TECHNICAL NOTE 66048

Quantification of total homocysteine in human plasma or serum by LC-HRAM-MS for clinical research

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Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass® kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- 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™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

Introduction

Determination of nutritional requirements that can help optimize metabolism for an individual or population poses some 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, much information has been obtained from broad and deep phenotyping 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. Mass spectrometry (MS)-based methods have

demonstrated robust, accurate, and precise quantitation of several homocysteine–methionine cycle biomarkers in diverse biological matrices.

A robust and accurate LC-MS analytical clinical research method for the quantification of total homocysteine in human plasma or serum is reported. Only a small amount of homocysteine (approx. 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. Sample preparation involved a reduction step to free homocysteine from the various forms followed by protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization (HESI-II). Detection was performed by Parallel Reaction Monitoring (PRM) acquisition mode. Method performance was evaluated using the MS2000 ClinMass Complete Kit Homocysteine in Plasma / Serum from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration range, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurements, and intra- and inter-assay precision.

Experimental

Target analytes

D_8-homocystine (reduced to d_4-homocysteine during sample preparation) was used as the internal standard.

Sample preparation

Reagents included four calibrators (MS2013 batch #1188) and two controls (23080 and 23081 batch #2360) from RECIPE, covering a concentration range between 0.837 and 6.85 µg/mL. 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 minutes at 10,000 × g. The supernatant was transferred to a clean plate or vial.

Liquid chromatography

A Vanquish Duo UHPLC system with binary Vanquish Flex pumps, a dual-channel instrument configured for both LC-only and online SPE applications (Figure 1), was used for chromatographic separation. The LC-only channel was used in this case for an isocratic elution at 0.7 mL/min utilizing mobile phase and analytical column (kept at 25 °C) provided by RECIPE. The injection volume was 5 µL. Total runtime was just 1.0 minute.

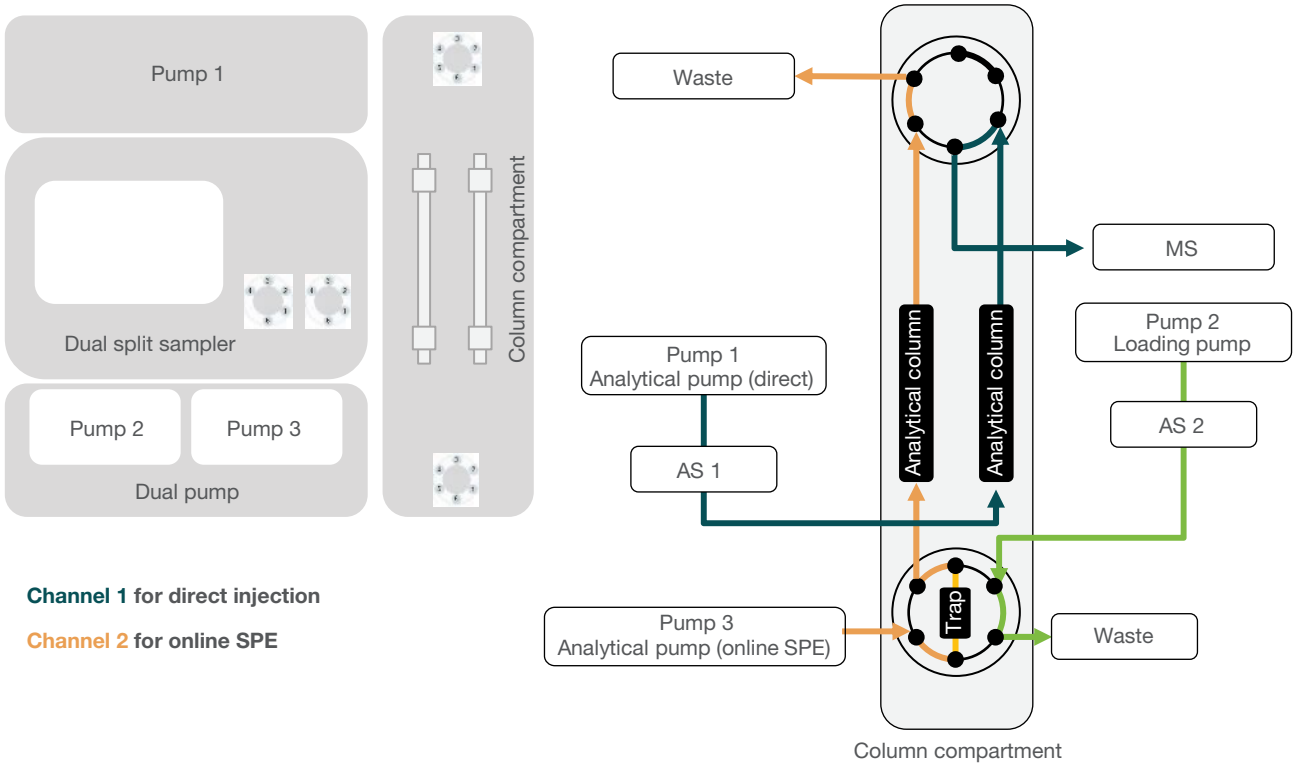

Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup

Mass spectrometry

Analyte and internal standard were detected by PRM acquisition mode using a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer equipped with a HESI-II source operated in positive ionization mode. A summary of the MS settings is reported in Table 1.

