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Research Article

Development and Validation of Quantification Method for Homocysteine in Human Plasma Using Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

We developed and validated a sensitive and robust method for quantifying homocysteine in human plasma using ultra-high-performance liquid chromatography-tandem mass spectrometry. Isocratic elution of the analyte and internal standard were achieved within 2 minutes using Zorbax Eclipse Plus C18 column, with 5% of mobile phase A (0.1% formic acid in water) and 95% of mobile phase B (0.1% formic acid in acetonitrile). Quantification was performed using multiple reaction monitoring (MRM) mode, based on parent and product ion transitions for L-Homocysteine (136 > 90.1, 56.2) and d8-DL-Homocysteine (140 > 94.1, 59.3) as the internal standard. The method was validated for linearity, sensitivity, accuracy, precision, recovery, and stability. Good linearity was observed within a range of 250-3000 ng^{-1}, with correlation coefficients (r^2 = 0.98). The extraction recovery ranged between 83% and 92%. Homocysteine remained stable under all five tested conditions, including 2 and 8 hours on the benchtop at room temperature, overnight storage in the autosampler (10 ºC), three freeze-thaw cycles (-20 ºC), and long-term storage (1 month) at -20 ºC. Based on these findings, the developed method met the criteria as stipulated by European Medicines Agency (EMA), Food and Drug Administration (FDA), and International Council of Harmonisation (ICH). In conclusion, this validated method can be used for quantitating homocysteine levels in human plasma.

Keywords: homocysteine, plasma, ultra-high performance liquid chromatography-tandem mass spectrometry

1. INTRODUCTION

Homocysteine, a sulfur containing amino acid produced endogenously during the metabolism of methionine was first discovered by Vincent DuVigneaud in 1932 (Ghassabian et al., 2014). Methionine is an important amino acid and an essential element of cellular

homeostasis in human. A variety of inherited and acquired conditions cause an increase in the concentration of this metabolite in blood (homocysteinemia) and other biofluids (Ghassabian et al., 2014). Homocysteine present in plasma in four different forms, 1% of homocysteine circulates in blood, 20%-30% combines with itself to form dimer homocysteine, 70%-80% is a disulphide bound to plasma homocysteine and the rest combines with other dimer as homocysteine mixed disulphide (Bostom et al., 1996; Schindler et al., 2000). The range of plasma total homocysteine varied depending on the age, gender and lifestyle such as smoking, alcohol consumption and vegetable intake. The normal range of plasma homocysteine is between 5-15 mmol/L and higher level is classified as hyperhomocysteinemia (Azzini et al., 2020). Hyperhomocysteinemia is categorized as moderate, intermediate and severe according to the levels at 15-30 mmol/L, 30-100 mmol/L and >100 mmol L^{-1}, respectively (Morris et al., 2017). Hyperhomocysteinemia has a high prevalence (16-55%) in critically-ill patients (Schindler et al., 2000). Hyperhomocysteinemia has been related to many diseases such as cardiovascular disease, Alzheimer disease, Parkinson disease, diabetes and migraine (Hou et al., 2012; Lea et al., 2009; Suliman et al., 1999). Increased levels of homocysteine present a considerable risk factor for cardiovascular diseases as they impede the activity of nitric oxide synthase, causing damage to endothelial cells. Moreover, elevated homocysteine levels, referred to as hyperhomocysteinemia, have been associated with disrupted methylation of proteins and DNA, leading to abnormal proliferation of vascular smooth muscle and heightened lipid peroxidation (Hou et al., 2012).

In the early stages, homocysteine was detected using ninhydrin reaction. However, this method was not sensitive and only suitable to detect homozygotes for genetic conditions leading to homocystinuria (Govindaraju et al., 2003; Alam et al., 2019). Subsequently, other methods such as fluorescence polarization immunoassays, chemiluminescent microparticle immunoassays and enzyme-linked immunoassays were developed. However, the use of these assays were limited due to high reagents cost (Govindaraju et al., 2003; Lea et al., 2009; Alam et al., 2019). The enzymatic assay which is based on the colorimetric measurement is easily performed on an automated clinical chemistry instrument. Despite the great advantages in terms of reagent costs and the availability of equipment to conduct colorimetric measurement in most laboratory, this method shows some limitations regarding pathological values HPLC with a fluorescence detector (HPLC-FL) is a robust method but it requires time-consuming procedures for derivatization of samples at 60ºC (Rasmussen & Moller, 2000). Thus, HPLC-FL has limited routine use due to low throughput as compared to completely automated immunometric methods. The detection using HPLC with electrochemical detection shows high sensitivity, high selectivity, and no derivation is required. However, the throughput is modest due to difficulty in stabilizing the detector (Tomaiuolo et al., 2009).

