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Development, validation and application of LC–MS/MS method for quantification of amino acids, kynurenine and serotonin in human plasma

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ABSTRACT

Altered serotonergic neurotransmission is a key factor in several neurologic and psychiatric disorders such as migraine. Human and animal studies suggest that chronically low interictal serotonin levels of plasma and brain may facilitate increased activity of the trigeminovascular pathway, and may contribute to development of repeated migraine attacks. However, brain serotonin synthesis is affected by the concentration of tryptophan, its metabolites and a number of amino acids. In this work a simple and robust LC–MS/MS method for the quantitative determination of valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, serotonin and kynurenine in human plasma has been developed and validated. Sample preparation was achieved by protein precipitation, using trifluoroacetic acid. Chromatographic separation was carried out on a Supelco Ascentis® Express C18 column (3.0 mm i.d. × 150 mm, 2.7 μm) equipped with an Agilent Zorbax Eclipse XDB C8 guard-column under isocratic conditions at a flow rate of 0.4 mL/min, over a 6.5 min run time. Mobile phase was 0.2% trifluoroacetic acid – acetonitrile (85:15, v/v). The eight analytes and two internal standards were ionized by positive electrospray ionization and detected in multiple reaction monitoring mode.

A "fit-for-purpose" validation approach was adopted using surrogate matrix for the preparation of calibration samples. The calibration curves of all analytes showed excellent linearities with a correlation coefficient (r^2) of 0.998 or better. Spiked surrogate matrix samples and pooled human plasma were used as quality control samples. Intra-day and inter-day precisions were less than 11.8% and 14.3%, and accuracies were within the ranges of 87.4–114.3% and 87.7–113.3%, respectively. Stability of the components in standard solutions, surrogate matrix, pooled plasma and processed samples were found to be acceptable under all relevant conditions. No significant carryover effect was observed. The surrogate matrix behaved parallel to human plasma when assessed by standard addition method and diluting the authentic matrix with surrogate matrix. The method was successfully applied for analysis of 800 human plasma samples to support a clinical study.

1. Introduction

Altered serotonergic neurotransmission is a key finding in several neurologic and psychiatric disorders including migraine [1]. Migraine is a common disabling primary headache disorder with a global prevalence of 15–18% [2]. It is characterized by throb-

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bing and usually unilateral moderate or severe pain accompanied by phono- and photophobia, nausea or vomiting, and worsening by routine physical activity [3]. Human and animal studies suggest that chronically low interictal serotonin level of plasma and brain predispose to increased sensitivity of the trigeminovascular pathway that may contribute to the development of repeated migraine attacks [1,2,4,5]. In line with the serotonergic dysfunction hypothesis, the 5-$HT_{1B/1D}$ (5-hydroxytryptamine receptor 1B and 1D subtypes) agonist triptans alleviate migraine pain, while tryptophan depletion, which acutely decreases the brain serotonin concentration, increases the excitability of the brain and symptoms of the migraine attacks [1,5,6]. However, the brain serotonin concentration is affected not only by tryptophan intake but also by the ratio of the plasma concentrations of tryptophan and other large neutral amino acids (LNAA) that are competing to occupy a transporter at the blood brain barrier, called L-type amino acid transporter 1 (LAT1). Fernstrom defined a ratio of plasma tryptophan and LNAAs, namely tyrosine, phenylalanine, leucine, isoleucine and valine, which influenced the brain tryptophan concentrations and serotonin synthesis in experimental studies. We selected these LNAAs for this measurement based on his study [7]. Investigation of these amino acids is important because dietary manipulation of tryptophan/LNAA ratio promptly able to elicit behavioral alterations, although the exact mechanism is not fully understood. For example, tryptophan depletion with ingestion of a tryptophan free amino acid mixture is acutely able to increase anxiety, lower mood and contribute to more severe course of migraine attacks in vulnerable subjects [6–8]. Thus our aim was to develop a method to simultaneously measure tryptophan, LNAAs and the peripheral concentrations of serotonin and kynurenine (another compound synthesized from tryptophan and have been implicated in migraine) in human plasma [9].

During a typical bioanalytical validation procedure, calibration and quality control (QC) samples are prepared by spiking the same sort of biological matrix as the study samples with known amounts of analyte. This ensures that the analyte of interest faces same conditions with respect to matrix effects and extraction recoveries in the validation samples as well as the study samples. In the case of endogenous compounds, preparation of validation samples are hampered by the absence of analyte-free (blank) matrix [10]. As accurate quantitative analysis of endogenous analytes is crucial for a number of clinical and nonclinical applications, increasing interest is arising for alternative, so-called "fit-for-purpose" quantitation/validation approaches. There are four commonly used approaches: standard addition, background subtraction, surrogate analyte in authentic matrix and authentic analyte in surrogate matrix [11–16]. Applicability, advantages and drawbacks of these methods have recently been reviewed [17].

