Rapid Determination of Glutamine in Human Plasma by High-Performance Liquid Chromatographic–Tandem Mass Spectrometry and Its Application in Pharmacokinetic Studies

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A rapid and accurate high-performance liquid chromatographic–tandem mass spectrometric method was developed and validated for the determination of glutamine in human plasma. Phenomenex EZ: faastTM amino acid analysis kit was used for sample pretreatment. Chromatographic separation was conducted on an EZ: faast amino acid analysis–mass spectrometry column (250 × 3.0 mm i.d., 4 μm). A binary gradient elution of mobile phases A (0.2% formic acid containing 5 mM ammonium acetate) and B (methanol, containing 0.2% formic acid and 5 mM ammonium acetate) was programmed at 0.4 mL/min. Multiple reaction monitoring was used for quantification by monitoring ion transitions of \( m/z 275.3/172.1 \) for derivatized glutamine and \( 317.3/84.1 \) for internal standard in the electrospray positive ionization mode. The standard curve was linear (\( r^2 > 0.99 \)) over the concentration range of 3.14–157.20 μg/mL. The intra- and inter-day precision values were <8.70% and the accuracy within −4.35 to 8.91% at three concentrations. The method was successfully applied to the pharmacokinetic study in Chinese healthy male subjects following oral administration of glutamine with doses of 2 and 4 g.

Introduction

Glutamine (2-amino-4-carbamoylbutanoic acid) is the most abundant free amino acid in the human body. It is synthesized by the glutamine synthetase from glutamate and ammonia in humans, and possesses many important physiological activities. In catabolic states of injury and illness, glutamine becomes conditionally essential (1). This natural amino acid supplement has been studied for the past couple of years and proved beneficial for human body. Nowadays, glutamine is widely used in clinical practice, e.g. gastrointestinal tract ulcer and dysfunction (2, 3), tumor therapy support (4, 5) and enhancement of immune system (6, 7). Recently, the therapeutic effect of glutamine in diabetes was also reported (8).

The pharmacokinetic properties of exogenous glutamine have been investigated in both patients and healthy subjects (9–11). The peak glutamine plasma concentration obtained within 45 min following oral administration and >50% was taken up by splanchnic tissues (12, 13). However, previous studies only reported a dose of glutamine over 4 g to each person, and we investigated the pharmacokinetic behavior of glutamine with doses of 2 and 4 g in healthy Chinese male people.

Several assay methods for the determination of glutamine concentration have been reported, such as fluorescence detection, ion-exchange chromatography (IEC), high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), gas chromatography (GC) and GC–MS. Only few of these methods were well validated and suitable for pharmacokinetic study. The HPLC (9, 10) and enzymatic method (11) have been used for plasma concentration determination, but a derivatization or enzymatic hydrolysis step was necessary and such step was time consuming and costly. Although the derivatization can be avoided by using the ion-pairing reagents, the residue of these reagents will cause impaired performance of mass spectrometer (14, 15).

To further improve the previous assay method, Phenomenex EZ: faast amino acid analysis kit was utilized to prepare the glutamine plasma sample (16, 17) for the determination of derivatization glutamine plasma concentration. A simple solid-phase extraction (SPE) and rapid derivatization was initially conducted, and then the derivatized samples were quickly analyzed by an HPLC–MS–MS method. The entire duration of the sample analysis was ~13,50 min.

The aim of this study is to develop an HPLC–MS–MS method for quantification of glutamine in human plasma. The analysis was performed using a modified protocol based on the EZ: faast amino acid analysis kit from Phenomenex. The method was applied to a clinical pharmacokinetic study in healthy Chinese male subjects following oral administration of glutamine with doses of 2 and 4 g.

