Liquid Chromatography–Tandem Mass Spectrometry Method for Determination of Homocysteine in Rat Plasma: Application to the Study of a Rat Model for Tauopathies

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Hyperhomocysteinemia is a common occurrence in many neurodegenerative diseases, including tauopathies. We developed and validated a simple and sensitive liquid chromatography-tandem mass spectrometry method for the analysis of homocysteine (Hcy) in rat plasma. Hcy was analyzed using ultra-performance liquid chromatography on a C8 column with detection by positive ESI tandem mass spectrometry. For optimal retention and separation, we used ion-pair reagent—heptadecafluorobutyric acid. The method utilizes heavy labeled internal standard and does not require any derivatization or extraction step. The procedure was validated in compliance with the European Medicines Agency guideline. The limit of detection was 0.15 μmol/L and the limit of quantification was 0.5 μmol/L. The method showed excellent linearity with regression coefficients higher than 0.99. The accuracy was in the range of 93–98%. The inter-day precision (n = 5 days), expressed as % relative standard deviation, was in the range 3–8%. Using this method, we analyzed plasma samples from two transgenic lines of the rat model for tauopathies.

Introduction

Tauopathies are a heterogeneous group of neurodegenerative diseases characterized by abnormal deposition of the tau protein in nervous system cells (1). They include around 20 different degenerative disorders such as Alzheimer’s disease (AD), progressive supranuclear palsy, Pick’s disease and others. Transgenic animal models of the neurodegenerative diseases provided information about the disease mechanisms and opportunities to test new therapeutic agents. Since metabolites are conserved during the evolution, detailed metabolomic studies of body fluids of these transgenic animal models could help to understand the neurodegenerative process better.

Moderate increase of plasma homocysteine (Hcy) has previously been linked to a variety of neurodegenerative conditions such as vascular and nonvascular dementia (e.g. AD), Parkinson’s disease, schizophrenia and depression (2–4). Increased concentration of Hcy in cerebrospinal fluid (CSF) usually correlates with that in serum.

There is a precise balance between Hcy synthesis and metabolism under normal metabolic circumstances. Hcy is formed by demethylation of the essential amino acid methionine. The metabolism of Hcy includes re-methylation to methionine (requires vitamins B12, B2 and folic acid), or a conversion to cysteine in transsulfuration pathway (requires vitamin B6) (5).

The brain has a limited capacity for Hcy metabolism. Neuronal cells are capable of producing Hcy under normal conditions, however, increased concentrations of extracellular Hcy has a neurotoxic effect (6, 7). Hcy is transported to and from the brain through the blood–brain barrier via specific bi-directional transporter (8). Elevated concentration of Hcy in plasma is associated with blood–brain barrier opening (9).

The link between hyperhomocysteinemia and tauopathies exists. It has been shown that an increased concentration of Hcy can induce AD-like phosphorylation of tau protein (10). Interestingly, the tau protein can undergo N-homocysteinylation, and this post-translational modification alters its binding to tubulin (11).

Several liquid chromatography-mass spectrometry (LC/MS) methods have been developed and used for the analysis of Hcy in plasma. These include simple reversed phase (RP) chromatography on C18 or C8 columns with MS detection (12, 13), cation exchange chromatography coupled to MS detection (14) and derivatization with Ellman’s reagent with subsequent RP chromatography coupled to MS (15). Most of the methods published have been validated for clinical use. One of the difficulties with LC/MS analysis of highly polar compounds, such as Hcy, is low retention of these compounds on RP columns. The use of ion-pair reagents in LC/MS analysis offers a simple solution for this problem. Classic ion-pair reagents such as tetrabutylammonium salts of phosphoric or sulfuric acid are not applicable for MS due to their low volatility. The pentadecafluorooctanoic acid and heptafluorobutyric acid (HFBA) have been previously used as ion-pair reagents for the LC/MS analysis of highly polar compounds such as glutamine, glutamate, γ-aminobutyric acid (16, 17) and metabolites related to the tryptophan–kynurenine pathway (18).