In the present article, we report a quick, accurate and reliable liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for quantitative determination of valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, serotonin and kynurenine in 800 human plasma samples. Two stable isotope-labeled internal standards (SIL-ISs) were used to reduce assay variation: leucine-5,5,5-d_3 (Leu-d_3) for aliphatic amino acids and tyrosine-(phenyl-3,5-d_2) (Tyr-d_2) for aromatic compounds, respectively. Due to the large number of samples and compounds to be analyzed, authentic analyte in surrogate matrix approach was adopted by using artificial plasma as a surrogate matrix for the construction of calibration standards and QC samples. Some assay validation issues of the method of choice such as parallelism and limit of quantitation (LOQ) are also discussed. Fig. 1 shows the eight analytes and the two SIL-ISs.

2. Materials and methods

2.1. Chemicals

LC–MS grade acetonitrile was purchased from VWR Chemicals (Pool, England). LC–MS grade trifluoroacetic acid (TFA) was purchased from Fisher Chemical (Loughborough, UK). Leucine (99.9%), isoleucine (99.5%), valine (99.3%), phenylalanine (99.0%), tyrosine (99.7%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Tryptophan (≥99%) was obtained from Alfa Aesar (Haverhill, MA, USA). Kynurenine (≥98%) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Serotonin (≥98%), L-leucine-5,5,5-d_3 (99% D atom), L-tyrosine-(phenyl-3,5-d_2) (98% D atom), phosphate buffered saline (PBS) and human serum albumin (HSA) (≥96%) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). K_3EDTA tubes were obtained from Greiner Bio-One International GmbH (Kremsmünster, Austria). Ultrapure water was prepared with an in-house Simplicity® Water Purification System (Merck Millipore, Burlington, MA, USA).

2.2. Standard solutions

Stock solutions of the analytes and internal standards were made up in 0.2% trifluoroacetic acid – acetonitrile (85:15, v/v) and stored at −20 °C. On the day of analysis, calibration and QC standard solutions were prepared by serial dilution of two separate primary stock solutions with water. IS working solution was prepared in water to give a concentration of 250 μg/mL for Leu-d_3 and 25 μg/mL for Tyr-d_2. Surrogate matrix was prepared by adding 4 g of HSA to 100 mL PBS solution.

2.3. Plasma samples

Human blood samples were collected into 3 mL K_3EDTA tubes from migraine patients without aura and healthy volunteers (total number of participants was 98, total number of blood samples was 800) by intravenous cannulation. The samples were immediately centrifuged, then plasma samples were frozen and kept at −80 °C until the assay. Pooled plasma was prepared by mixing equal volumes of plasmas obtained from 6 healthy individuals. The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council, Budapest, Hungary.

2.4. Sample preparation

Sample preparation was achieved by simple protein precipitation. For the preparation of calibration samples and QC samples in surrogate matrix, 100 μL of spiking standard solutions and 20 μL of IS working solution were mixed with 900 μL of surrogate matrix. In case of study samples, 100 μL of water and 20 μL of IS working solution were mixed with 900 μL of human plasma. For protein precipitation, 200 μL of trifluoroacetic acid was added to each sample and vortex mixed. After centrifugation at 4000*g* for 10 min at 4 °C, aliquots (150 μL) of the supernatant were transferred to autosampler vials. 5 μL of the resulting solutions were injected into the LC–MS/MS system.

2.5. Liquid chromatography-tandem mass spectrometry

The chromatographic separations were performed on an Agilent 1260 Infinity LC system (Agilent Technologies, CA, USA). The analytes were separated on a Supelco Ascentis® Express C18 column (3.0 mm i.d. × 150 mm, 2.7 μm) equipped with an Agilent Zorbax Eclipse XDB C8 guard-column (4.6 mm i.d. × 12.5 mm, 5 μm). The column and the autosampler were maintained at 25 °C and 4 °C, respectively. The aliquots of samples were eluted under isocratic

