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Development and validation of a UPLC-MS/MS method for the simultaneous determination of gamma-aminobutyric acid and glutamic acid in human plasma

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ABSTRACT

Gamma-aminobutyric acid (GABA) and its precursor glutamic acid are important neurotransmitters. Both are also present in peripheral tissues and the circulation, where abnormal plasma concentrations have been linked to specific mental disorders. In addition to endogenous synthesis, GABA and glutamic acid can be obtained from dietary sources. An increasing number of studies suggest beneficial cardio-metabolic effects of GABA intake, and therefore GABA is being marketed as a food supplement. The need for further research into their health effects merits accurate and sensitive methods to analyze GABA and glutamic acid in plasma. To this end, an ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for the quantification of GABA and glutamic acid in human plasma. Samples were prepared by a protein precipitation step and subsequent solid phase extraction using acetonitrile. Chromatographic separation was achieved on an Acquity UPLC HSS reversed phase C18 column using gradient elution. Analytes were detected using electrospray ionization and selective reaction monitoring. Standard curve concentrations for GABA ranged from 3.4 to 2500 ng/mL and for glutamic acid from 30.9 ng/mL to 22,500 ng/mL. Within- and between-day accuracy and precision were <10% in quality control samples at low, medium and high concentrations for both GABA and glutamic acid. GABA and glutamic acid were found to be stable in plasma after freeze–thaw cycles and up to 12 months of storage. The validated method was applied to human plasma from 17 volunteers. The observed concentrations ranged between 11.5 and 20.0 ng/ml and 2269 and 7625 ng/ml for respectively GABA and glutamic acid. The reported method is well suited for the measurement of plasma GABA and glutamic acid in pre-clinical or clinical studies.

1. Introduction

Gamma-amino butyric acid (GABA) and glutamic acid (Fig. 1) are non-essential amino acids and mainly known as inhibitory and excitatory neurotransmitters, respectively. Next to this, glutamic acid has a central position in amino acid metabolism and has a signalling function in for example the pancreas and the gut where it regulates postprandial responses after protein ingestion [1–3]. GABA can be synthesized from glutamic acid via glutamic acid decarboxylase. Next to its actions as a neurotransmitter, GABA plays a role as a signalling molecule in peripheral tissues. For example, GABA is involved in regulating hormone secretion in the pancreas and its receptors are found on immune cells [4–6]. In addition to their localization in tissues, GABA and glutamic acid are circulating in the blood stream [7,8]. In healthy volunteers, mean (±SD) GABA concentrations of 13.40 ± 2.75 ng/mL are reported [9]. Lower Plasma GABA concentrations have been associated with mental disorders like bipolar disorder, depression and schizophrenia [9-13], while autism has been associated with higher Plasma GABA concentrations [14,15]. Literature regarding normal plasma glutamic acid concentrations is inconsistent [16]. However, significantly higher plasma glutamic acid concentrations compared to control values have been reported for patients with depression and autism [16–19]. At the same time, lower glutamic acid concentrations are associated with schizophrenia [20].

GABA and glutamic acid are both available from dietary sources. GABA is only present as free amino acid, while glutamic acid is also one

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of the most abundant amino acids in dietary protein. Food products rich in GABA and glutamic acid include fermented foods and tomatoes [21,22]. In addition, monosodium glutamic acid is added as a flavouring agent to many food products. Oral administration of GABA and glutamic acid has acute effects on their circulating concentrations, presumably reflecting the plasma kinetics of uptake and distribution. Although 96% of dietary glutamic acid is instantly used as metabolic substrate by the intestine, a single oral dose of 150 mg/kg body weight has been reported to increase the plasma glutamic acid concentrations 10-fold over baseline within 50 min after ingestion [8,23]. GABA was also found to be rapidly absorbed in humans, producing a peak concentration 10 to 350 fold above baseline at 1–1.5 h after ingestion of a dose of 2 g [7]. Both amino acids are most likely unable to pass the blood–brain barrier, so oral administration is unlikely to influence brain GABA and glutamic acid concentrations directly [24,25].

More recently, oral administration of GABA was shown to have beneficial effects in animal models of diabetes. GABA administration was able to induce β-cell replication and to reverse chemically induced diabetes [26–28]. The potential health effects of GABA in humans are currently under investigation (NCT04144439, NCT03635437, NCT03721991, NCT02002130, NCT04144439; ClinicalTrials.gov). To better comprehend the physiological effects of exogenously administered GABA, more insight in its plasma kinetics is needed. This requires sensitive methods that allow for the reliable quantification of GABA and its precursor glutamic acid in plasma. The development of chromatographic methods for small polar analytes is challenging. Many methods, using reversed phase or HILIC chromatography for example, are already available that measure these analytes in plant material, brain tissue, cerebrospinal fluid and urine [29–33].

Although some methods have been described that measure GABA in plasma, these have specific disadvantages, such as the need for derivatization or insufficient sensitivity to detect GABA in the bloodstream [34–36]. The present paper describes an ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method that has been optimized to simultaneously measure GABA and glutamic acid in human plasma. This method is simple and robust and sufficiently sensitive, accurate and precise to quantify endogenous plasma concentrations of GABA and glutamic acid.