The aim of our study was to investigate the effect of tau-induced neurodegeneration on plasma levels of Hcy. For this, we developed and validated a rapid and sensitive ultra-performance liquid chromatography (UPLC)/MS method for the analysis of Hcy in rat plasma. To achieve optimal separation and retention on a reverse phase column, we used an ion-pair reagent, HFBA. The benefit of using UPLC/MS is a short analysis period and thus, a very good sample throughput.

Experimental

Chemicals and materials

n-Homocysteine, HFBA, formic acid (FA), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and LC/MS grade acetonitrile were purchased from Sigma-Aldrich (St Louis, MO, USA). n-Homocysteine-3,3,4,4-d4 was from C/D/N...
isotopes (Quebec, Canada). Tris(2-carboxyethyl)phosphine (TCEP HCl) was produced in Thermo Scientific (Rockford, IL, USA). Deionized water (MPW) was purified using the in-house Millipore system (Bedford, MA, USA). All other reagents used in the study were of analytical grade.

**Mass spectrometry**

A Waters (Waters, Praha, CZ) Quattro Premier XE mass spectrometer system was used. Mass spectra were acquired using positive electrospray ionization and selected reaction monitoring (SRM). The capillary voltage was 0.5 kV and the source temperature and desolvation temperature were 120 and 450°C, respectively. The cone gas and desolvation gas flowed at 50 and 600 L/h, respectively. Argon was used as collision gas at a manifold pressure of 3 × 10^{-3} mbar. The collision energies and source cone voltages were manually optimized for each SRM transition. Data were acquired with MassLynx 4.0, calibrated and quantified by QuanLynx software.

**Ultra-performance liquid chromatography**

Waters ACQUITY UPLC system was used. An Acquity UPLC BEH C8 column (2.1 mm × 50 mm, 1.7 μm particle size) with VanGuard pre-column was used for analysis. Column temperature was set for 30°C. Mobile phase A consisted of 0.5% HFBA/0.1% FA in MPW, and mobile phase B was 0.5% HFBA/0.1% FA in acetonitrile. Mobile phase gradient program was as follows: 3% B for 0.5 min; increased to 50% B from 0.5 to 2 min; decreased to 3% B at 2.1 min and then 3% B from 2.1 to 3 min. The flow rate was 0.5 mL/min and the injection volume was 5 μL.

**Preparation of standards and quality control samples**

Individual stock solutions of dL-homocysteine and dL-homocysteine-3,5,4-d4 were freshly prepared in water. Working solutions of seven standards (0.5, 1, 5, 10, 25, 50 and 100 μmol/L) were prepared from the stock solution every day by spiking into blank rat plasma. The quality control samples (1, 10 and 50 μmol/L) were prepared from the stock solution every day by spiking into blank rat plasma. The quality control samples (1, 10 and 50 μmol/L) were prepared in bulk and stored at −20°C. The sample preparation consisted from a reduction and protein precipitation step. The 60 μL of plasma and 10 μL of TCEP (100 mg/mL) were incubated at 37°C for 15 min. After this reduction step, proteins were precipitated by 90 μL of TCA (100 mg/mL) containing EDTA (1 mmol/L). The samples were vortexed and centrifuged for 10 min at 30,000 × g. Subsequently, supernatant was transferred to sample vials and analyzed with UPLC/MS.

**Method validation**

The method was tested according to the European Medicines Agency (EMA) guideline (19). The inter-day precision and accuracy of the method were determined by analyzing the three different quality control (QC) samples over 5 days. Intra-day accuracy and precision were calculated from six repeat injections. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from standard chromatograms. The matrix effect (ME) and extraction recovery (RE) were assessed using the post-extraction addition method. Freeze–thaw stability was tested by analyzing the QC samples stored at −20°C. Three 24 h freeze thaw cycles were performed.