Table 1. MS settings

Ion source parameters	
Source type	Heated Electrospray Source Ionization (HESI-II)
Spray voltage - Positive (V)	3,500
Sheath gas (Arb)	50
Aux gas (Arb)	15
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	350
Vaporizer temp. (°C)	450
S-lens RF level	60
PRM scan settings	
Orbitrap resolution (@ *m/z* 200)	17,500
Isolation window (*m/z*)	4.0
AGC target	1e6
Maximum IT (ms)	100
Normalized stepped collision energy	20, 30, 40

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration range, LLOQ, carryover, accuracy, trueness of measurements, and intra- and inter-assay precision.

The lowest calibrator was diluted 20-fold with water to determine the LLOQ; a full set of calibrators (four levels), diluted calibrators (four levels), and controls (two levels) were extracted in replicates of five (n=5), injected in a single batch and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a percentage coefficient of variation (%CV) below 20% across the entire batch of samples.

Carryover was estimated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it.

Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations at two different levels using the quality control samples provided by RECIPE and prepared and analyzed in replicates of five on three different days.

Trueness of measurements was also evaluated as the percentage bias using a certified external quality control from NIST (SRM 1950) prepared and analyzed in replicates of five on a single day.

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™ 5.1 software.

Results and discussion

A linear response with equal weighting was obtained not only in the calibration range covered by the calibrators, but also down to a LLOQ of 0.419 ng/mL The percentage bias between nominal and back-calculated concentration was always within ±15% for all the calibrators (±20% for the lowest calibrator) in all the runs. Representative chromatograms at the LLOQ for both the analyte and its internal standard are depicted in Figure 2. A representative calibration curve in the concentration range covered by the kit is shown in Figure 3.

No carryover was registered, with no peak detected in the blank sample injected just after the highest calibrator.

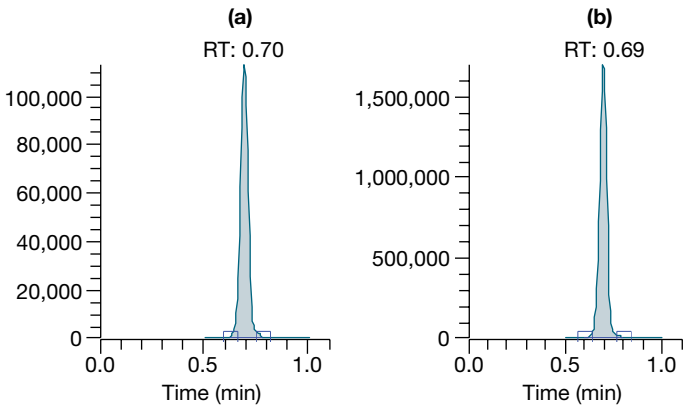

Figure 2. Representative chromatograms of the LLOQ for (a) homocysteine and (b) d_4-homocysteine

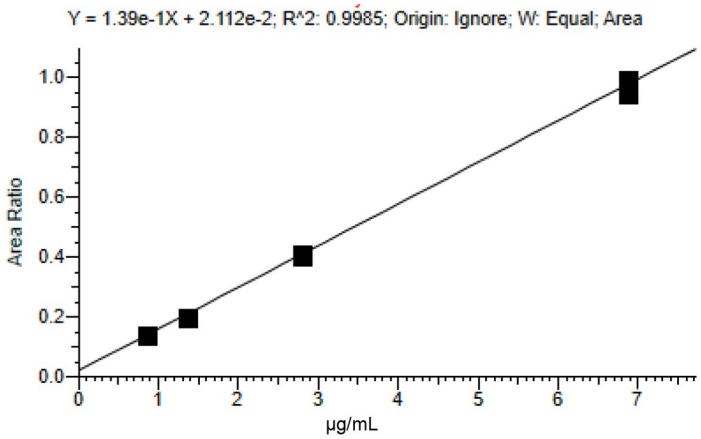

Figure 3. Representative calibration curve

An outstanding accuracy was obtained for this method on both the internal and external control samples, with the percentage bias between nominal and average back-calculated concentration ranging between 4.1% and 5.3% (Table 2).

The method also proved to be extremely reproducible, with a maximum %CV of 2.6% and 2.5% for intra-and inter-assay precision, respectively (Table 3).

Table 2. Analytical accuracy results for controls 23080 and 23081 batch #2360 and NIST SRM 1950

Control sample	Nominal conc. (µg/mL)	Average conc. (µg/mL)	Bias (%)
23080 batch #2360	1.56	1.63	4.7
23081 batch #2360	3.69	3.89	5.3
NIST SRM 1950	1.15	1.20	4.1

Conclusions

An LC-HRAM-MS-based method for reliable and accurate quantification of total homocysteine in human plasma or serum is reported here. The power of Orbitrap technology in performing accurate and efficient qualitative analyses and quantitation in an applied environment is demonstrated. The ClinMass Complete Kit Homocysteine in Plasma / Serum from RECIPE was used, enabling easy offline sample preparation with a reduction step followed by protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

Table 3. Intra- and inter-assay precision results for controls 23080 and 23081 batch #2360

Control sample	Intra-assay						Inter-assay	
	Day 1		Day 2		Day 3			
	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)
23080 batch #2360	1.66	1.7	1.63	2.6	1.61	2.3	1.63	2.5
23081 batch #2360	3.89	1.5	3.89	0.9	3.87	0.6	3.89	1.0

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