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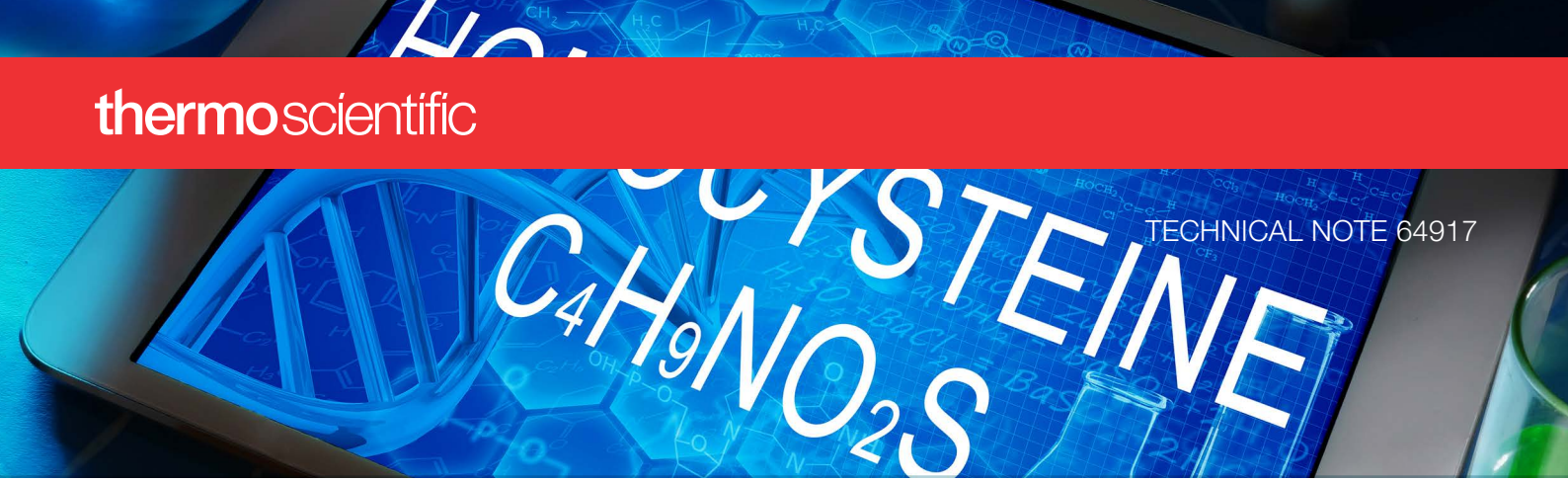

Quantification of total homocysteine in human plasma or serum by liquid chromatography tandem mass spectrometry for clinical research

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Keywords
Total homocysteine, plasma, serum, mass spectrometry

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 Endura™ triple quadrupole mass spectrometer.

Introduction

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 (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. 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 TLX-1 system coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. Detection is performed by selected-reaction

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monitoring (SRM) using d8-homocystine as the internal standard, which is reduced during sample preparation and therefore detected as homocysteine-d4 for quantification. Method performance was evaluated using the MS2000 ClinMass® Complete Kit Homocysteine in Plasma / Serum from RECIPE®, to obtain limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision.

Experimental

Target analytes

- Homocysteine
- d4-homocysteine

Sample preparation

Reagents included four calibrators and two controls from RECIPE covering a concentration range of 5.87–50.7 µmol/L. Additional reagents were a d8-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 × g. The supernatant was transferred to a clean plate or vial.

Liquid chromatography

Chromatographic separation was achieved by isocratic elution using the mobile phase and analytical column provided by RECIPE; details of the analytical method are reported in Figure 1. Total runtime was 70 seconds.

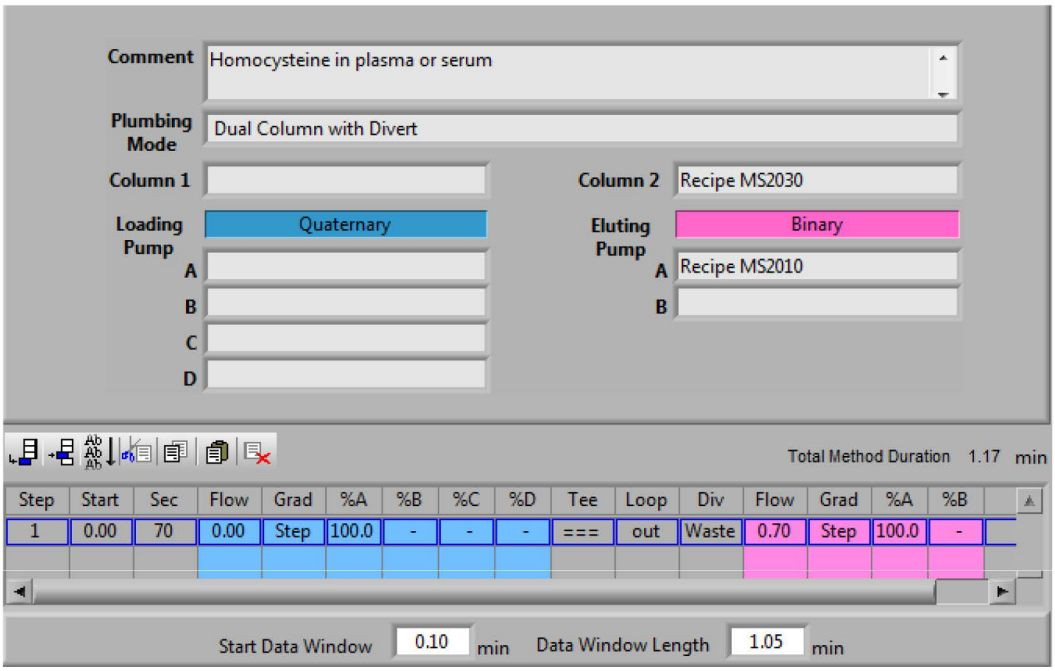

Figure 1. Method description for the Transcend II TLX-1 system.