2. Materials and methods

2.1. Chemicals, materials and reagents

GABA, L-glutamic acid and internal standards gamma-aminobutyric acid-2,2-d2 (GABA-d2, 98% atom % D, 99% chemical purity) and glutamic acid-2,3,3,4,4-d5 (97 atom % D, 98% chemical purity) were purchased from Sigma–Aldrich (Munich, Germany). Acetonitrile (ACN), methanol and formic acid (ULC–MS grade) were purchased from Biosolve (Valkenswaard, the Netherlands). Milli-Q purified (MQ) water was used for all relevant preparations (ultrapure water system, arium 611UF, Sartorius Stedim Biotech GmbH, Göttingen, Germany). Blood samples were collected from apparently healthy volunteers in vacutainer plastic K2EDTA tubes purchased from Becton Dickinson (Etten-Leur, the Netherlands). Plasma (EDTA) was separated by centrifugation at 3000*g* for 10 min at 4 °C. A pooled plasma sample obtained by mixing plasma from 5 volunteers was snap-frozen, aliquoted and stored at −80 °C until further analysis.

2.2. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions of GABA (10 mg/mL) and internal standard GABA-d2 (1 mg/mL), glutamic acid (1 mg/mL) and internal standard glutamic acid-d5 (1 mg/mL) were prepared in MQ water. Working solutions of 1 mg/mL GABA and glutamic acid were diluted to prepare a 7-point calibration curve in MQ water. The 7 calibration standards were prepared by 3-fold serial dilutions with a starting concentration of 2500 ng/mL for GABA and 22,500 ng/mL for glutamic acid. Highest calibrator concentrations were based on the expected range in plasma concentrations after oral intake. All calibration standards contained 200 ng/mL of internal standard GABA-d2 and 2000 ng/mL of internal standard glutamic acid-d5.

Working solutions of 10 μg/mL, 1 μg/mL and 0.1 μg/mL for GABA and working solutions of 100 μg/ml, 10 μg/mL and 1 μg/mL of glutamic acid in MQ water were used for the preparation of the quality control (QC) samples. The QC samples were prepared by spiking GABA and glutamic acid into 100 μL human plasma from the human plasma pool. All stock solutions and calibration standards were stored at −20 °C. Pooled human plasma was stored at −80 °C in aliquots.

2.3. Sample preparation

To 100 μL of plasma, 400 μL of ACN containing 0.1% v/v formic acid (FA) was added. Internal standards were added to correspond to a concentration of 200 ng/mL GABA-d2 and 2000 ng/mL glutamic acid-d5 in the final extract. Samples were mixed at 1400 rpm (Eppendorf thermomixer, Eppendorf, Hamburg, Germany) for 5 min and centrifuged at 15,000*g* for 15 min. The supernatant was diluted to 95% ACN with 1500 μL ACN. The extract was purified with solid phase extraction (SPE) using BondElut C8 200 mg SPE cartridges (Agilent, Santa Clara, United States). The samples were loaded onto cartridges, previously conditioned with 1 mL methanol and 1 mL 50% methanol in MQ water. Cartridges were washed with 2 mL 95% ACN and the analytes were eluted with 2 mL 80% ACN. Samples were evaporated to dryness in a vacuum concentrator (RVC 2–33 CDplus, Martin Christ, Osterode am Harz, Germany) for 3 h at 30 °C and dissolved in 100 μL MQ water with 0.1% v/v FA. The extracts were stored at −80 °C until analysis.

2.4. UPLC-MS/MS

Extracts were analysed for GABA and glutamic acid concentrations using an Acquity I-class UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp, Etten-leur, the Netherlands). Chromatographic separation was performed using an Acquity UPLC HSS reversed phase C18 column 2.1 × 150 mm with 1.8 μm particle size (Waters Corp, Milford, MA, United States). Column temperature was set

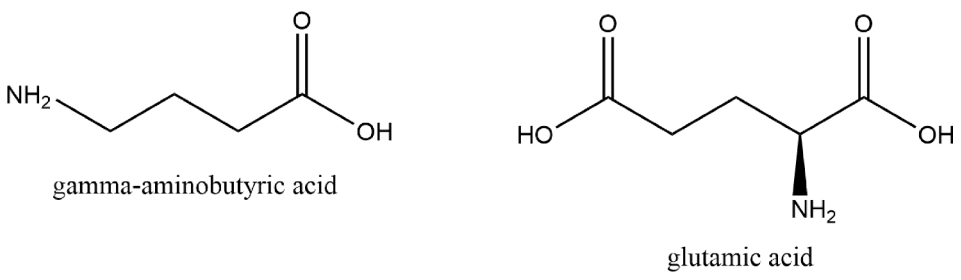

Fig. 1. Molecular structures of the amino acids GABA and glutamic acid.

to 30 °C, the sample temperature was set to 10 °C and the injection volume was 5 μL. Eluent A consisted of MQ water with 0.1% v/v FA, eluent B consisted of 100% methanol. The following gradient was applied: 98% A; 0.0–3.0 min, 98–50% A; 3.0–6.0 min, 50% A; 6.0–8.0 min, 98% A; 8.0–10.0 min. Total runtime was 10 min and the eluent flowed at a rate of 0.15 mL/min. Electrospray ionization in positive mode (ESI-pos) was used for all analytes. Compounds were tuned individually to obtain optimal signals. The MS was run in selective reaction mode (SRM). Settings were used as follows: capillary voltage 3.05 kV, desolvation temperature 600 °C, source temperature 150 °C, cone gas flow 150 L/h, desolvation gas flow 1000 L/h and collision gas flow was 0.13 mL/min. The SRM settings are shown in Table 1. Data acquisition and quantification were performed using MassLynx version 4.1. Quantification was performed against a linear, 1/x weighted, regression curve based on the duplicate injection of calibration standards.