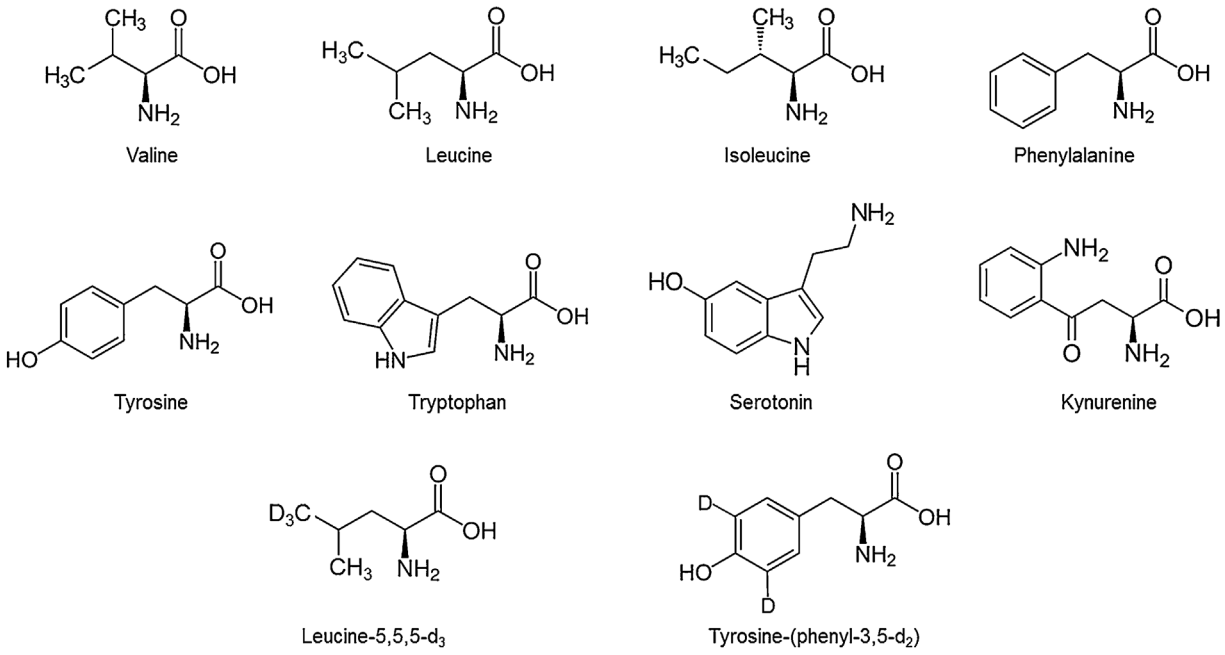

Fig. 1. Chemical structure of the eight analytes and two internal standards.

conditions over 6.5 min at a flow rate of 0.4 mL/min. The mobile phase was composed of 0.2% trifluoroacetic acid – acetonitrile (85:15, v/v). The needle was washed for 10 s in the flush port before every injection in order to minimize carryover effect.

Samples were analyzed by an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using positive electrospray ionization (ESI) and scheduled multiple reaction monitoring (MRM) mode. Agilent MassHunter Data Acquisition software (version B.04.01) was used to control the equipment, MassHunter Quantitative QQQ Analysis (version B.05.01) and Qualitative Analysis software (version B.05.00) were applied for quantitation and data processing. Settings were as follows: capillary voltage, +3.5 kV; nozzle voltage, +400 V. Nitrogen was applied as a nebulizer gas of 45 psi, a carrier gas of 10 L/min at 350 °C, and a sheath gas of 11 L/min at 350 °C. MRM transitions, collision energies (CE) and fragmentor voltages for all compounds were auto optimized by Optimizer of Agilent MassHunter workstation.

2.6. Validation

2.6.1. Calibration curves, LOQ

The spiking standard solutions of calibration standards were diluted from the stock solution to obtain eight calibration levels, and ran in duplicate at the beginning and the end of each batch. The lowest and highest points of the calibration curve coincided with the lower limit of quantitation (LLOQ) as well as the upper limit of quantitation (ULOQ). The calibration curve was derived by plotting the concentration of the standards versus the analyte to IS peak area ratios using 1/x weighted least-squares linear regressions.

2.6.2. Accuracy and precision

Four concentration levels of QC samples were used for amino acids: LLOQ, low QC, medium QC, high QC, and three concentration levels for serotonin and kynurenine (LLOQ, low QC, high QC) in order to cover the calibration range. Concentrations were set according to the EMA Guideline on bioanalytical method validation [18]. Furthermore, pooled plasma was applied as an additional level of QC to evaluate potential errors caused by matrix differences [10]. Endogenous levels of these samples were determined by the method of standard addition. Calibration ranges and concentrations of the QC samples are summarized in Table 1. Intra-day accuracy and precision were assessed by evaluating five replicates of each QC samples described above. The inter-day accuracy and precision were established by the repetition of the intra-day validation procedure on five consecutive days. Accuracy was expressed as percentage of the nominal concentration and precision was calculated as the coefficient variation (%CV). The acceptance criteria for both parameters were set at ±15% except for the LLOQ for which it should be within ±20% [18].