Mass spectrometry

Homocysteine and its internal standard were detected on a TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. Two SRM transitions for both homocysteine and its internal standard were included in the acquisition method for quantification and confirmation, respectively, as reported in Table 1. Mass spectrometric conditions are reported in Table 2.

Table 1. SRM transitions.

Analyte	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)
Homocysteine	136.2	90.3	35
		56.2	24
d4-homocysteine	140.3	94.2	24
		60.3	20

Table 2. Mass spectrometric parameters.

Parameter	*Value*
Source Type:	Heated electrospray ionization (HESI) in positive mode
Vaporizer Temp:	440 °C
Capillary Temp:	275 °C
Spray Voltage:	3500 V
Sheath Gas:	54 AU
Sweep Gas:	2 AU
Auxiliary Gas:	17 AU
Data Acquisition Mode:	Selected reaction monitoring (SRM)
Chrom Filter Peak Width:	3.0 s
Collision Gas Pressure:	0.5 mTorr
Cycle Time:	0.200 s
Q1 (FWMH):	0.7
Q3 (FWMH):	0.7

Method evaluation

The method performance was evaluated by obtaining limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentration using the Standard Reference Material® #1955 from NIST at three different levels prepared and analyzed on five different days in a single run each day. Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the controls from RECIPE at two different levels in replicates of eight (n=8), prepared and analyzed in one batch. Inter-assay precision was evaluated on the same controls in replicates of three (n=3) prepared and analyzed on five different days.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 3.3 software.

Results and discussion

The method proved to be linear not only in the calibration ranges covered by the calibrators but also on a wider range obtained by diluting the lowest calibrator up to 50-fold. The reported method covered a concentration range between 0.117 and 50.7 µmol/L with a linear response and a correlation factor (R^2) of 0.991. A representative chromatogram of both homocysteine and d4-homocysteine at the lowest calibration level is reported in Figure 2. A representative calibration curve is reported in Figure 3.

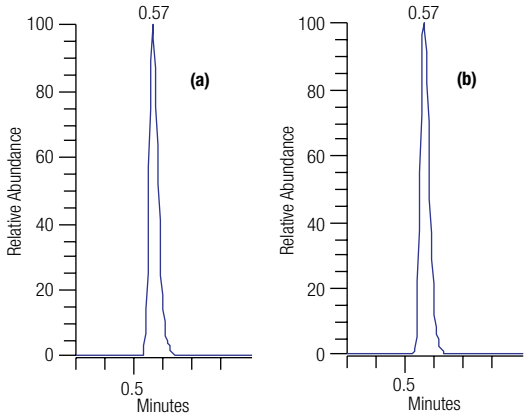

Figure 2. Representative chromatogram for (a) homocysteine and (b) d4-homocysteine at the lowest calibration level.

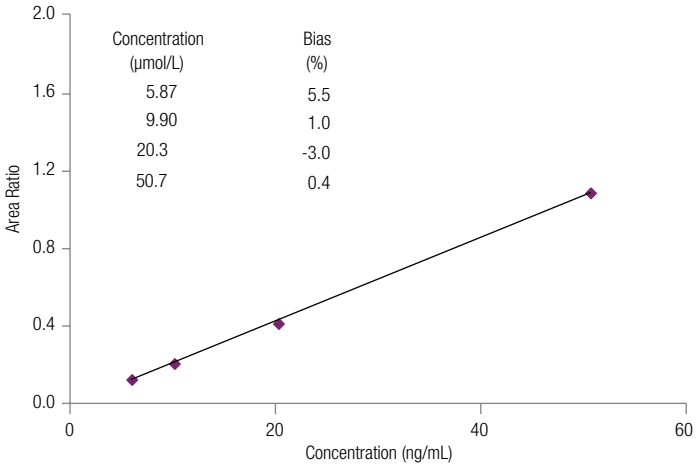

Figure 3. Representative calibration curve for homocysteine.

Analytical accuracy was exceptional, with the percentage bias between nominal and average back-calculated concentration for the used control samples always between 3.7% and 5.2% at all levels (Table 3).

Table 3. Analytical accuracy results.

Control	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)
Level I	3.98	4.15	4.3
Level II	8.85	9.31	5.2
Level III	17.7	18.4	3.7

A maximum %CV of 2.0% and 3.3% was obtained for intra- and inter-assay precision, respectively (Table 4).

Table 4. Intra- and inter-assay precision results.

Analyte	n	MS23080 #234		MS23081 #234	
		Average Concentration (ng/mL)	CV (%)	Average Concentration (ng/mL)	CV (%)
Intra-assay precision	8	9.07	2.0	26.5	1.4
Inter-assay precision	15	9.25	3.1	26.5	3.3

Conclusions

A 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 validated on a Transcend II TLX-1 system coupled to a TSQ Endura triple quadrupole mass spectrometer. The method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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