2.5. Method validation

2.5.1. Calibration, linearity, sensitivity and carry-over

Quantification was performed using a 7-point calibration curve (see Section 2.2.) which was based on the duplicate analysis of calibrators using regression analysis with 1/x weighing. The peak area ratio of GABA/GABA-d2 and glutamic acid/glutamic acid-d5 were plotted against the analyte concentrations. The lower limit of quantification (LLOQ) was determined as the lowest concentration of the calibrator curve (n = 3 curves) which did not deviate more than 20% from the actual concentration. The limit of detection (LOD) was assessed using a dilution series of GABA and glutamic acid in a range from 3.43 to 0.04 ng/mL for GABA and in a range from 277.8 to 3.43 ng/mL for glutamic acid. The LOD was defined as the concentration of analyte with a signal to noise ratio equal to 3. With linear regression (GraphPad Prism 5) the concentrations of GABA and glutamic acid with a S/N ratio of 3 were determined. Carry-over was determined by injecting a blank containing MQ water and 0.1% v/v FA immediately after the highest calibration standard. Carry-over was found acceptable if the peak area was ≤20% of the LLOQ.

2.5.2. Matrix effects and recovery

Since GABA and glutamic acid are endogenously present in human plasma, deuterated internal standards were spiked before and after extraction to establish matrix effects and recovery. To assess recovery, human plasma samples were spiked prior to sample extraction (pre-spiked) with 200 ng/mL GABA-d2 and 2000 ng/mL glutamic acid-d5 or were spiked with the internal standards only after extraction (post-spiked). For each condition 3 replicates were prepared. The average internal standard peak area of the pre-spiked samples was expressed as a percentage of the post-spiked samples to determine the percentage recovery. To determine the matrix effect, the average peak area of the post-spiked plasma samples was compared to direct analysis of the internal standard solutions in clean solvent with the same concentration. Both the internal standard solution and the post-spiked plasma samples were prepared with solid phase extraction as is described in Section 2.3.

2.5.3. Accuracy and precision

The within- and between-day accuracy and precision of the method were determined with low, medium and high QC concentrations spiked to a pooled human plasma sample. No GABA/glutamic acid free plasma is available since they are endogenously present in plasma. On top of the endogenously present GABA, plasma was spiked with 15 ng/mL (low), 150 ng/mL (medium), and 1500 ng/mL (high) GABA. These concentrations were chosen based on a previous report which showed that mean plasma GABA concentrations may range between ~10 up to ~1000 ng/mL after the administration of GABA supplements, with outliers at >5000 ng/mL [7]. For glutamic acid, plasma was spiked with 1000 ng/mL (low), 5000 ng/mL (medium) and 15,000 ng/mL (high). Five replicates of unspiked plasma and (spiked) QC samples were prepared, which was repeated on three days. Each analytical batch consisted of validation samples from one day and a duplicate calibration curve. Accuracy was calculated as [accuracy (Δ%) = ((measured concentration − endogenous concentration) * 100)/spiked concentration]. To determine precision, the relative standard deviation (RSD) was used. RSD was calculated as [RSD = (SD * 100)/mean]. Accuracy and precision were calculated both within- and between-day, to calculate the between-day accuracy and precision the average value of each day was taken.

Table 1
SRM settings for GABA, glutamic acid, and internal standards.

Analyte	Precursor (*m/z*)	Product (*m/z*)	Collision energy
GABA	104.1	87.0	8
GABA-d2	106.1	89.1	10
Glutamic acid	148.0	130.0	10
Glutamic acid-d5	153.0	135.0	10

2.5.4. Stability and re-injection reproducibility

Since GABA and/or glutamic acid are commonly determined in plasma, we determined pre-analytical stability only in plasma. The effects of one, two and three freeze–thaw cycles were assessed with three replicates of human plasma from a pool that was frozen at −80 °C. After each freeze–thaw cycle the samples were frozen again at −80 °C for 24 h. Storage stability was assessed using the same human plasma pool. Three replicates were stored at −80 °C for either 1 month, 3 months, 6 months or 12 months. GABA and glutamic acid concentrations after freeze–thaw cycles and after storage were compared to plasma from the same plasma pool that was prepared immediately after blood drawing, without freezing and storage of the plasma sample. Prepared extracts were stored at −80 °C overnight for all conditions. The re-injection reproducibility of extracts was determined by re-injection after one week of storage at −80 °C. The GABA and glutamic acid concentrations of the re-injected extracts were calculated against both the original calibration curve as well as a freshly prepared calibration curve. For stability analyses, a deviation of <15% from the original concentration is considered acceptable.

