Among all solvents tested, methanol with 0.1 % formic acid as additive showed the best characteristics by reducing peak tailing and improving separation. Finally, the flow gradient was improved to accelerate overall throughput without compromising reproducibility or the chromatographic separation of isomers.

Optimization of MS/MS conditions

MS/MS operation parameters were carefully optimized for the determination of six analytes. It was found that BCAAs and BCKAs could be ionized only in positive and negative ESI mode, respectively. Therefore, the ESI source was operated with polarity switching between positive and negative mode in a single run. The first segment with positive ion mode was designed between 0 and 3.5 min to detect BCAAs and IS-1. The second with negative ion mode was used for detection of BCKAs and IS-2 at the time period of 3.51–8 min.

Owing to the limited mass resolution of quadrupole-based mass spectrometers, we treated all analytes of their own nominal mass and injected them separately to characterize their MS/MS properties (Table 1). The non-isomeric analytes with identical nominal mass and different molecular fragments are easily distinguishable, i.e. Val and KIVA. The isomers, such as KICA and KMVA, were

Fig. 2 MRM chromatograms and possible fragmentations of standard (1 $\mu\text{g/mL}$) Leu and Ile (selected reaction monitoring transitions are **a** m/z 132.2–86.0 for Ile and Leu, **b** m/z 132.2–69.0 for Ile and m/z 132.2–43.0 for Leu)



indistinguishable by MS because of their identical elemental composition and similar fragmentation pattern. Hence, the separation of them in this study was primarily achieved by chromatographic means. Noticeably, instead of the fragment at m/z 86.0 which were used by some reported works (Wang et al. 2011; Qi et al. 2015), we found two unique fragments at m/z 43.0 and 69.0 for the quantification of Leu and Ile, respectively (Fig. 2). As a result, the resolution of these isomers in the total ion chromatogram (TIC) was improved from 0.99 to 1.1 and the selectivity of this method was further enhanced.

Selection of internal standards

During LC–MS/MS bioanalysis, there is a risk that matrix effects (of either the analytes or the internal standards) would give the inaccurate concentration value. The effects can be minimized by the use of isotopically labeled internal standards and/or adequate chromatography. However, not all isotopic standards for BCAAs and BCKAs were commercially available and, to also minimize costs, two internal standards were chosen for this analytical method to correct potential matrix effects, with one assigned to each monitoring window.

Sample preparation

As BCAAs and BCKAs are polar metabolites with good solubility in water, protein precipitation is the first chosen for the pretreatment of plasma samples. The kinds and pH of precipitation solvent could play vital roles in protein

precipitation process. It was assumed that if there was more macromolecular material (i.e. unremoved proteins/peptides from the precipitation reaction) injected onto the analytical column, the shorter the columns lifetime and the lower the quality of the resulting data would be. Thus, the one-dimensional (1D) gel electrophoresis was performed on the extracts (supernatant after protein precipitation) of human plasma to assess protein removal efficiency of different organic solvents, including methanol, acetonitrile, methanol/acetonitrile, methanol/ethanol and methanol/acetonitrile/acetone. The gel results (Supporting Information Figure S1) clearly showed that the supernatant after protein precipitation using acetonitrile contained lowest level of protein/large peptide material. This was in agreement with Bruce's study (Bruce et al. 2009) and suggested that acetonitrile would be a good selection for LC column prolongation and data quality. Then, various concentrations of acids (formic acid, acetic acid, etc.) in acetonitrile were tested. It was found that the increasing amount of volatilizable acids in acetonitrile from 0 to 0.5 % can give the increasing recoveries of BCKAs, but decreasing the recoveries of BCAAs. According to Kato's study (Kato et al. 2011), amino acids might be non-enzymatically hydrolyzed to α -keto acids in acidic aqueous conditions. Thus, we chose the simple but efficient protein precipitation method using only 160 μL acetonitrile to extract analytes from human plasma.

Method validation

Eight-point calibration curve of each analyte was constructed using linear regression with a weighted factor $1/x^2$.

