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Robust, reliable assay to quantify total homocysteine in human plasma or serum by liquid chromatography-tandem mass spectrometry for use in clinical research

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Application benefits

- Simple sample preparation
- Quantification of total homocysteine by reduction of its various forms into the free form during sample preparation

Goal

Development and implementation of an analytical method for the quantification of total homocysteine in human plasma using a Thermo Scientific™ TSQ Quantis™ triple-stage quadrupole mass spectrometer.

Introduction

Determination of nutritional requirements that can help optimize metabolism for an individual or population poses serious challenges due to the complexity of food macro- and micronutrient composition, inter-subject variability in physiological responses, and environmental and genetic factors. In the last couple of decades, owing to some broad and deep biological phenotyping, much information has been obtained that provides deeper insight into the physiological and pathological processes.

The methionine cycle is one of the critical pathways that contributes towards human health, and dysfunction of this pathway has been linked to cardiovascular disease, mild cognitive decline, vascular dementia, and Alzheimer's disease. In addition, co-factors derived from diet are crucial for proper functioning of the methionine cycle. Monitoring the activity of this pathway in response to nutrition through metabolomics analysis (nutritional metabolomics) would generate a more comprehensive understanding of the interplay between host, environment, and nutrient interactions.

A thorough understanding of the methionine cycle can be obtained by monitoring and quantifying critical homocysteine-methionine cycle biomarkers. In particular, mass spectrometry (MS)-based methods have demonstrated robust, accurate, and precise quantitation of several homocysteine–methionine cycle biomarkers in diverse biological matrices.

In this report, we present development and implementation of an analytical method for clinical research for the quantification of total homocysteine in human plasma. Only a small amount of homocysteine (approx. 1–2%) is present in plasma as free homocysteine (reduced form). The predominant part is bound to proteins, dimerized via disulfide bonds (homocystine), or forms a mixed disulfide bond with cysteine. In the reported approach, the various forms of homocysteine are reduced and transformed into free homocysteine during sample preparation. This involves reduction of analyte and internal standard to free homocysteine followed by protein precipitation and injection onto a Thermo Scientific™ Transcend™ II UHPLC system connected to a TSQ Quantis triple-stage quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. Detection is performed by selected-reaction monitoring (SRM) using d_8-homocystine as the internal standard, which is reduced during sample preparation and, therefore, detected as d_4-homocysteine for quantification. Method performance was evaluated using calibrators and controls from RECIPE® Chemicals + Instruments GmbH (Munich, Germany), to obtain limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision.

Experimental

Sample preparation

Reagents included four calibrators (MS2013 batch #1188) and two controls (MS23080 and MS23081 batch #234) from RECIPE, covering a concentration range of 0.794–6.86 ng/mL. Additional reagents were a d_8-homocystine solution as the internal standard for quantification and a 1 M reduction solution of DL-dithiothreitol (DTT). A sample of 50 µL of plasma was mixed with 20 µL of internal standard solution and 20 µL of reduction solution and incubated at 35 °C for 15 minutes. Then, 200 µL of methanol were added to the reduced sample, followed by vortex-mixing, incubation for 15 minutes at 4 °C and centrifugation for 5 minutes at 10,000 × g. The supernatant was transferred to a clean plate or vial.

Liquid chromatography

Extracted samples were injected onto a Transcend II UHPLC system. LC separation was achieved on a Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2) analytical column 100 × 3.0 mm (3 µm) kept at 30 °C. Mobile phases A and B consisted of water and methanol, respectively, both containing 0.1% formic acid. Details of the analytical method are reported in Table 1. Total runtime was 3.5 minutes.

Table 1. LC method description

Gradient Profile		
Time (min)	**Flow Rate (mL/min)**	**B (%)**
0.00	0.6	5
1.00	0.6	15
1.01	0.6	100
2.00	0.6	100
2.01	0.6	5
3.50	0.6	5
Other Parameters		
Injection volume (µL)		5
Column temperature (°C)		30

Mass spectrometry

Analyte and internal standard were detected by SRM on a TSQ Quantis triple-stage quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. Two SRM transitions for each compound were included in the acquisition method for quantification and confirmation, respectively. Mass spectrometric conditions are reported in Table 2.