2.6. Measurement of the analytes in human plasma

For the measurement of GABA and glutamic acid, human blood samples were obtained from 17 volunteers by venepuncture. Sample collection was approved by the medical ethical committee of Wageningen University. Volunteers gave written informed consent before donation. Plasma (EDTA) was separated by centrifugation at 3000g for 10 min at 4 °C, snap-frozen and stored at −80 °C until further analysis.

3. Results and discussion

3.1. Method development and optimization

The initial method was based on previously published methods that measure GABA in plasma [7,34]. For our purpose, the sample preparation was optimised to reach sufficient sensitivity to detect endogenously present GABA. In our hands, a simple extraction step of protein precipitation with ACN, as described by others, did not result in acceptable performance [7,34]. Endogenously present GABA was not detected, due to an apparent lack of sensitivity.

Therefore, we explored the effectiveness of sample preconcentration and clean-up steps. Multiple SPE cartridges (such as Strata X-C and X-A cartridges) were tested for their suitability to achieve a near 100% recovery of GABA and glutamic acid (dissolved in MQ water) (data not shown). Among these, only BondElut C8 200 mg SPE cartridges were capable of retaining GABA. Elution with 20% ACN showed near 100% recovery of GABA as compared to a pure standard which did not go

through the process of SPE. However, when a plasma sample containing GABA (endogenously present or spiked) was extracted with the same protocol, extraction was optimal when using 80% ACN during the elution step (Fig. 2). When eluting with 20% ACN during plasma extraction, barely any GABA is recovered. This shows that the matrix has a critical effect on the extraction behaviour of GABA. Various evaporation methods were compared, including vacuum concentration and evaporation under a gentle stream of nitrogen using the TurboVap system (Biotage, Uppsala, Sweden). Both methods displayed comparable performance (n = 5, 5.3% average difference in internal standard area) and are equally useful, with the TurboVap system allowing shorter drying times.

Next to the optimization of the sample preparation, the chromatographic separation was optimized. Reducing the UPLC flow rate to 150 µL/min increased the elution time and peak areas of GABA and glutamic acid. Chromatograms of GABA and glutamic acid at different flow rates are shown in Fig. 3. By decreasing the flow rate from 0.3 mL/min to 0.15 mL/min the peak areas increased with 84% and 74% for GABA and glutamic acid respectively. Reversed-phase columns poorly retain polar analytes, this may lead to matrix effects and signal suppression. In method development we did not explore other chromatographic options. HILIC chromatography would be a valid option for these polar analytes and could increase retention time [32]. Derivatization of the analytes could also aid in increasing retention time and therefore reduce matrix effects [37]. While the current method is simple in use and has a robust quality, future research could focus on these methods if increased retention time would be called for.

In the method described, stability issues have required some attention. Previous reports have shown that glutamine can, under specific conditions, be converted to glutamic acid [38]. We have evaluated whether the glutamine present in plasma could lead to artefacts in our method by analyzing a pure glutamine standard solution. The glutamine sample showed a peak in the expected mass transition (147 > 130.1) for glutamine but showed no peak in the glutamic acid trace (Fig. S1). It would therefore be unlikely that the glutamine present in plasma is a cause of artefacts in the glutamic acid analysis.

In addition, glutamic acid may undergo in-source conversion to pyroglutamic acid [39]. When injecting glutamic acid we did indeed detect the mass (130 > 84.1) that would be expected for pyroglutamic acid. A considerable amount of glutamic acid is converted in-source to pyroglutamic acid (pyroglutamic acid peak area is 1.4 times the glutamic acid peak area) (Fig. S2). As investigated by Purwaha et al. (2014), this conversion to pyroglutamic acid is fully corrected for by the use of an appropriate internal standard, such as deuterium-labeled glutamic acid. Meaning that the [M + H] transition is suitable for glutamic acid quantification when glutamic acid-d5 is used as an internal standard [39].

Although internal standards with higher deuterium number are usually preferred, we chose GABA-d2 as an internal standard for GABA quantification. GABA-d6 was found unsuitable for the present application. Analyzed plasma samples that were not spiked with GABA-d6, contained peaks in the GABA-d6 trace (data not shown). We concluded that no significant conversion or contamination of GABA and GABA-d2 was detected in our analyses (Figs. S3–S5).

3.2. Method validation and performance

3.2.1. Standard curve, sensitivity, linearity and carry-over

Calibration curves (n = 3) were made with duplicate analysis of calibrators using regression analysis with 1/x weighing. The calibration curve for GABA ranged from 3.4 ng/mL to 2500 ng/mL, and for glutamic acid from 30.9 ng/mL to 22,500 ng/mL. The r^2 values for the 3 obtained calibration curves were ≥0.997 for GABA and ≥0.998 for glutamic acid. The deviation of calculated concentrations was <20% from the actual concentration for all calibration points. At the LLOQ, accuracy was 6.7 Δ% for GABA and 2.0 Δ% for glutamic acid. Precision at the LLOQ was 10.5% (RSD) for GABA and 6.1% (RSD) for glutamic acid. Carry-over was assessed by injecting a blank after the highest calibrators; 2500 ng/mL GABA and 22,500 ng/mL glutamic acid. Based on peak areas, carry-over was <7% of the LLOQ peak area. The sensitivity, matrix effects and recovery are shown in Table 2. At 0.12 ng/mL GABA can be detected with a signal to noise ratio of 3 (LOD). At 3.4 ng/mL GABA can reliably be quantified (LLOQ). The method is less sensitive for the detection of glutamic acid. The LOD of glutamic acid is 4.4 ng/mL at a signal to noise ratio of 3 and the LLOQ is at 30.9 ng/mL. This method is more sensitive than a comparable method by Busardo et al. (2017) which was not able to measure endogenously present GABA [34]. Other available methods to measure GABA in plasma have a comparable or higher sensitivity [7,35,36]. The sensitivity of other available methods for the determination of glutamic acid are equally sensitive or slightly more sensitive than the current method [40–43]. With the achieved sensitivity this method is sufficiently sensitive to quantify endogenously present levels of GABA (~15 ng/mL) and glutamic acid (~4600 ng/mL) in human plasma (Fig. 4).