**Animals**

The generation and characterization of transgenic rat models for tauopathies expressing human truncated tau 151–391 were described in details elsewhere (20). For this study, heterozygous transgenic rats (6–7 month old) and non-transgenic spontaneously hypertensive rat (SHR)/Wistar age-matched controls were used. All animals were housed under standard laboratory conditions with free access to water and food and were kept under diurnal lighting conditions (12 h light/dark cycles with light starting at 7:00 am). All experiments on animals were carried out according to the institutional animal care guidelines conforming to international standards and were approved by the State Veterinary and Food Committee of Slovak Republic (permission number RO-1101/14-221C; 9 April 2014) and by Ethics Committee of Institute of Neuroimmunology, Bratislava, Slovak Republic (11 December 2013). Efforts were made to minimize the number of animals utilized and to limit discomfort, pain or any other suffering of the experimental animals used in this study.

**Collection of plasma**

Whole blood was collected from the tail vein. Animals were fixed in a plastic holder and the tail was warmed up in a water bath. Approximately 500 μL of the blood was collected by a 22G needle. After a short centrifugation step (10 min at 5,000 g; 4°C), all plasma samples were immediately flash frozen in liquid nitrogen and stored at −80°C until used.

**Data statistics**

The data from the animal samples were analyzed by an one-way ANOVA, followed by the Tukey’s multiple comparison test (Prism 5.0 software, GraphPad, Inc., San Diego, CA). Values are expressed as mean ± SEM. Differences at P < 0.05 were accepted as statistically significant.

**Results**

**UPLC/MS method development**

The single protonated molecular ions (m/z 136.1) for Hcy or (m/z 140.1) for internal standard (homocysteine-d4) were the most intensive peaks in the MS spectra and were used as parent ions for SRM method development. Both compounds were detected by MS/MS, under collision-activated dissociation conditions. Similar to previous reports, the fragment resulting from the loss of FA (m/z 89.9) and the corresponding homocysteine-d4 fragment (m/z 93.9) were the most abundant product ions and were therefore used for SRM. A representative MS and MS/MS spectra of Hcy are shown in Figure 1.

Table I demonstrates the MS parameters used for the analysis of Hcy and its internal standard—homocysteine-d4. The UPLC BEH C8 column with UPLC BEH C8 VanGuard pre-column was used for analysis. For the optimal separation, the gradient elution program was established, using ion-pair reagents—HFBA as an additive into the mobile phase A and B. The retention time for Hcy under these conditions was 1.29 min. Figure 2 displays a typical chromatogram of Hcy and homocysteine-d4 in rat plasma.
All quantitative analyses were performed by an internal standard procedure using corresponding deuterated homocysteine \((3,3,4,4\text{-d}_4)\) as internal standard. The linear calibration curve was obtained within the concentration range studied \((0.5–100\ \mu\text{mol/L})\), with the correlation coefficient >0.99. The method proves to be sensitive enough to detect physiological concentrations of Hcy and potential changes due to the pathological processes. The LOD was 0.15 \(\mu\text{mol/L}\) and LOQ was 0.5 \(\mu\text{mol/L}\). The QC had acceptable intra-day and inter-day precision \(<10\%\) and accuracy \((93.7–98.7\%)\). These results are summarized in a Table II.
The analytical recovery for QC standards was 102.3–114.8%, and the ME was <10% (4.2–9.3%). The stability of analyte in the biological matrix was analyzed during the three freeze/thaw cycles at −20°C. There were no significant differences in Hcy responses in QC standards (6.9–8.9%, relative standard deviation).