Table 2. Mass spectrometric parameters

Parameter	Value
Source type	Heated electrospray ionization (HESI)
Vaporizer temperature	350 °C
Capillary temperature	350 °C
Spray voltage (positive mode)	4000 V (positive mode)
Sheath gas	50 AU
Sweep gas	4 AU
Auxiliary gas	20 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Collision gas pressure	1.5 mTorr
Cycle time	0.300 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration range, carryover, accuracy, and intra- and inter-assay precision. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected just after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at two different levels provided by RECIPE (MS23080 and MS 23081 batch #234), prepared and analyzed in replicates of five on three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

The method proved to be linear in the calibration range covered by the calibrators with a correlation factor (R^2) always above 0.999. A representative chromatogram of both homocysteine and d_4-homocysteine at the lowest calibration level is reported in Figure 1. A representative calibration curve is reported in Figure 2.

No significant carryover was observed, with no signal detected in the blank injected just after the highest calibrator.

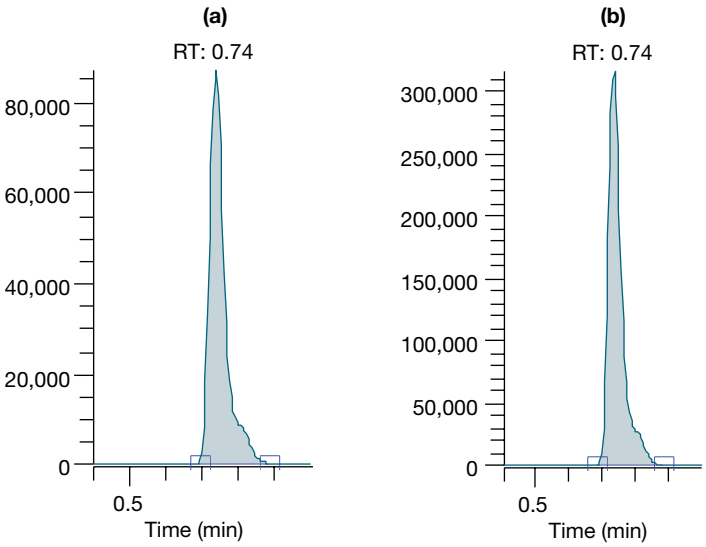

Figure 1. Representative chromatogram for (a) homocysteine and (b) d_4-homocysteine at the lowest calibration level

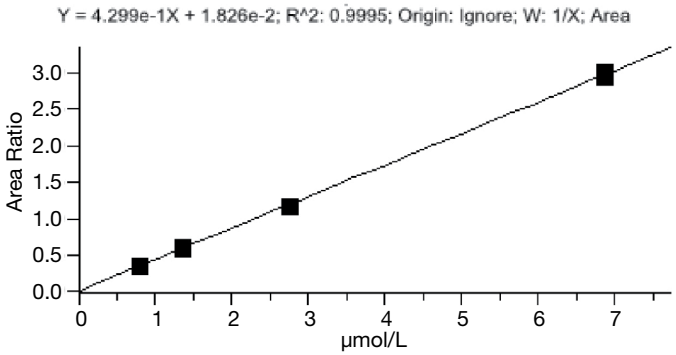

Figure 2. Representative calibration curve for homocysteine (day 3)

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples of -0.3% at both levels. The %CV for intra-assay precision was always below 3.3%. The maximum %CV for inter-assay precision was 2.7%. Results are reported in Table 3.

Table 3. Analytical accuracy and intra- and inter-assay precision results for controls MS23080 and MS23081 batch #234

		MS23080				MS23081			
		Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	CV (%)	Nominal concentration (ng/mL)	Average Calculated concentration (ng/mL	Bias (%)	CV (%)
Accuracy		1.22	1.22	-0.3		3.49	3.48	-0.3	
Intra-assay precision	Day 1		1.21		3.3		3.47		2.2
	Day 2		1.20		2.7		3.48		2.3
	Day 3		1.23		2.3		3.48		1.1
Inter-assay precision			1.21		2.7		3.48		1.8

Conclusions

A robust, reproducible, sensitive and easy-to-implement liquid chromatography-tandem mass spectrometry method for clinical research for the quantification of total homocysteine in human plasma was developed and implemented on a Transcend II UHPLC system coupled to a TSQ Quantis triple-stage mass spectrometer. The calibrators and controls were obtained from RECIPE, and a simple and robust sample extraction procedure was used for upfront purification of the samples. The data obtained with the described method successfully met sensitivity, reliability, accuracy, and precision expectations typically demanded by clinical research laboratories.

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