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Quantification of 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

Implementation of an analytical method for the quantification of total homocysteine in human plasma or serum on a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer.

Introduction

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

physiological and pathological processes. The methionine cycle is among the many central pathways that contribute 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 (i.e., nutritional metabolomics) would generate a more comprehensive understanding of the interplay between host, environment, and nutrient interactions. In particular, mass spectrometry (MS)-based methods have demonstrated robust, accurate, and precise quantitation of several homocysteine–methionine cycle biomarkers in diverse biological matrices. However, no single and high-throughput method currently exists to monitor both metabolites and co-factors in the methionine pathway.

An analytical method for clinical research for the quantification of total homocysteine in human plasma or serum is reported. Only a small amount of homocysteine (approximately 1–2%) is present in plasma or serum as free homocysteine (reduced form). The predominant part is bound to proteins, dimerized via disulfide bonds (homocystine), or forms a mixed disulfide 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 system connected to a TSQ Quantis triple 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 the MS2000 ClinMass® Complete Kit Homocysteine in Plasma / Serum 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 and two controls from RECIPE, covering a concentration range of 0.794 to 6.86 ng/mL. Additional reagents were a d_8-homocystine solution as the internal standard for quantification and a solution to reduce analyte and internal standard into the corresponding free forms. A sample of 50 µL of plasma or serum was mixed with 50 µL of reduction solution and 50 µL of internal standard solution and incubated at room temperature for 5 minutes. Then, 200 µL of the precipitation solution were added to the reduced sample, followed by vortex-mixing, incubation for 5 minutes at 4 °C and centrifugation for 5 min at 10,000 x g. The supernatant was transferred to a clean plate or vial.

Liquid chromatography

Liquid chromatography was achieved by isocratic elution at 0.55 mL/min using the mobile phase and analytical column provided by RECIPE. Total runtime was 1.2 minutes. Injection volume was 5 µL.

Mass spectrometry

Analyte and internal standard were detected by SRM on a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively. Mass spectrometric conditions are reported in Table 1.

Table 1. Mass spectrometric parameters

Parameter	Value
Source type	Heated electrospray ionization (HESI)
Vaporizer temperature	350 °C
Capillary temperature	350 °C
Spray voltage	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	0.350 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 the percentage ratio between the 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 MS23081 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 for either analyte, with no signal detected in the blank injected just after the highest calibrator.

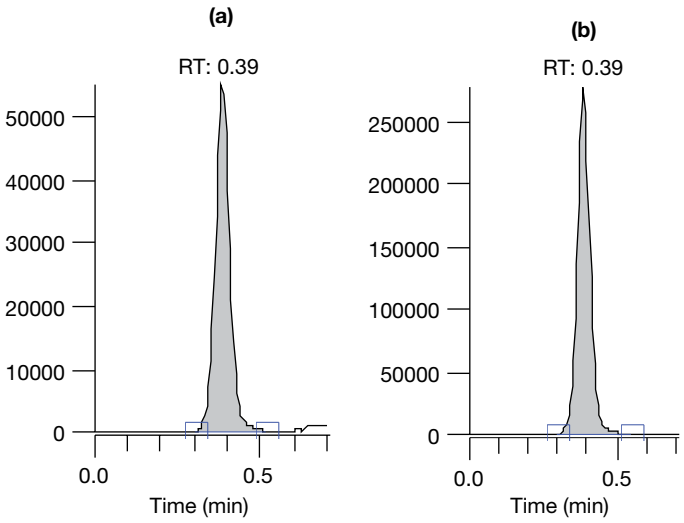

Figure 1. Representative chromatogram for (a) homocysteine and (b) d_4-homocysteine at the lowest calibration level

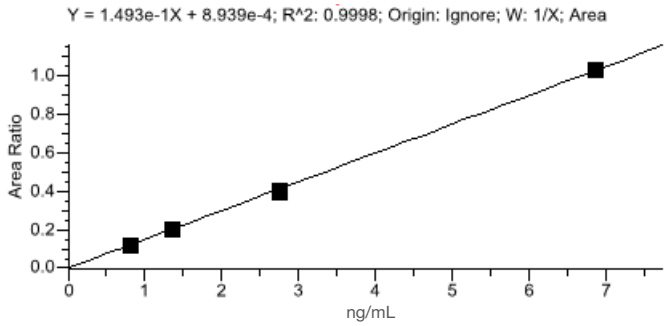

Figure 2. Representative calibration curve for homocysteine (day 3)

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -2.2% and -1.7%. The %CV for intra-assay precision was always below 2.1%. The maximum %CV for inter-assay precision was 2.0%. Results are reported in Table 2.

Table 2. 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	1.20	-1.7		3.49	3.41	-2.2	
	Day 1		1.16		0.8		3.39		1.4
Intra-assay precision	Day 2		1.19		2.1		3.38		1.1
	Day 3		1.19		1.9		3.46		0.9
Inter-assay precision			1.18		2.0		3.41		1.6

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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 or serum using the MS2000 ClinMass Complete Kit Homocysteine in Plasma / Serum from RECIPE, was implemented and analytically evaluated on a Transcend II system connected to a TSQ Quantis triple quadrupole mass spectrometer. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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