Table 2 Linearity, LOD and LOQ of each analyte in plasma using the proposed method ($n = 5$)

Analyte	Linear range ($\mu\text{g/mL}$)	Slope	Intercept	Regression coefficient	LOQ ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)
Leu	0.2–50	45.848	0.46537	0.99689	0.2	0.07
Ile	0.2–50	41.645	0.67223	0.99162	0.2	0.07
Val	0.2–20	14.088	0.19018	0.99592	0.2	0.07
KICA	0.1–20	1.6675	0.10410	0.99275	0.1	0.04
KMVA	0.1–20	1.8076	0.016717	0.99682	0.1	0.04
KIVA	0.1–20	3.6451	0.018895	0.99007	0.1	0.04

Table 3 Summary of accuracy, precision, recovery and matrix effect of analytes in plasma

Analyte	Concentration added ($\mu\text{g/mL}$)	Intra-day ($n = 6$)		Inter-day ($n = 18$)		Recovery ($n = 6$)		Matrix effect ($n = 6$)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
Leu	0.5	93.7	2.5	97.2	6.6	88.0	13.0	92.0	11.2
	5	103.6	4.9	102.9	4.9	87.7	8.5	92.3	6.7
	40	101.9	3.2	98.6	4.2	92.1	1.9	89.5	10.9
Ile	0.5	106.6	2.4	104.2	4.2	84.4	13.6	109.2	12.8
	5	103.8	4.7	100.9	5.3	89.7	10.9	96.9	14.1
	40	98.3	1.7	99.5	5.8	90.1	10.2	97.5	7.5
Val	0.5	100.8	12.9	100.0	7.3	94.3	3.0	111.1	9.2
	5	99.9	6.5	99.3	4.0	91.9	3.4	90.9	6.2
	40	98.0	2.8	99.4	5.7	93.2	1.9	96.9	6.7
KICA	0.2	95.3	11.1	98.7	7.1	95.9	6.3	113.1	6.8
	4	97.7	4.1	100.4	4.4	97.9	3.8	104.3	7.1
	16	98.8	2.9	100.9	3.9	92.9	6.2	112.5	6.9
KMVA	0.2	104.3	8.0	101.6	6.2	81.5	7.6	109.8	10.7
	4	108.4	5.3	103.2	6.6	83.1	8.0	90.2	6.7
	16	102.9	5.7	101.7	4.5	90.9	5.9	103.6	5.8
KIVA	0.2	99.1	8.6	101.1	6.8	97.0	3.3	102.2	8.6
	4	100.1	5.6	100.6	5.6	90.4	3.1	101.3	8.8
	16	103.0	0.8	100.5	6.4	95.7	2.2	98.5	5.5
IS-1	50				91.8	2.4	88.6	12.3	
IS-2	5				86.6	4.9	105.4	3.3	

The regression parameters, such as linear range, slope, intercept, and correlation coefficients (R^2), are tabulated in Table 2. Good linear responses ($R^2 > 0.99$) were observed for BCAAs and BCKAs over the range of 0.2–50 and 0.1–20 $\mu\text{g/mL}$, respectively. LOD and LOQ of each analyte were calculated and are also shown in Table 2. The obtained values were much lower than the limits required for biological samples and demonstrated a good sensitivity of this method.

The intra-day and inter-day performance data are summarized in Table 3. All values obtained were well within internationally recognized acceptance criteria for assay validations and were within the pre-defined 15 % limits required. Recovery and matrix effect obtained at the different tested concentrations are also presented in Table 3. Mean recovery for all analytes ranged from 81 % to 98 %

with a maximum RSD less than 15 %, which proved the process of extraction was stable and effective. No significant suppression or enhancement effects were observed in this study.

The stability tests were designed to cover the anticipated conditions of handling of the real samples. The accuracy, precision, and sensitivity of processed human plasma QC samples were found to be acceptable (precision and accuracy within the pre-defined 15 % limits) on re-injection with freshly prepared calibration standards, after storage at $-80\text{ }^\circ\text{C}$ for 3 months, room temperature for 10 h, $15\text{ }^\circ\text{C}$ in auto-sampler tray for 12 h, and three freeze–thaw cycles (Supporting Information Table S2). This demonstrated that plasma samples were stable when stored under these conditions.

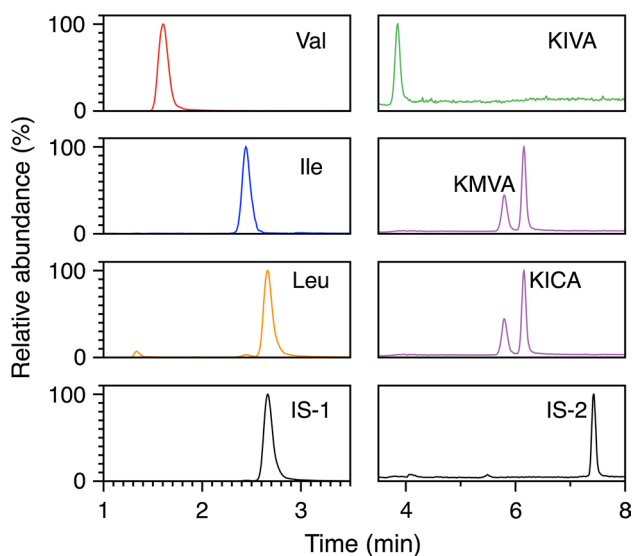


Fig. 3 Representative MRM chromatogram of each analyte and IS in a plasma sample of patient with cerebral infarction (Leu: 28.11 $\mu\text{g}/\text{mL}$; Ile: 11.71 $\mu\text{g}/\text{mL}$; Val: 30.18 $\mu\text{g}/\text{mL}$; KICA: 2.40 $\mu\text{g}/\text{mL}$; KMVA: 1.36 $\mu\text{g}/\text{mL}$; KIVA: 0.83 $\mu\text{g}/\text{mL}$)