3.2.2. Matrix effects and recovery

Matrix effects were assessed by comparing the post-spiked plasma samples with the internal standard in clean solution. In the plasma

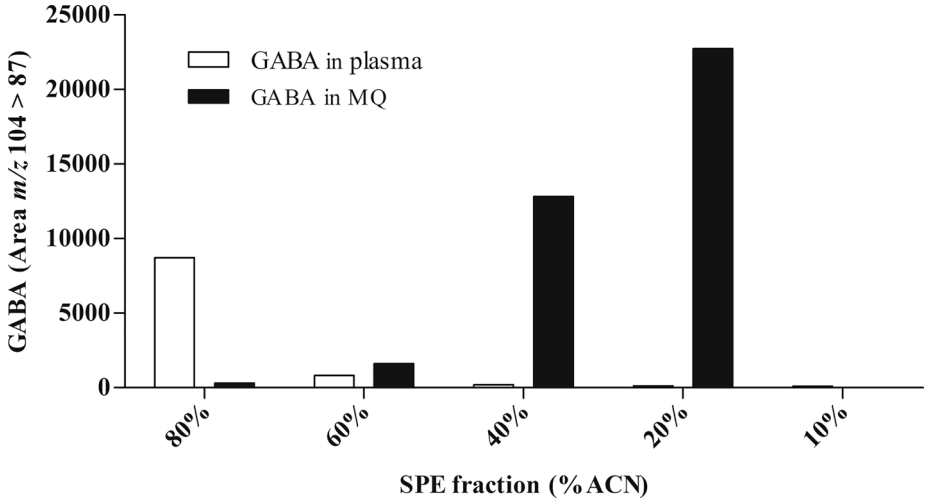

Fig. 2. Relative abundance of GABA in different SPE fractions. Plasma or MQ water was spiked with 200 ng/mL GABA. Samples were prepared according to section 2.3. During elution, decreasing percentages of ACN were used.

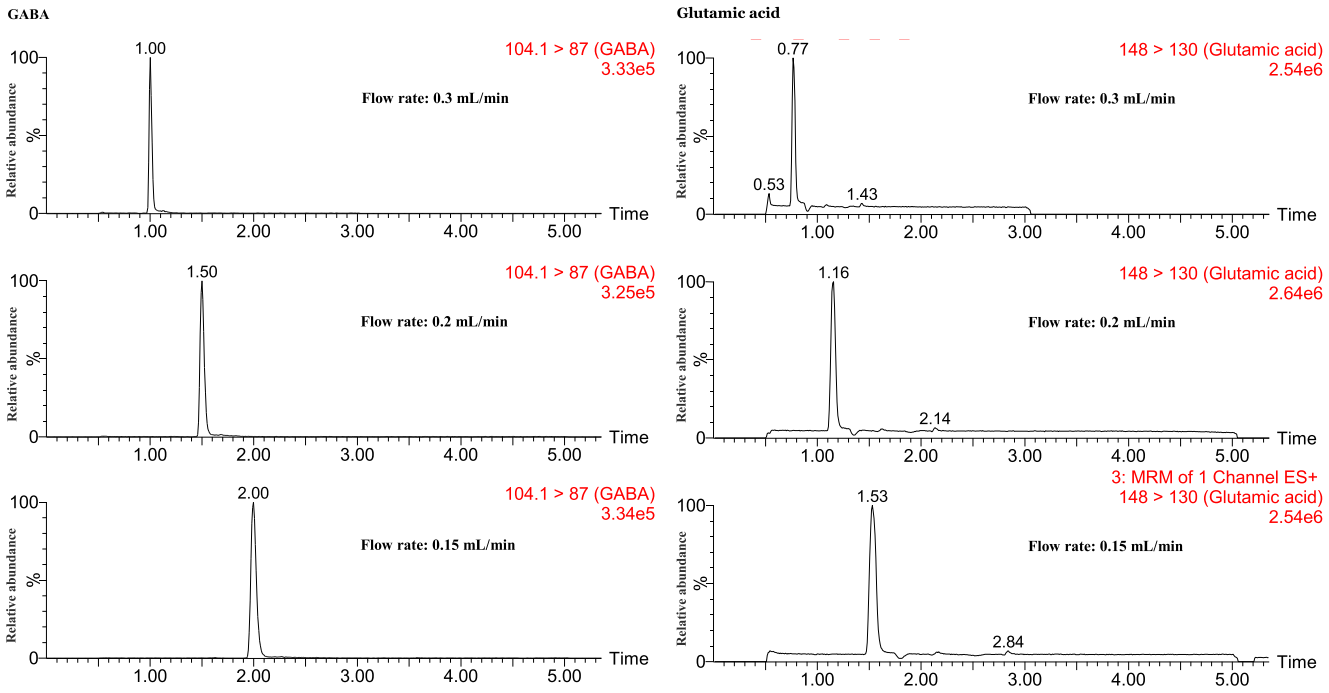

Fig. 3. SRM chromatograms of GABA (200 ng/mL) and glutamic acid (2000 ng/mL) obtained by UPLC-MS/MS at different UPLC flow rates.