2.6.3. Extraction recovery, parallelism, dilution integrity

Extraction recoveries were evaluated by comparing the analyte responses observed in pre-spiked and post-spiked samples. The experiment was performed in six replicates. Pre-spiked samples were prepared by adding 100 μL of high QC standard solution and 20 μL of IS standard working solution to 900 μL of surrogate matrix, then precipitated with TFA and centrifuged. In the case of post-spiked samples, 1000 μL of surrogate matrix (blank sample) was processed in the same way. 90 μL of the resulting supernatant were mixed with 10 μL of high QC standard solution and 2 μL of IS standard working solution. Extraction recovery (%) was calculated as peak area of pre-spiked samples/peak area of post-spiked samples x 100. IS-normalized extraction recovery (%) was also expressed for the analytes as extraction recovery of the analyte/extraction recovery of the IS x 100.

The term parallelism is generally understood to mean how well a set of calibration standards track the response of the analyte of interest in the biological matrix [19]. Evaluation of parallelism is of critical relevance when using surrogate matrix, as high degree of such parameter indicates that there is no significant difference between the authentic and the surrogate matrix with respect to extraction recoveries and matrix effects. Determination of parallelism was carried out based upon the experiment proposed by Houghton et al. [20]. Endogenous concentrations of six individual plasma samples were determined in triplicates by standard addition. The same samples were spiked with medium QC standard solution and were serially diluted with surrogate matrix two,

Table 1
Calibration ranges and QC levels of the analytes.

QC type		Surrogate matrix				Pooled plasma
Analyte	Calibration range (μg/ml)	QC level (μg/ml)				
		LLOQ	Low	Medium	High	
Valine	1–100	1	3	20	80	24.05
Leucine	1–100	1	3	20	80	13.09
Isoleucine	0.5–50	0.5	1.5	10	40	7.82
Phenylalanine	1–100	1	3	20	80	6.98
Tyrosine	0.5–50	0.5	1.5	10	40	11.88
Tryptophan	0.5–50	0.5	1.5	10	40	7.81
Serotonin	0.1–2.5	0.1	0.3	–	2	0.20
Kynurenine	0.1–2.5	0.1	0.3	–	2	0.44

five and ten times. Then the diluted and undiluted samples were measured against surrogate calibrators. The %RE and %CV of the back-calculated concentrations were maximized in ±15% [20]. By tracking the effect of diluting the plasma with surrogate matrix, the method can also be considered as a dilution integrity test.

2.6.4. Stability

Stability of the stock solutions and working solutions were assessed at room temperature (20 °C) for 24 h and at 10 °C for ten days. Working solutions of the eight analytes were studied using bracketing approach: only the lowest and the highest calibration standards were measured. The latter was also the stock solution. Stability of the analytes in matrix were evaluated using low and high QC samples prepared in surrogate plasma and pooled human plasma samples. Each levels were analyzed in triplicates after being exposed to different conditions: room temperature for 24 h, −20 °C for two months and three freeze-thaw cycles. Stability of processed samples were determined at room temperature for 12 h and autosampler temperature (4 °C) for 24 h. Samples were considered stable when the %RE was within 15% [18].

2.6.5. Carryover

Carryover was analyzed by assaying blank samples (processed surrogate matrix samples) injected after the analysis of a pooled plasma sample spiked with the highest calibration standard. The acceptance criteria were set at 20% analyte response of the LLOQ for the analytes of interest and 5% for the ISs [18].

2.7. Application of the method to clinical human plasma samples

The validated method was applied to 800 samples collected during the clinical study described earlier. The samples were measured in ten separate analytical runs. Surrogate calibrators were run at the beginning and the end of each batch. Low, medium, high QCs and pooled plasma samples were analyzed in duplicates at each batch to keep track of accuracy and precision. Reproducibility of the described method for incurred study samples was tested by reanalysis of 10% of the samples (n = 80). Reanalyzed samples were considered acceptable when the difference between the pairs of the results were within 20% for at least 66.7% of the samples [18].