In this study, Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS) was used. It is a chemistry technique that combines the capabilities of liquid chromatography in physical separation of chemical entities with the capabilities of mass spectrometry for mass and charge analysis. This is a powerful technique that has found extensive application due to its extremely high sensitivity. Generally, it accurately identifies and quantifies the amount of chemicals or analytes in complex biological fluid mixtures, such as plasma. Additionally, this technique offers undeniable advantages in terms of result quality due to its specificity, sensitivity, and the utilization of labelled internal standards. Furthermore, it increases sample throughput and reduces solvent consumption. Moreover, the cost of reagents is notably lower compared to that of immunoassays and enzymatic assays (Rasmussen & Moller, 2000; Tomaiuolo et al., 2009). Other than that, it also required less time for sample extraction. The extraction method only involved samples mixed with internal standard of d8-homocysteine. Then, dithiothreitol helped the conversion of the disulfide forms into reduced homocysteine. Protein is precipitated by the addition of formic

acid in acetonitrile. The supernatant was then loaded into liquid chromatograph. For these reasons, we developed a fast and robust method for quantification of homocysteine by UHPLC-MS/MS which is applicable in a clinical chemistry laboratory (Zappacosta et al., 2006).

The aim of this study was to develop a robust method for the quantification of plasma homocysteine based on the guidelines by European Medical Agency (EMA) 2011, US Department of Health and Human Services, Food and Drug Administration (FDA) 2018, and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2005 (Rafii et al., 2009; Stefanini-Oresic, 2022; Timmerman et al., 2020).

2. MATERIALS AND METHODS

2.1 Materials and reagent

Reference standards for L-homocysteine was obtained from Sigma Aldrich (USA). dL homocysteine was obtained from Cambridge Isotope laboratory (USA). Formic acid was obtained from Sigma Aldrich (USA) whereas HPLC grade methanol and acetonitrile were obtained from Merck (Germany) and freshly purified water obtained from an ELGA PURELAB ultra purification system (UK).

2.2 Liquid Chromatography Mass Spectrometry conditions

The equipment used was 1290 Infinity UPLC system coupled to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, USA)) which was equipped with an ESI source using Agilent Jet Stream Technology (Agilent Technologies, USA). Chromatographic separation was conducted using Zorbax Eclipse Plus C18 (2.1×50 mm i.d; 1.8 µm, Agilent Technologies) coupled with a C18 guard column. The mobile phases A consisting of 0.1% (v/v) formic acid in water while mobile phase B consisting of 0.1% formic acid in methanol. The flow rate of liquid chromatography (LC) was maintained at 0.4 mL/min, and the total time to run the test was 2 min. For mass spectrometry (MS), a Jet Stream electrospray source was used. Instrument parameters were set as follows: drying gas temperature 320°C, drying gas flow at 5 L/min, nebulizer at 45 psi, sheath gas temperature 380°C, sheath gas flow at 11 L/min, capillary voltage at 4000 V, nozzle voltage at 1000 V, and dwell time of 25 ms. Instrument control, data acquisition, and quantification were performed by MassHunter Workstation software version B.08.02. The multiple reaction monitoring (MRM) transitions and other quantification settings for analytes are presented in Table 1.