Table 2
Method sensitivity, matrix effects and recovery in human plasma.

	GABA	Glutamic acid
LOD[1] (ng/mL)	0.12	4.43
LLOQ[2] (ng/mL)	3.43	30.9
Matrix effect[3]	78.4%	70.9%
Recovery[4]	41%	22%

[1] The concentration with a S/N ratio of 3.
[2] The lowest calibrator which deviates more than 20% from the actual concentration.
[3] Percentage decrease in peak area in post-spiked samples as compared to the peak area of the spike solution.
[4] Percentage of the pre-spiked samples peak area relative to the post-spiked samples peak area.

samples relative to the internal standard solution, the peak area is decreased by 78.4% for GABA and for glutamic acid the peak area is decreased by 70.9% (Table 2). To determine the recovery, the peak area of the plasma samples that were pre-spiked with internal standard were compared with the post-spiked plasma samples. For GABA, 41% is recovered from the matrix while this is 22% for glutamic acid. In contrast, internal standard solution prepared similarly to the plasma samples has near 100% recovery as compared to a directly injected internal standard solution. This shows that the matrix has substantial influence on the recovery of the analytes. Future efforts may focus on improving recovery from plasma. Despite poor recovery and substantial matrix effects, the method is sufficiently sensitive as it is able to quantify endogenous levels of GABA and glutamic acid with sufficient sensitivity, accuracy and precision in human plasma (Section 3.2.3).

3.2.3. *Within- and between-day accuracy and precision*

To determine the within- and between-day variation of the method, a pool was prepared from plasma of 5 volunteers. The precision and accuracy of the method were determined on 3 different days. On each day GABA and glutamic acid were spiked in five-fold into plasma at low, medium and high concentrations (see Table 3). Endogenous concentrations of GABA and glutamic acid were determined in the unspiked samples (average of 20 (SD 2.6) and 3864 (SD 188) ng/mL respectively),

Table 3
Within- and between-day accuracy and precision for GABA and Glutamic acid in quality control samples, presented concentrations are corrected for endogenous concentrations.

	GABA			Glutamic acid		
Spike (ng/mL)	15	150	1500	1000	5000	15,000
Day 1						
Mean (SD), ng/mL[1]	16.0 (1.0)	140.8 (3.0)	1429.6 (15.7)	995.9 (99.7)	5256.3 (132.5)	15,572.8 (974.1)
Δ%[2]	7.0	−6.1	−4.7	−0.4	5.1	3.8
RSD (%)[3]	6.2	2.2	1.1	10	2.5	6.3
Day 2						
Mean (SD), ng/mL	15.1 (1.3)	141.5 (2.6)	1435.6 (39.2)	1030.6 (37.4)	5159.4 (149.8)	15,428.3 (348.6)
Δ%	0.6	−5.7	−4.3	3.1	3.2	2.9
RSD (%)	8.4	1.9	2.7	3.6	2.9	2.2
Day 3						
Mean (SD), ng/mL	14.3 (0.8)	137.6 (8.1)	1422.4 (33.2)	1047.4 (24.2)	4915.4 (235.2)	15,033.5 (197.9)
Δ%	−4.5	−8.3	−5.2	4.7	−1.7	0.2
RSD (%)	5.4	5.9	2.3	2.3	4.8	1.3
Between-day						
Mean (SD), ng/mL	15.2 (0.9)	140.0 (2.1)	1429.2 (6.6)	1024.6 (26.3)	5110.4 (175.7)	15,344.9 (279.2)
Δ%	1.0	−6.7	−4.7	2.5	2.2	2.3
RSD (%)	5.8	1.4	0.4	2.6	3.5	1.9

[1] The mean is the average concentration measured in the spiked samples subtracted by the mean endogenous concentration of the plasma pool.
[2] Δ% is the percentage deviation of the measured concentration from the actual spiked value.
[3] RSD (%) is the relative standard deviation, the standard deviation is expressed as a percentage of the mean.

and these values were subtracted from the spiked samples to allow determination of accuracy. The mean concentration and the within- and between-day accuracy (Δ%) and precision (RSD (%)) are shown in Table 3. The method is capable of accurately and precisely quantifying GABA. Accuracy deviations ranged between 0.6% and 8.3% from the expected values, and precision ranged from 1.1% to 8.4%. The between-day precision and accuracy for GABA measurement is lower than 6.7% for all concentration levels. The within-day accuracy measurements of glutamic acid for all concentrations ranged from 0.2% to 5.1% from the nominal value and the precision ranged from 1.3% to 10%. The between-day deviation in accuracy and precision was lower than 3.5% for all concentration levels. It can be concluded that the method is accurate and precise for the determination of both GABA and glutamic acid in plasma.