3. Results and discussion

3.1. Liquid chromatography-tandem mass spectrometry

There is a vast amount of literature on the quantitative determination of amino acids in plants, animal and human samples [21–23]. However, to the best of our knowledge, no bioanalytical LC–MS/MS method has been reported for the simultaneous analysis of amino acids, serotonin and kynurenine in human plasma. Amino acids are very often derivatized in order to enhance fluorescent detection selectivity, sensitivity or chromatographic resolution [23,24]. However, the derivatization agent can bring an additional source of interferences and errors into the system complicating method validation [24]. It should be also taken into account that derivatization can significantly increase the labor intensity of the method, which can be crucial when the number of samples to be analyzed is large. In this particular case, where the aim was the measurement of 800 (with validation samples far over 1000) plasma samples, we primarily focused on speed, simplicity and high throughput during method development. MS conditions including MRM transitions, collision energies (CE) and fragmentor voltages were individually optimized for all analytes and SIL-ISs in positive ion mode by Optimizer of Agilent MassHunter workstation in order to enhance selectivity and sensitivity of the method. As shown in Table 2, the optimization procedure resulted in same MRM transitions for isomeric amino acids leucine and isoleucine. Because of the insufficient mass spectrometric selectivity, proper chromatographic resolution was necessary to separate such compounds without compromising peak shape or a reasonably short analytical run time. As representative MRM chromatograms in Fig. 2 demonstrates, a Supelco Ascentis® Express C18 column (3.0 mm i.d. × 150 mm, 2.7 μm) provided satisfactory separation of the analytes within a 6.5 min run. An isocratic condition consisting of 0.2% TFA-acetonitrile (85:15, v/v) at flow rate of 0.4 mL/min was chosen in order to avoid the need for column reequilibration between consecutive injections. A Zorbax Eclipse XDB C8 guard-column was used to protect the analytical column, therefore, performance of the separation in terms of peak shapes and retention times was consistent throughout the study.

Table 2
MRM parameters of the analytes and internal standards.

Compound	Transition (m/z)	Fragmentorvoltage (V)	Collisionenergy (eV)
Valine	118 → 72	10	8
Leucine	132 → 86	2	8
Isoleucine	132 → 86	2	8
Phenylalanine	166 → 120	2	8
Tyrosine	182 → 136	30	12
Tryptophan	205 → 188	40	4
Serotonin	177 → 160	45	4
Kynurenine	209 → 192	45	4
Leu-d_3	135 → 89	45	4
Tyr-d_2	184 → 138	45	10

3.2. Validation

3.2.1. Calibration curves, LLOQ

The mean regression coefficients (r^2) for all the analytes were over 0.998 indicating excellent linearities. The calibration ranges were sufficient to cover analyte concentrations in almost all human