**Table I.** SRM Conditions of Homocysteine and Internal Standard (Homocysteine-d₄)

<table>
<thead>
<tr>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Dwell time (s)</th>
<th>Cone voltage (V)</th>
<th>Collision energy</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Dwell time (s)</th>
<th>Cone voltage (V)</th>
<th>Collision energy</th>
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<tr>
<td>Homocysteine</td>
<td>136</td>
<td>89.9</td>
<td>0.05</td>
<td>21</td>
<td>Used for quantification</td>
<td>136</td>
<td>118</td>
<td>0.05</td>
<td>21</td>
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<tr>
<td>Homocysteine-d₄</td>
<td>140</td>
<td>93.9</td>
<td>0.05</td>
<td>21</td>
<td>12</td>
<td>140</td>
<td>93.9</td>
<td>0.05</td>
<td>21</td>
</tr>
</tbody>
</table>

**Figure 2.** Extracted ion chromatogram of homocysteine (A) and internal standard (homocysteine-d₄; B) in rat plasma. Selected reaction monitoring (SRM) was used to detect analytes. Chromatographic conditions: column, Acquity UPLC BEH C8 column (2.1 mm × 50 mm, 1.7 μm particle size) with VanGuard pre-column; flow rate = 0.5 mL/min; column temperature 30°C; gradient elution conditions are described in ‘Experimental’ section. Homocysteine and homocysteine-d₄ peak at 1.29 min.
The increased plasma concentration of Hcy is common in many neurodegenerative diseases including tauopathies, such as AD (21). The method was subsequently used for the analysis of plasma samples from two transgenic rat models for tauopathies. The expression of truncated tau in our transgenic rat models does not have any impact on Hcy levels in plasma. We did not detect any significant changes in plasma of transgenic animals in comparison to control animals; the only significant difference we found was a difference in plasma Hcy levels between animals from different rat strain (SHR vs Wistar). The difference between Hcy concentrations in different ages, strains or colonies of rats exists. Interestingly, Hcy concentration can also vary based on circadian rhythm and it is therefore important to compare data from samples collected at the same time of the day (22). The data we measured by the developed method are within the previously published ranges for Wistar or SHR rats (23, 24).

Conclusion

In this study, we developed and validated UPLC/MS for analysis of total Hcy in rat plasma. To achieve good retention, we used ion-pair reagent, HFBA. The developed method is easy and sensitive and has good accuracy, precision and extraction recovery. Using this method, we analyzed Hcy in the plasma of two transgenic rat models for human tauopathies.

Acknowledgements

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References


**Table II**

<table>
<thead>
<tr>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 5)</th>
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<tr>
<td></td>
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<tr>
<td><strong>Nominal</strong></td>
<td><strong>Mean</strong></td>
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<tr>
<td>(μmol/L)</td>
<td>(μmol/L)</td>
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<tr>
<td>Homocysteine</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>10</td>
<td>9.87</td>
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<tr>
<td>50</td>
<td>48.61</td>
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</table>

Analysis of samples from transgenic rats

The developed method was used for analysis of plasma samples from transgenic and age-matched control animals. We detected a statistically significant difference in plasma levels of Hcy between SHR and Wistar control animals (1.96 ± 0.22 μmol/L in SHR, n = 7 vs 4.17 ± 0.26 μmol/L in Wistar, n = 9; P < 0.001). Similarly, we detected a statistically significant difference in plasma levels of Hcy between SHR72 and Wistar72 transgenic animals (1.57 ± 0.28 μmol/L in SHR72, n = 30 vs 4.09 ± 0.9 μmol/L in Wistar72, n = 18; P < 0.001). There were no differences between control and transgenic animals from the same rat strain (Figure 3).

Discussion

We developed and validated the UPLC/MS method for determination of total Hcy in rat plasma. The method was validated in compliance with the EMA guideline. In comparison to previous reports, the use of ion-pair reagent in our method resulted in good retention of Hcy on the C8 RP column (retention factor 2.2). We did not observe any changes in column separation properties, life time or any build up of ion-pair reagent on the column. The method is simple. The samples are prepared by reduction and protein precipitation without any additional derivatization or purification step. The use of UPLC allows for a rapid throughput with a 3-min run time. The assay showed good linearity over the analytical range that is necessary to cover reference values from rats reported in the literature.