3.2.4. Stability and re-injection reproducibility

The freeze–thaw stability was assessed by comparing the analyte concentrations in plasma after up to three freeze–thaw cycles to plasma samples that were prepared immediately following venepuncture (n = 3 for each condition). The concentrations of both analytes slightly increase after freeze–thaw cycles. The GABA concentration does not change more than 11.5% and the glutamic acid concentration does not change more than 3.3% as is shown in Table 4. Therefore, GABA and glutamic acid can be considered stable after multiple freeze thaw cycles. Long term storage was assessed by storing the plasma pool in aliquots for either 1 month, 3 months, 6 months or 12 months at −80 °C. Analyte concentrations were compared between the stored plasma samples and samples that were prepared immediately following venepuncture. As is shown in Table 4, GABA concentrations changed no more than 7.6% and glutamic acid concentrations changed no more than 6.9% during storage. Therefore, plasma can be stored for up to 12 months at −80 °C without any substantial concentration change of the analytes. However, it should be taken into account that plasma concentrations at higher than basal concentrations have not been tested for stability, and we therefore recommend to reduce storage time before sample preparation as much as possible when higher concentrations are expected.

Re-injection reproducibility was determined to assess the possibility of re-injecting the extracts in case of machine failure. QC samples and calibrators from the same batch were re-injected after 7 days of storage at −80 °C. Concentrations were calculated with the initial calibrator curve that was stored alongside the QC samples and a freshly prepared calibrator curve. The difference between the GABA and glutamic acid concentration calculated with the two different concentrations was negligible (<3%). Therefore, the initial calibrator does not have to be stored for re-injection. After re-injection, GABA concentrations changed no more than 6.9%, 8.2% and 2.8% for low, medium and high QC's respectively. Glutamic acid concentrations in low, medium and high QC's changed no more than 10.4%, 12.4% and 14.7% after re-injection. Thus, post-preparative storage for 7 days is considered acceptable since the results do not deviate more than 15% from the original analysis.

3.3. Application of the method to biological samples

The optimised method was applied to the measurement of GABA and glutamic acid in human plasma from 17 different apparently healthy volunteers. The values obtained are shown in Fig. 4. An average (±SD) Plasma GABA concentration of 16.1 ± 2.5 ng/mL (range: 11.5–20.0 ng/mL) was found. The average plasma glutamic acid concentration was 3968 ± 1400 ng/mL (range: 2269–7625 ng/mL). Differences between individuals are larger for glutamic acid (RSD 35.3%) than for GABA (RSD 15.5%). Most individuals have glutamic acid concentrations between 2000 and 4000 ng/mL, a few individuals have higher concentrations of up to ~8000 ng/mL as is shown in Fig. 4. Since glutamic acid is one of the most abundant amino acids in dietary protein, differences in diet between individuals could have introduced additional variation. In these individuals, plasma GABA and glutamic acid levels are not correlated (R^2 = 0.13). Representative chromatograms of GABA and glutamic acid and their respective deuterium labelled internal standards are shown in Fig. 5.

The plasma glutamic acid concentrations are consistent with other validated methods [40,41,43]. The Plasma GABA concentrations are also consistent with values reported in literature [7,9,13,14]. However, it is good to note that two validated methods for the determination of GABA in plasma show substantially higher GABA levels in healthy volunteers [35,36]. Since there is no gold standard method available, only speculations are possible regarding the cause of these differences. As opposed to the current method several methods require a derivatization step, which increases retention of the analytes but on the other hand complicates sample preparation, reduces reliability or may create artefacts [37,44]. In addition, different calibrators could have been used. Song et al. (2004) speculates that the often low recovery could explain the discrepancy between their results and others. However, our method

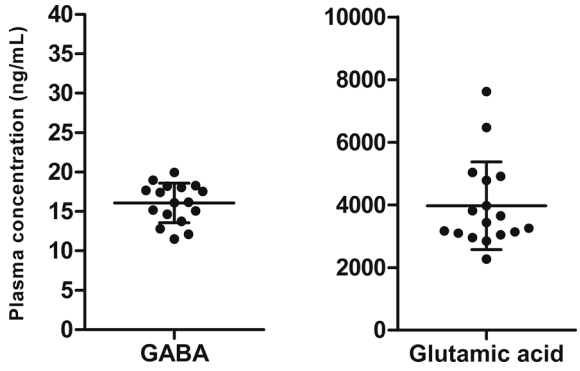

Fig. 4. Concentrations of GABA and glutamic acid in human plasma samples from 17 volunteers. Individual values as well as mean ± error bars representing SD are presented in the figure.

Table 4
Stability of GABA and glutamic acid in human plasma.