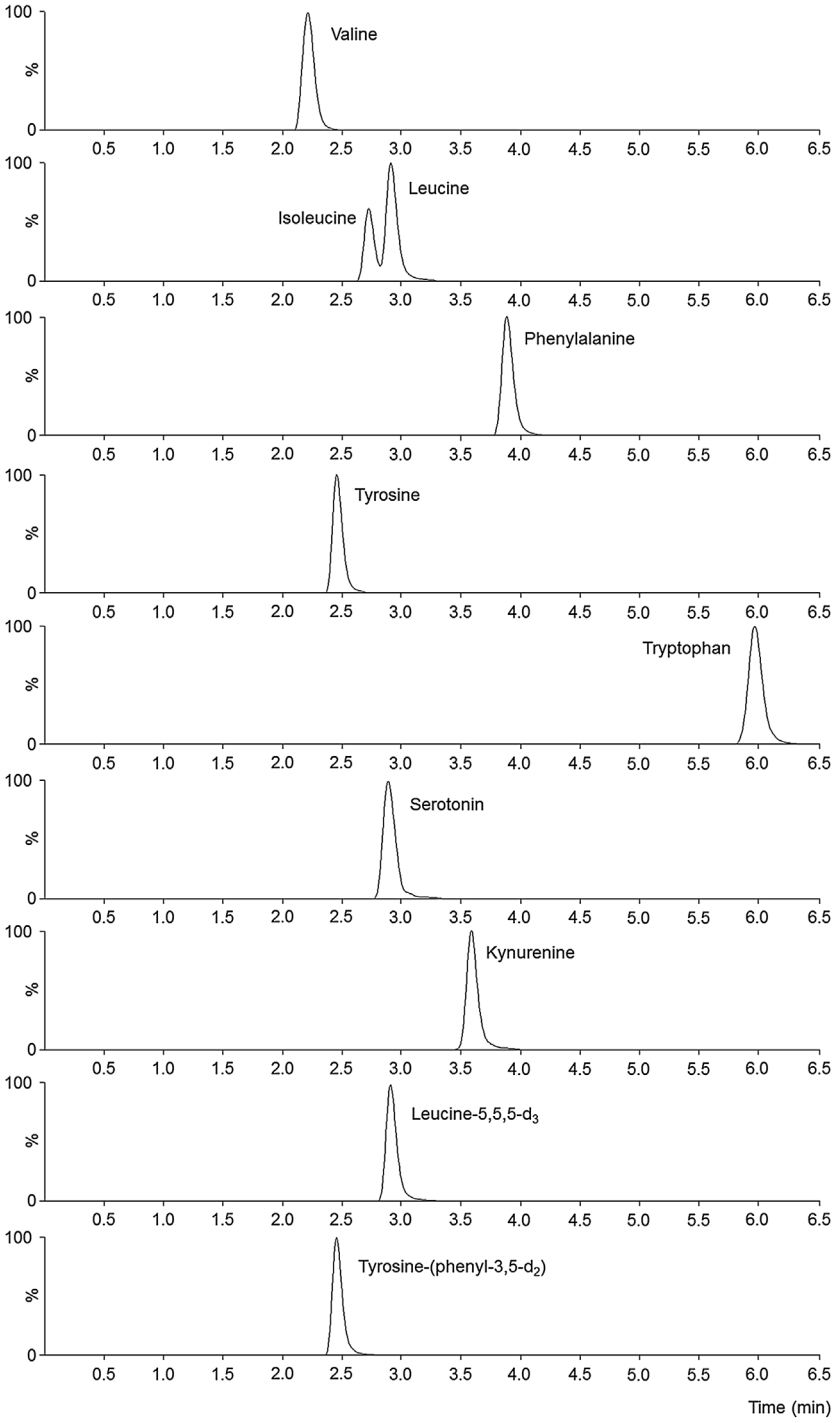

Fig. 2. Extracted MRM chromatograms of the analytes and internal standards.

plasma samples. LLOQ is described as the lowest concentration with acceptable accuracy and precision which largely depends on the sensitivity of the instrument [18]. However, in this study, only the LLOQ of serotonin and kynurenine were determined with this approach. In the case of amino acids, measurement of accuracy and precision at the LLOQ may not be adequate, considering that such concentrations are outside the physiologically relevant range [25]. Thus the lowest calibration levels, following a more pragmatic approach, were set to be approximately one tenth of the concentrations measured in pooled plasma. This method simplifies the preparation and dilution of calibration standards when working with a large number of analytes, while covering the relevant concentration range, even if downregulation of the analytes is expected [20,25].

3.2.2. Accuracy and precision

Intra-day and inter-day accuracies and precisions for surrogate matrix and pooled plasma QC samples are summarized in Table 3. In this study, the intra-day and inter-day precisions were less than 11.8% and 14.3%, and accuracies were within the ranges of 87.4–114.3% and 87.7–113.3%, respectively. The results indicate that the method is accurate and precise enough for the measurement of plasma samples.

Table 3
Intra-day and inter-day accuracy and precision of the QC samples.

	QC type	Surrogate matrix								Pooled plasma	
	QC level	LLOQ		Low		Medium		High			
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Intra-day	Valine	111.7	7.4	93.0	11.8	100.4	11.3	110.8	8.8	92.4	3.7
	Leucine	102.7	5.7	95.3	2.5	93.8	4.9	89.7	3.3	90.9	4.0
	Isoleucine	114.3	7.2	104.8	3.3	108	3.2	113.3	7.0	101.3	3.9
	Phenylalanine	100.2	5.2	102.1	5.6	99.8	6.4	102.9	2.8	102.3	6.4
	Tyrosine	98.6	4.3	87.4	2.5	88.9	10.5	103.7	4.2	107.0	3.3
	Tryptophan	106.8	6.8	106.0	6.5	95.8	10.2	108.0	4.6	97.2	6.7
	Serotonin	101.0	8.1	92.7	2.9	–	–	87.8	4.7	95.1	7.2
	Kynurenine	110.7	8.2	114.3	10.1	–	–	113.4	9.4	110.9	9.7
Inter-day	Valine	107.6	10.2	96.5	11.9	97.0	9.7	111.7	9.0	91.5	5.3
	Leucine	102.5	6.4	95.6	7.8	91.5	5.6	92.4	7.1	95.8	8.2
	Isoleucine	114.4	8.2	106.7	9.6	103.0	6.8	112.6	8.3	106.4	8.7
	Phenylalanine	102.1	7,4	100.1	7.2	96.5	6.9	104.6	8.8	108.8	5.9
	Tyrosine	111.2	14.3	102.3	13.3	102.9	10.7	109.5	7.6	104.5	3.5
	Tryptophan	102.9	8.1	96.7	7.6	95.4	6.3	113.3	4.7	109.5	7.0
	Serotonin	99.5	14.2	87.7	7.7	–	–	91.1	7.4	89.2	9.1
	Kynurenine	92.4	13.8	90.7	10.6	–	–	95.0	12.8	91.2	11.4

3.2.3. Extraction recovery, parallelism, dilution integrity

Extraction recovery of the method was evaluated by comparing the mean peak area of pre-extracted and post-extracted surrogate matrix samples. The sample preparation process did not influence considerably the concentrations of the analytes an ISs (extraction recoveries varied between 85.2–101.4%). Furthermore, the IS-normalized extraction recovery values highlight that the selected SIL-ISs are appropriate references of the analytes (IS-normalized extraction recoveries varied between 93.6–111.8%). Results of extraction efficiency evaluation are detailed in Table 4.