Experimental

Chemicals and reagents

Glutamine standard (98.7% purity) (Figure 1) was purchased from the National Institutes for Food and Drug Control (Beijing, China). The EZ:faastTM amino acid analysis kit was obtained from Phenomenex, Inc. (Torrance, CA, USA). Homoarginine (Figure 1) in the analysis kit was used as the internal standard (IS). HPLC grade methanol was obtained from Merck (Darmstadt, Germany). Ammonium acetate and formic acid of analytical grade were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA) with purity greater than 99%. Water was deionized and purified using an Milli-Q system (Millipore, Bedford, MA, USA) and used to prepare all aqueous solutions. Blank plasma was supplied by Chengdu Blood Center (Chengdu, China) and stored at −20°C until assayed. Glutamine capsules (0.25 g/capsule, lot no. 20

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deionized water was spiked instead of the corresponding working solutions as mentioned above. Briefly, 100 µL of IS (homoaarginine at 0.20 mM) and 100 µL of sample were combined in a glass vial. The mixture should have a pH between 1.5 and 6.0 for successful loading onto the sorbent tip. A sorbent tip was attached to a 1.5-mL syringe, and the mixture was passed through the tip by slowly pulling back the plunger. The sorbent tip was then washed by washing solution of 30% n-propanol. Afterwards, the sample was eluted by 200 µL of freshly prepared elution buffer (0.33 M NaOH : n-propanol : 3-picoline : H2O = 3: 3: 2: 0.8). The eluted glutamine was derivatized using 50 µL of derivatizing reagent chloroform, and the mixture was incubated for 1 min. Both the NH2 and COOH groups were modified. The glutamine derivative was extracted using 100 µL of iso-octane by vortexing vigorously for 5 s, and the mixture was allowed to react for 1 min. Fifty microliters of the upper organic layer were transferred into an autosampler vial and evaporated to dryness under a stream of nitrogen at room temperature. The residue was redissolved in 100 µL of HPLC mobile phase and 2 µL was injected into the HPLC–MS–MS system.

**Method validation**

Since glutamine is an endogenous compound, the endogenous interference exists when using chromatographic assay. A standard addition method was utilized to determine the plasma concentration contributed by exogenous glutamine in this study.

The calibration curve was constructed by plotting the peak area ratios of each analyte to IS against the corresponding nominal concentrations in plasma. The linearity of the calibration curves were evaluated using weighted least-squares linear regression with the weighing factor of $1/x^2$. The correlation ($r^2$) of >0.99 was considered to be acceptable.

The sensitivity of the method was evaluated in terms of the lower limit of quantification (LLOQ). The precision of the LLOQ was assessed by the relative standard deviation (RSD) of five measurements which should be <20%, and accuracy was evaluated by the deviation from the nominal concentration which should be within ±20%.

To assess the intra- and inter-day precision and accuracy, glutamine QC samples at low (6.29 µg/mL), medium (31.44 µg/mL) and high (125.76 µg/mL) concentrations were prepared as described above. The intra-day precision was assessed by calculating the RSD values for the analysis of the QC samples in five replicates in a single day, while inter-day precision was determined by analyzing the QC samples on 3 separate days. Accuracy was evaluated by comparing the measurements with the nominal values. In addition, the glutamine concentrations were corrected by subtracting the amount of endogenous glutamine at baseline level. The criteria for acceptability of the precision should not exceed 15% except at LLOQ, which should not exceed 20%. The average values of accuracy should be within ±15% of the nominal concentration except the LLOQ which should be ±20%.

The extraction recovery for glutamine was determined at three QC concentrations (6.29, 31.44 and 125.76 µg/mL) by comparing the peak area ratios of the analyte spiked in plasma with those in deionized water at equivalent concentrations.

Although a matrix effect by ionization competition between the analytes and co-eluents may exist in mass spectrometer,
the method of standard addition can effectively rule out matrix effects (18).

The stability of glutamine at all experimental conditions was tested. The short-term stability of glutamine in plasma was evaluated by analyzing three aliquots of each QC sample after leaving at room temperature for 9 h. The stability of the QC samples from plasma was also assessed by reanalyzing the same sample after being stored in an autosampler (4°C) for 27 h. Freeze–thaw stability was evaluated by analyzing the QC samples after three freeze and thaw cycles along with the freshly prepared QC samples. The long-term stability was estimated by assessing the QC samples stored at −20°C for 30 days. Samples were considered to be stable if the deviations were within 15% from those fresh samples.