Table 1. MRM transitions parameter used for quantification of homocysteine

Analyte	MRM transition	Fragmentor (V)	Collision energy (eV)
d8-DL-Homocysteine	140 > 94.1	100	15
	140 > 59.3	100	20
L-Homocysteine	136 > 90.1	135	15
	136 > 56.2	135	15

2.3 Sample preparation

Artificial plasma fluid was used in this study. Aliquot of 100 µL of artificial plasma was spiked with 10 µL of 50 000 ng/mL of internal standard (d8-dL-Homocysteine). The mixture was then vortexed for 10 seconds. 30 µL of 0.5 M dithiothreitol was added and mixed by vortexing for 10 sec. The mixture was then incubated for 30 min at room temperature between 25-28°C. After that, 200 µL of acetonitrile containing 0.1% formic acid was added and mixed

by vortexing the samples for 10 seconds. Subsequently, the samples were centrifuged at 14,000 RPM (UK) at 25°C for 10 min before transferring 100 µL of the mixture into an insert for analysis.

2.4 Method validation

The method was validated in accordance with the guidelines of Bioanalytical Method Validation from EMA, Bioanalytical Method Validation Guideline from Industry by FDA, Validation of Chromatographic Methods by Centre for Drug Evaluation and Research (CDER)-FDA as well as Validation of Analytical Procedure by ICH. The validation parameters included the measure of linearity, recovery, accuracy, precision, stability, matrix effect and dilution integrity. Linearity, limit of detection (LOD) and limit of quantification (LOQ) were evaluated using the guidelines from EMA, FDA and ICH. The standard curves were calibrated using 7 seeded concentrations (250-3000 ng/mL) which were prepared in triplicates. The seeded concentrations were prepared by serial dilution of the homocysteine stock solution to produce a calibration curve in the range 250-3000 ng/L. Each concentration was prepared and assayed in triplicates on 3 different days. The linearity was evaluated by analysing the slope, intercept and correlation coefficient-. Intra-day and 3 inter-day precision and accuracy were done by analysing three replicates of quality control samples spiked with analytes at low, medium and high concentration level (625-3000 ng/mL). The accuracy was evaluated by comparing the measured concentration with the true value and the precision was expressed as the relative standard deviation percentage (RSD%). The stability of the analytes in plasma was assessed by analysing the spiked plasma samples that were kept at different settings which include (i) room temperature for 2 hours and (ii) 8 h, (iii) in autosampler at 4ºC for overnight and (iv) three times freeze-thaw cycles at -20 ºC and (v) long-term storage (1 month) at -20ºC. The results were compared with the freshly prepared QC samples. Three replicates were analysed for each storage condition at low and high concentrations. 3-Deazaadenosine were added to the samples as stabilizer. The concentration for the stabilizer was 50 µm/L in 3 mL EDTA blood tube (Hill et al., 2002; Marín et al., 2001).

3. RESULTS AND DISCUSSION

The method was developed and validated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantitate the levels of homocysteine in human plasma. We intend to use the method for monitoring homocysteine levels in individuals who are at risks of cardiovascular diseases and to allow personalising diets such as folate and vitamin B12. This is indeed in parallel to the strategies to realise the practise of precision health in our local setting. The analytes were separated using isocratic elution of 95% of mobile phase A. The total run time for this method was 2 min. Both analyte and internal standard were eluted at the same retention time 0.65 min with sharp and symmetrical peak shapes as shown in Figure 1.

Linearity of homocysteine was expressed over a six-point calibration curve ranging from the lower limit of quantitation (LLOQ) of 250 ng/mL to the upper limit of quantitation (ULOQ) of 3000 ng mL^{-1} as presented in Table 2 and Figure 2. The least-square linear regression analysis used to study the relationship between the relative concentrations (x) to the relative response (y) between the ratios of internal standard to the target analyte. The equation derived from the graph is $y = 0.007611x + 0.001232$. The correlation coefficients (r^2) of the calibration curve are 0.98 which indicates good linearity. The developed method demonstrates sensitivity by detecting as low as 250 ng/mL (1.85 µmol/L) compared to ninhydrin reaction, fluorescence polarization immunoassays, chemiluminescent microparticle immunoassays, and enzyme-linked immunoassays. Additionally, the method exhibits excellent selectivity by utilizing

multiple reaction monitoring (MRM), allowing precise selection and monitoring of specific precursor and product ion pairs for homocysteine. This targeted approach significantly enhances the analysis's specificity by focusing solely on selected ions of interest, minimizing interference from other compounds present in the sample (Anderson & Hunter, 2006; Li et al., 2021).