For the parallelism experiment, background concentrations of the individual plasma samples were determined by standard addition. Evaluation of the same samples with surrogate calibrators resulted very similar concentrations for all the analytes (RE is less than 10%). Fig. 3 illustrates the use of standard addition to assess parallelism with surrogate matrix method in the case of isoleucine. Moreover, the measured analyte concentrations in the spiked samples at each dilution levels were within the acceptance criteria, which indicates that the surrogate matrix behaves similarly to the authentic biological matrix. Fig. 4 illustrates the relatively balanced accuracy profile of a representative plasma sample when diluted zero- two-, five-, ten-times with surrogate matrix.

3.2.4. Stability

The stock solutions and working solutions of the analytes and internal standards were stable for at least 24 h at 20 °C and for 10 days at 10 °C, respectively. Human plasma samples frozen to −20 °C and thawed to room temperature for three cycles did not change considerably the concentrations of the analytes. Human plasma and surrogate matrix spiked with QC standards were stable at room temperature for 24 h and −20 °C for 2 months. No apparent change was observed in the concentrations of the processed samples after being 24 h at autosampler temperature (4 °C) and 12 h at room temperature.

3.2.5. Carryover

Carryover was analyzed by injecting blank samples after pooled plasma spiked with the highest calibration standard. As Table 5 demonstrates, responses of the analytes were 0.7–17.2% and 0.8–1.0% for the internal standards, respectively.

3.3. Application of the method to clinical human plasma samples

The validated method was successfully applied in support of a clinical study to measure the plasma concentration of the eight analytes in 800 human plasma samples obtained from migraine patients and healthy individuals. Valine, isoleucine and tyrosine concentrations were above the ULOQ in less than 1% of the samples. In such cases, another aliquot of the sample were diluted two times with surrogate matrix, then processed and measured again. Accuracy and precision of the QC samples did not change considerably compared to the results of the method validation, incurred sample reanalysis was also within the acceptance criteria. Table 6 summarizes the concentration ranges in 800 study samples.

3.4. Strategies for quantitative analysis of endogenous compounds

Regulatory guidances are designed primarily to address validation of drug molecules as well as their metabolites. However, reflecting on the challenges of endogenous analytes, the draft version of the ICH guideline of bioanalytical method validation dedicates a section for the quantitative determination of these compounds, in which the above-mentioned quantitation strategies are detailed [26]. Considering that both methods have advantages and limitations, their applicability is determined by the specific analytical challenge. In the present case, when the number of samples to be analyzed is large and the amount of each sample is relatively low (in some cases 1 mL or less), the use of standard addition is narrowed by the requirement for multiple measurements of every study samples. Compared to healthy individuals, downregulation of certain analytes is expected in migraine patients, which means that samples with lower concentration than the control matrix should be taken into account. Therefore, the use of background subtraction is not recommended due to the potential measurement errors introduced by extrapolation beyond the calibration range [20]. The situation is further complicated when there are multiple analytes with variable endogenous levels [17]. Surrogate analyte in authentic matrix typically requires two versions of SIL-ISs of the analyte of interest [10]. Despite being a well-accepted approach, its application is limited by the availability and high price of stable isotope-labeled analogs, especially when the analysis of numerous analytes is needed. Under such circumstances, surrogate matrix approach may be the method of choice, which enables reliable quantitation of multiple analytes with relatively low labor

Table 4
Mean extraction recoveries of the analytes and internal standards in surrogate matrix.

	Valine	Leucine	Isoleucine	Phenylalanine	Tyrosine	Tryptophan	Serotonin	Kynurenine	Leu-d_3	Tyr-d_2
Extraction recovery (%)	87.5	101.3	88.6	101.4	95.0	95.1	85.2	97.2	90.6	91.0
IS-normalized extraction recovery (%)	96.6	111.8	97.8	111.3	104.4	104.5	93.6	106.7	–	–