**Pharmacokinetic study**

The HPLC–MS-MS method was applied to plasma samples collected from the clinical trial. The study was performed in accordance with the principles of the Declaration of Helsinki and

![Typical mass spectrometry chromatograms of glutamine and IS in human plasma.](image)

**Figure 2.** Typical mass spectrometry chromatograms of glutamine and IS in human plasma. (a) Blank plasma with endogenous glutamine; (b) blank plasma spiked with glutamine and IS; (c) a plasma sample obtained from a subject at 4 h post dosing 2 g drug with its IS.
approved by the independent Ethics Committee of The First Affiliated Hospital of Fourth Military Medical University (Xi’an, China) (permission number: 20120426-2). The approval is valid for 1 year. Twelve healthy male subjects aged between 18 and 40 years with a body mass index of 19–24 kg/m² were included in this study. Written informed consent was obtained from each subject after explanation of the design and objectives of the trial prior to any study procedures.

In this trial, subjects were allowed to drink a small quantity of water only 2 h after taking the medicine and eating standard meal 4 h later. As glutamine comes from high-protein diet, protein food such as meat, egg, bean products and milk were forbidden throughout the entire study. Smoking and drinking (drinks containing alcohol, caffeine, grapefruit, etc.) were also forbidden in the study. Additionally, the subjects were not taking any other medications.

An open-label, randomized, single-dose, three-way crossover study was designed. Twelve subjects were assigned according to a computer-generated randomization schedule to receive a single dose of glutamine (0, 2 and 4 g) administered in each session separated by a 7-day washout period. Venous blood samples (3 mL) were immediately collected into heparinized tubes before and after dose at 0.15, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h. The plasma was separated by centrifuging at 4,000 rpm for 5 min, and the supernatant was collected and stored at −20°C until analysis.

The endogenous plasma glutamine concentration was subtracted before the pharmacokinetic data analysis. Pharmacokinetic parameters were calculated by the non-compartmental method with aid of Phoenix WinNonlin version 6.3 (Pharsight Corporation, Mountain View, CA, USA). The paired t-test (SPSS Statistics version 19, SPSS, Inc., Chicago, IL, USA) was used to determine statistically significant differences between results. A P-value of <0.05 was considered to be statistically different.

Results

Method validation
The detection of glutamine was acquired by positive ion mode in ESI source. The representative chromatograms of glutamine and IS in plasma are shown in Figure 2. The typical retention times were ~4.22 and 3.58 min for glutamine and IS, respectively. No interference from the blank plasma was observed surrounding IS.

One common challenge for endogenous substance assay is to find an appropriate surrogate matrix, which should be free of target analytes and with identical or similar physicochemical properties as the real sample matrix (18, 19). However, such matrix similar to human plasma is not available at present. Thus, standard addition method was chosen as an alternative approach for quantification of glutamine in human plasma. By adding serial concentrations of the analyte to the blank sample, calibration curves were created and the endogenous concentration in the blank sample (~90 µg/mL) was then determined. The calibration curves for aqueous solution and for plasma after standard addition were shown in Figure 3. The regresion equations were $y = 0.029x + 0.06$ for aqueous solution and $y = 0.018x + 0.96$ for plasma after standard addition with good linearity over a concentration range of 3.14–157.20 µg/mL. The LLOQ was 3.14 µg/mL for the plasma samples.

The precision and accuracy for the analysis of glutamine in human plasma are summarized in Table I. At the LLOQ, low, medium and high concentration levels, the intra- and inter-day precision (RSD) were within 7.58 and 8.70% except for the LLOQ (within 11.04%). The accuracy, presented as percent deviation from nominal concentration, was found to be within <15%.

The extraction recoveries for the glutamine ranged from 82.62 to 89.75%, with the mean recovery of 84.35% at the three QC concentration levels. The RSDs of these values were <15%. The method of standard addition was used to eliminate the matrix effect as reported before (20).

The plasma samples at three QC concentrations were found to be stable in all the tested conditions, including short term (9 h at room temperature), autosampler (27 h in autosampler at 4°C), three freeze–thaw cycles at −20°C and long term (30 days at −20°C) (Table II).