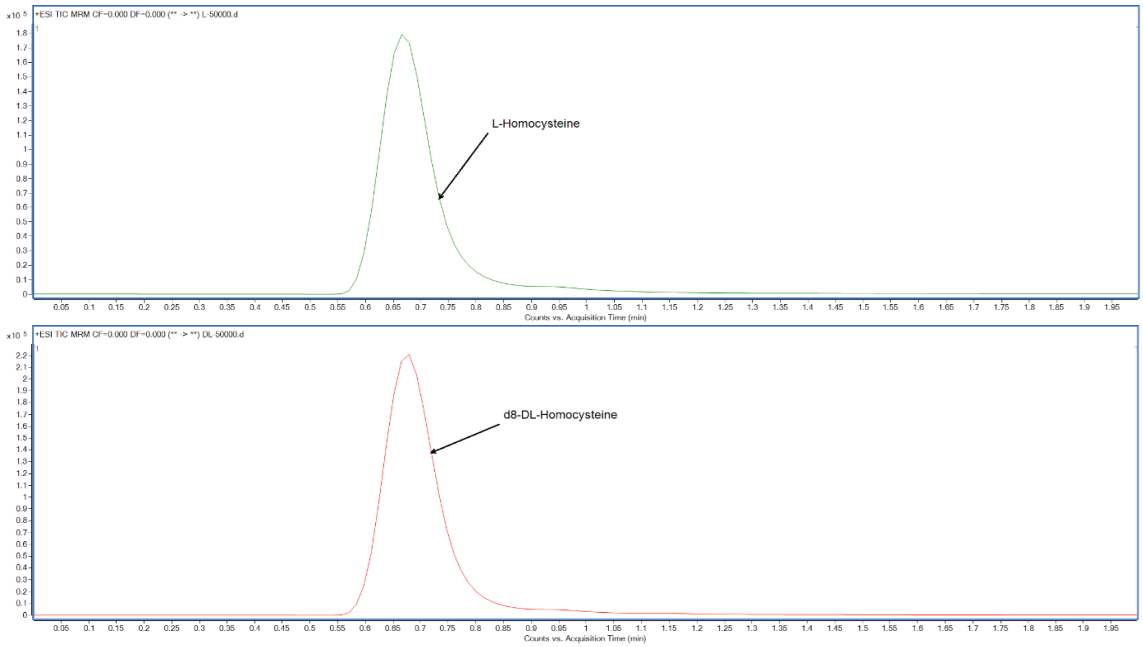

Figure 1. Chromatograms of L-homocysteine and d8-DL-homocysteine

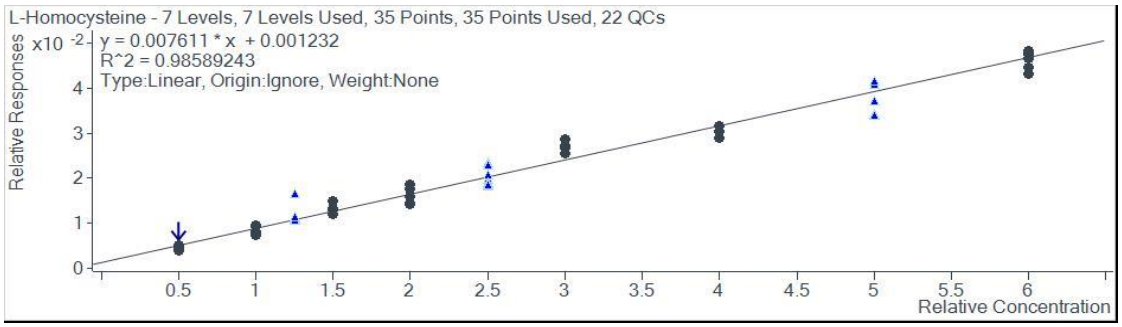

Figure 2. The linearity of the calibration levels with 3 QC levels

Table 2. The regression equation, correlation coefficients, linear range and lower limit of quantitation