Table 4 Results of LC–MS/MS analysis of plasma samples from healthy volunteers and patients with cerebral infarction

Analyte	Plasma concentration (mean \pm SD, $\mu\text{g}/\text{L}$)		<i>p</i> Value
	Controls (<i>n</i> = 32)	Patients (<i>n</i> = 33)	
Leu	15.88 \pm 2.26	18.48 \pm 4.95	0.0089
Ile	7.06 \pm 1.43	8.61 \pm 2.77	0.0063
Val	20.97 \pm 3.18	23.67 \pm 4.28	0.0054
KICA	2.24 \pm 0.80	2.63 \pm 0.73	0.0433
KMVA	1.18 \pm 0.43	1.48 \pm 0.45	0.0084
KIVA	0.70 \pm 0.29	0.90 \pm 0.36	0.0186

^a *p* < 0.05 was considered statistically significant

The proposed LC-ESI^{+/−}-MS/MS method overcomes most of the disadvantages of those reported methods (Kand'ár et al. 2009; Nguyen et al. 2013; Qi et al. 2015; Ghosh et al. 2015; Ehling and Reddy 2015). Sample preparation was quick and simple avoiding the complex derivatization step. The running time was only 8 min with LOD in the range of 0.1–0.2 $\mu\text{g}/\text{mL}$ which made it faster and more sensitive than most GC/LC–MS methods. Most importantly, it was the first analytical method developed and validated for simultaneous separation and quantitation of BCAAs and BCKAs in human plasma.

Method application

To show the utility of the validated method, an application to simultaneously quantify BCAAs and BCKAs in human

plasma from 65 participants was performed. Although it has been reported that BCAAs and BCKAs can influence brain function, such as blood pressure and affective state, by modifying large, neutral amino acid transport at the blood–brain barrier (Fernstrom 2005; Ribeiro et al. 2008), the information of them in patients with cerebral infarction is limited. In this study, both BCAAs and BCKAs were well separated (Fig. 3) by the proposed method and the concentration levels of them were calculated from calibration curves according to the peak area ratios of analytes and IS. As shown in Table 4, the concentration of each analyte is statistically significantly different between control and case group, revealing an increased content of both BCAAs and BCKAs in patients' plasma. It is well known that an excess amount of free BCAA or their catabolic products BCKAs can be cytotoxic (Harris et al. 2005; Tom and Nair 2006). Hence, the results of this study were encouraging and may provide profound insight into complete metabolic pathways of cerebral infarction. However, further study is definitely needed to fully elucidate the origin of BCAAs and BCKAs increase in plasma of patients suffering cerebral infarction.

Conclusions

A reliable, simple and selective LC-ESI^{+/−}-MS/MS method was developed and validated to simultaneously determine BCAAs (leucine, isoleucine and valine) and BCKAs (KICA, KIVA, KMVA) in human plasma. In an 8-min gradient, adequate separation of all six analytes including charged isomers without requiring tedious and time-consuming derivatization procedure was attained. Further acceleration of the gradient is possible at the costs of isomers separation. In comparison to reported works, the developed method is easier and faster due to minimal sample pre-treatment and rapid chromatographic separation.

Notably, the method was also sufficiently sensitive to routinely and accurately quantify metabolites in as little as 40 μL plasma. The applicability of the method was demonstrated by determining BCAAs and BCKAs in human plasma from healthy individuals and cerebral infarction patients, and significant difference of each analyte between case and control group was observed. Furthermore, with simple modification, this method could easily be used to study the dynamics of BCAAs and BCKAs metabolism in other diseases.

Acknowledgments This study was financially supported by the Open Project Program of Key Laboratory of Myocardial Ischemia (Harbin Medical University), Ministry of Education (No. KF201414); the Open Project Program of Key Laboratory of Drug Quality Control and Pharmacovigilance (China Pharmaceutical University), Ministry

of Education (No. MKLDP2013QN04); the Innovative Scientific Research Team Fund of Jiangsu Province; and the Priority Academic Program Development of Jiangsu Higher Education Institutions. The authors would like to thank the patients for their participation in our project.

Compliance of ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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