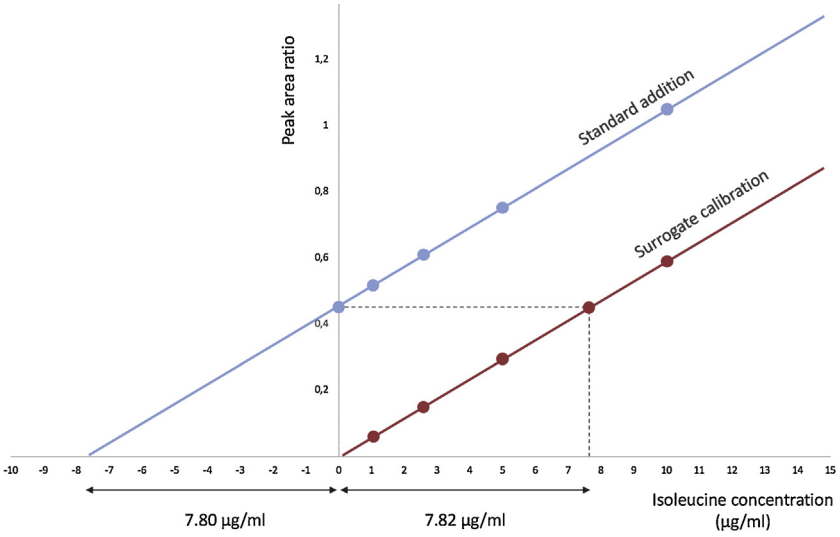

Fig. 3. Illustration of the use of standard addition to assess parallelism. Endogenous concentrations were determined by surrogate calibration and by extrapolating to the negative x-intercept from the standard addition calibration curve. Similarity of the results indicating parallelism between human plasma and the artificial matrix.

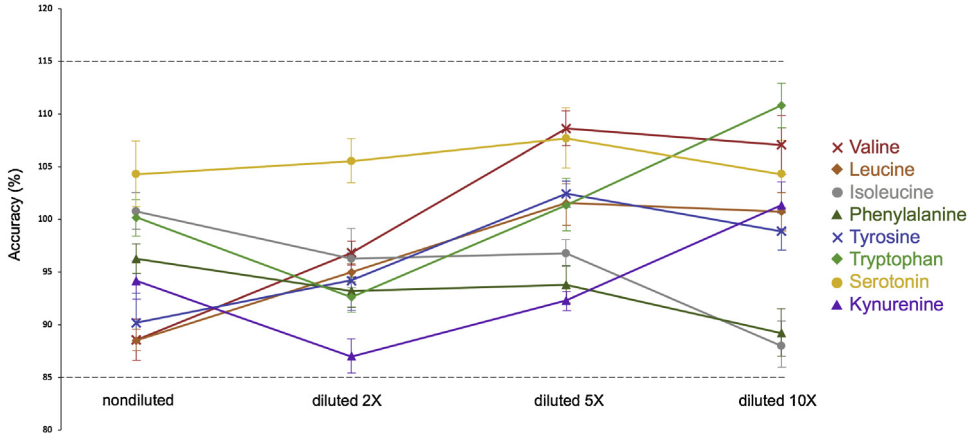

Fig. 4. Assessment of parallelism using dilution linearity test. Spiked human plasma samples were measured against surrogate calibrators when diluted two-, five-, ten-times and nondiluted. The data was the mean of triplicates. The analyte concentrations were multiplied by the dilution factors.

Table 5
Carryover of the analytes and internal standards.

	Valine	Leucine	Isoleucine	Phenylalanine	Tyrosine	Tryptophan	Serotonin	Kynurenine	Leu-d_3	Tyr-d_2
Carry over (%)	17.2	4.8	8.3	2.1	8.7	0.7	2.6	2.7	1.0	0.8

intensity, even when downregulation of the analytes is expected [1,7,9]. A potential drawback of the method is the systematic error that may be introduced by the lack of parallelism between the biofluid and the surrogate matrix [19,20]. However, parallelism can be ensured by the proper composition of the surrogate matrix and monitored by dilution linearity testing or standard addition [19]. Using standard addition to investigate parallelism clearly indicates that fit-for-purpose strategies should be considered as components of an analytical toolbox, those can be applied individually and in combination to make sure that none of the validation criteria are compromised.

Table 6
Concentration ranges of the analytes in 800 study samples.

Analyte	Concentration range (µg/ml)
Valine	4.63–115.58
Leucine	6.31–71.42
Isoleucine	1.68–97.66
Phenylalanine	1.65–36.76
Tyrosine	1.16–82.20
Tryptophan	1.17–39.35
Serotonin	0.27–1.70
Kynurenine	0.10–2.04

4. Conclusion

A simple, fast and reproducible LC–MS/MS based bioanalytical method has been developed for simultaneous quantitation of valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, serotonin and kynurenine in human plasma. The chromatographic method was capable to separate isomeric amino acids leucine and isoleucine. A fit-for-purpose validation approach was adopted by employing surrogate matrix for calibration, and partly for the construction of QC samples. The surrogate matrix was found to be parallel with human plasma, when investigated by standard addition and dilution integrity testing. Some quantitation and validation issues of endogenous compounds have been discussed. The method was able to meet the criteria imposed by the regulatory guidances of EMA and FDA [18,27], and demonstrated its value in the analysis of a total number 800 human plasma samples.

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Declaration of Competing Interest

Authors declare no conflict of interest.

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