Pharmacokinetic study
This method was successfully applied to the pharmacokinetic study of glutamine. The plasma concentration–time profiles for the subjects following an oral single dose (0, 2 and 4 g) of glutamine are shown in Figure 4. The basic pharmacokinetic parameters are listed in Table III. Since the endogenous glutamine exists in human plasma at a substantial concentration, the pharmacokinetic

Table I

<table>
<thead>
<tr>
<th>Nominal concentration determination (µg/mL)</th>
<th>Determined concentration (µg/mL)</th>
<th>Precision (% RSD)</th>
<th>Accuracy (% bias)</th>
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<tbody>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.14</td>
<td>3.42 ± 0.22</td>
<td>6.51</td>
<td>8.91</td>
</tr>
<tr>
<td>6.29</td>
<td>6.59 ± 0.49</td>
<td>7.36</td>
<td>4.78</td>
</tr>
<tr>
<td>31.44</td>
<td>30.98 ± 1.76</td>
<td>5.69</td>
<td>−1.48</td>
</tr>
<tr>
<td>125.76</td>
<td>120.32 ± 9.14</td>
<td>7.58</td>
<td>−4.35</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.14</td>
<td>3.27 ± 0.36</td>
<td>11.04</td>
<td>4.11</td>
</tr>
<tr>
<td>6.29</td>
<td>6.21 ± 0.54</td>
<td>8.70</td>
<td>−1.31</td>
</tr>
<tr>
<td>31.44</td>
<td>30.69 ± 2.29</td>
<td>7.42</td>
<td>−1.74</td>
</tr>
<tr>
<td>125.76</td>
<td>124.21 ± 7.80</td>
<td>6.37</td>
<td>−3.56</td>
</tr>
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</table>
properties were presented by adjusting the baseline level. A nonlinear pharmacokinetic behavior of glutamine was observed at the dose from 2 to 4 g by comparing $\ln(C_{\text{max}}/\text{dose})$, $\ln(AUC_{0-12}/\text{dose})$ and $t_{1/2}$ ($P < 0.05$, paired sample $t$-test), which could be caused by incomplete absorption of glutamine in gastrointestinal tract and the alteration of splanchnic tissue distribution at high dose (9).

Discussion

The EZ: faast amino acid analysis kit was used in this study, and the procedure consisted of SPE followed by rapid derivatization and liquid–liquid phase extraction. Total sample preparation time took $\sim$8 min, which is faster than the other reported method (21–23). The chromatographic conditions used were selected based on the optimization of peak separation, and the MS conditions were set up based on the maximum signal of the analytes and the assay reproducibility. The chromatographic and MS conditions in our study were further optimized based on the method described in the user manual. The gradient program could be completed within 5.5 min in our study in comparison with 17 min in the manual. In addition, we for the first time reported the pharmacokinetic profiles of glutamine in human following oral administration (2 and 4 g). This method can be applied to other studies in different bio-fluids or experimental media as well in the future.

Table II

<table>
<thead>
<tr>
<th>Parameters</th>
<th>2 g</th>
<th>4 g</th>
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<tbody>
<tr>
<td>AUC$_{0-12}$ (h µg/mL)</td>
<td>30.28 ± 7.01</td>
<td>53.27 ± 7.83</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (h µg/mL)</td>
<td>31.83 ± 7.51</td>
<td>57.13 ± 9.99</td>
</tr>
<tr>
<td>MRT$_{0-\infty}$ (h)</td>
<td>1.81 ± 0.35</td>
<td>2.01 ± 0.56</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>1.50 ± 0.50</td>
<td>1.77 ± 0.67</td>
</tr>
<tr>
<td>$t_{\lambda}$ (h)</td>
<td>0.73 ± 0.20</td>
<td>0.71 ± 0.21</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>22.67 ± 5.09</td>
<td>35.34 ± 4.70</td>
</tr>
<tr>
<td>$V_{ef}$ (L)</td>
<td>146.95 ± 71.97</td>
<td>178.85 ± 57.43</td>
</tr>
<tr>
<td>$Cl_{2}$ (L/h)</td>
<td>66.78 ± 19.14</td>
<td>72.36 ± 14.65</td>
</tr>
</tbody>
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Table III

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AUC, area under concentration–time curve; MRT, mean residence time; $t_{1/2}$, half-life; $T_{\lambda}$, peak time; $C_{\text{max}}$, peak concentration; $V_{ef}$, apparent volume of distribution; $Cl_{2}$, clearance.

Conclusion

A rapid HPLC–MS-MS analytical method was developed for the quantification of glutamine in human plasma based on the EZ: faast amino acid analysis kit. The method showed satisfactory accuracy and precision. This method was successfully applied to a pharmacokinetic study following a single oral administration of glutamine capsules with doses of 2 and 4 g in healthy male Chinese subjects.

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