	GABA			Glutamic acid		
	ng/mL	% of original concentration	RSD (%)	ng/mL	% of original concentration	RSD (%)
Freeze-thaw cycles						
No storage	21.6	100	2.0	4087.8	100	5.7
Freeze-thaw 1	24.1	111.5	6.0	4169.6	102.0	0.3
Freeze-thaw 2	23.5	108.6	6.5	4175.3	102.1	2.0
Freeze-thaw 3	24.0	110.8	6.9	4222.4	103.3	1.6
Long-term storage						
No storage	19.1	100	5.3	4340.7	100	6.4
1 month	18.5	96.7	4.3	4639.2	106.9	1.9
3 months	18.1	94.7	4.7	4348.4	100.2	0.9
6 months	19.2	100.4	2.4	4143.8	95.5	2.8
12 months	20.6	107.6	1.7	4295.1	99.0	0.4

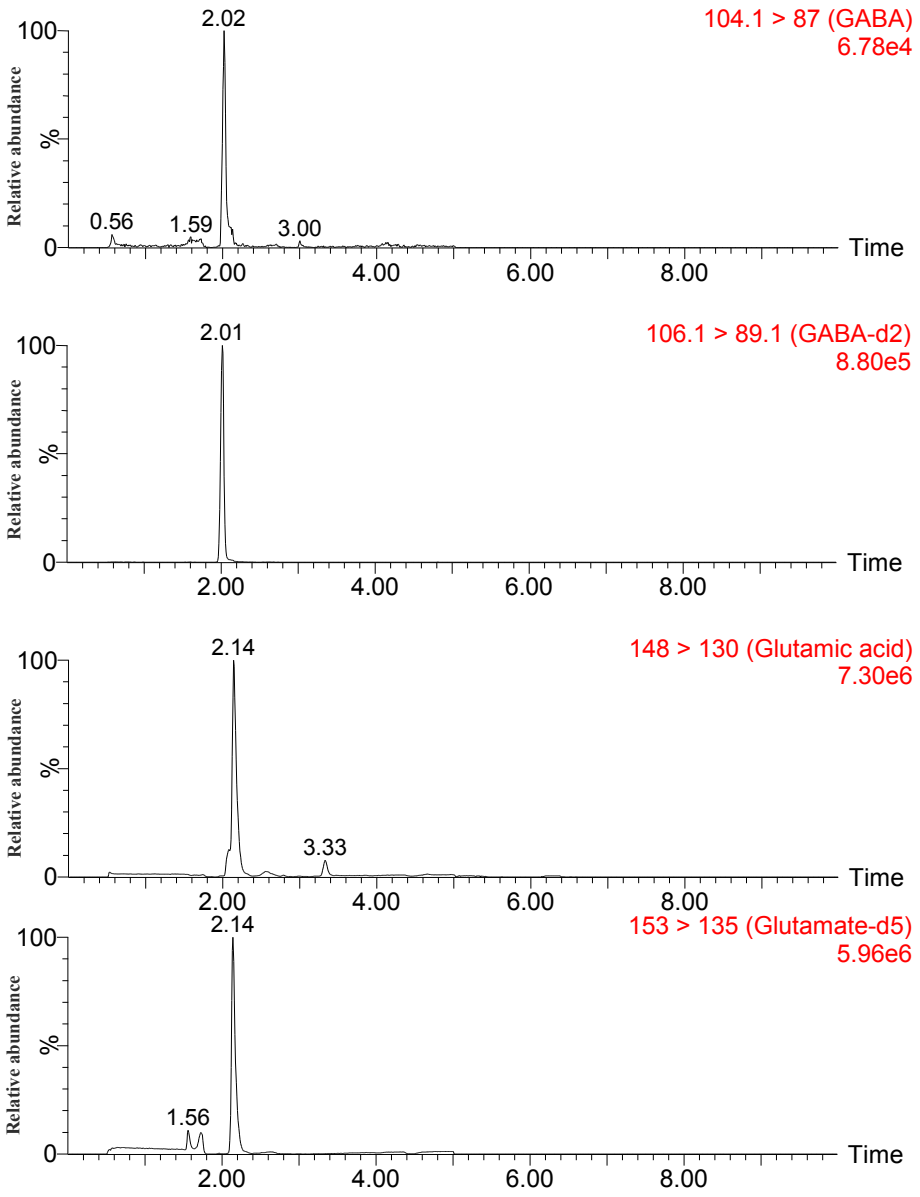

Fig. 5. Representative SRM chromatograms obtained from a human plasma sample of GABA and glutamic acid and their respective deuterium labelled internal standards.

corrects for losses in recovery by using internal standards. Future work should shed light on the causes of these discrepancies.

4. Conclusion

A UPLC-MS/MS method was successfully developed and validated for the simultaneous quantification of endogenous GABA and glutamic acid in human plasma. The analytical performance was found to be well within generally acceptable ranges. In addition, stability was for the first time thoroughly investigated. GABA and glutamic acid were found to be stable through multiple freeze–thaw cycles and long-term storage for up to 1 year. With the current method, we determined average GABA and glutamic acid concentrations of 16.1 ng/mL and 3968 ng/mL, respectively, which was in agreement with values reported by other groups. We conclude that the reported method is therefore well suited for the quantification of GABA and glutamic acid in human plasma.

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CRediT authorship contribution statement

Tessa H. de Bie: Investigation, Methodology, Validation, Visualization, Writing - original draft. **Renger F. Witkamp:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing. **Maarten A. Jongsma:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing. **Michiel G.J. Balvers:** Supervision, Methodology, Investigation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2020.122519.

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