Compound	Regression Equation	Correlation Coefficients (r^2)	Linear Range (ng/mL)	LLOQ (ng/mL)
Homocysteine	0.007611x + 0.001232	0.98	250 - 3000	250

EMA's guidelines for method validation parameters ensure that the methods used to analyze drugs or their metabolites in biological samples are accurate, specific, precise, and reliable. These parameters encompass selectivity, accuracy, precision, sensitivity, linearity, and stability. The FDA's method validation parameters largely mirror EMA's standards, while ICH guidelines cover broader principles, emphasizing harmonization across different regions. They mainly include parameters similar to those specified by EMA and FDA. The overall accuracy and precision were assessed by calculating the difference between the ratio of the response between the samples and are presented in the form of percentage accuracy, standard deviation and percentage of relative standard deviation (RSD) as tabulated in Table 3.

All the accuracy results are within the limit of ±15% and precision results are below 15%. This agrees with the reference guidelines from EMA, FDA and ICH (Committee for Medicinal Products for Human Use, 2023; FDA, 2018; Kaza et al., 2019; Stefanini-Oresic, 2022).

Table 3. Precision and accuracy data

Analyte	Conc. (ng/L)	Intra-day			Inter-day		
		Mean Measured Concentration (ng/mL)	Precision (RSD)	Accuracy (%)	Mean Measured Concentration (ng/mL)	Precision (RSD)	Accuracy (%)
Homo-cysteine	250	246 ± 15.35	6.22	98.66	218 ± 27.35	12.52	87.36
	625	644 ± 15.93	2.47	104.06	662 ± 24.20	3.65	105.98
	1250	1352 ± 107.16	7.93	108.16	1286 ± 130.84	10.17	102.91
	2000	2369 ± 307.95	12.99	94.79	2476 ± 317.62	12.82	99.07

Data on extraction recovery of homocysteine are shown in Table 4. The mean recoveries of plasma samples spiked with standards at concentration 250 and 500 ng/mL were 92.4% and 83.79%, respectively. This method managed to achieve high analyte recovery using a simple and cost-effective method.

Table 4. Recovery of Homocysteine from the plasma

Analyte	Endogenous (ng/mL)	Added (ng/mL)	Mean ± SD measured (ng/mL)	RSD (%)	Mean Recovery (%)
Homocysteine	727.41	250	958.52 ± 17.86	0.02	92.4
	727.41	500	1146.37 ± 107.05	9.33	83.79

Stability assessments of homocysteine are crucial as they ensure the reliability and accuracy of the analytical method, preventing inaccurate measurements that may result from the degradation or alteration of unstable homocysteine over time. Based on the findings (Table 5), it was observed that homocysteine remained stable under in all tested conditions which are 2 and 8 h at room temperature, overnight on the autosampler, three cycles of freeze and thaw at -20^{o}C, and one month of storage at -20^{o}C.

Table 5. Stability of the analyte at 2 and 8 h at room temperature

Analyte	Freshly sample[a]	2 h at room temperature		8 h at room temperature	
L-Homo-cysteine	Conc.[a]	*Conc.[a]	Stability[b]	*Conc.	Stability[b]
	1010.22	1048.30	103.77	1048.09	103.75
	807.34	80.96	100.31	777.75	96.33
	Overnight in autosampler		3 Times Freeze and Thaw Cycle		Long Term (-20°C)
	Conc.[a]	Stability[b]	Conc.[a]	Conc.[a]	Conc.[a]	Stability[b]
	962.17	95.24	1038.68	1038.68	969.98	96.02
	789.95	97.85	875.36	875.36	814.92	100.94

[a]concentration in ng/mL; [b]stability in %

4. CONCLUSION

The quantitative method developed using UHPLC-MS/MS exhibited excellent selectivity and accuracy for the quantitation of plasma homocysteine. The short run time of 2 minutes also suggested that there is an opportunity for high sample throughput. The method is reliable and

reproducible and has fulfilled the validation criteria of EMA, FDA and ICH guidelines. It is suitable for use in clinical settings as the method is high throughput and cost effective.

Declaration of Interest
The authors declare that there is no conflict of interest.

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