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POSTER NOTE

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Quantification of Total Homocysteine in Human Plasma or Serum by Liquid Chromatography Tandem Mass Spectrometry

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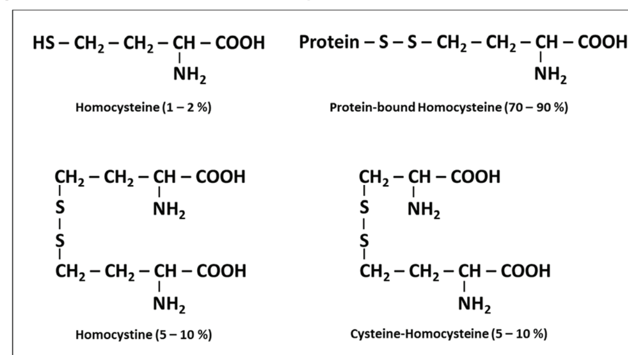
ABSTRACT

An analytical method for clinical research for the quantification of total homocysteine in human plasma or serum is reported. The method involves a fast and simple sample preparation followed by injection onto a Thermo Scientific™ Transcend™ II system. Mass spectrometric detection is performed by single reaction monitoring on a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer using heated electrospray ionization in positive mode. The method was analytically validated using the MS2000 ClinMass® Complete Kit Homocysteine in Plasma / Serum from RECIPE and limit of quantification, linearity range, accuracy and intra-day and inter-assay precision were evaluated.

Figure 1. Forms of Homocysteine



Different forms of homocysteine and their prevalence in circulating blood

INTRODUCTION

Homocysteine is a sulfurous α-amino acid of the methionine metabolism. Hyperhomocysteinemia, a medical condition in which an enhanced blood homocysteine concentration (i.e. > 10 µmol/l [1]) is present, is considered as an independent risk factor for degenerative vascular diseases.

As reported in Figure 1, only a small amount of homocysteine (approx. 1 - 2 %) is present as free homocysteine in a reduced form. The predominant part is bound to proteins, dimerised via disulfide bonds (homocystine) or forms a mixed disulfide with cysteine [1]. Therefore, total homocysteine (tHcys) is determined in order to evaluate the homocysteine level. For this, the various forms of homocysteine are reduced and transformed into free homocysteine during sample preparation.

Here we report a simple, rapid, and economical analytical method for the quantification of the total homocysteine (tHcys) in plasma or serum. The method, developed on a TSQ Endura triple quadrupole mass spectrometer equipped with a heated electrospray ionization source working in positive mode, has been analytically validated using the MS2000 ClinMass® Complete Kit Homocysteine in Plasma / Serum from RECIPE.

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MATERIALS AND METHODS

This analytical method for clinical research is based on the MS2000 ClinMass® Complete Kit Homocysteine in Plasma / Serum from RECIPE and allows for the quantification of homocysteine in human plasma or serum. The kit includes calibrators and controls at four and two different levels, respectively, covering a concentration range between 5.87 and 50.7 μmol/L.

In this analytical method homocysteine (total homocysteine) is determined by HPLC with tandem mass spectrometry (LC-MS/MS) after a sample clean-up, performed by a simple reduction and protein precipitation step.

SAMPLE PREPARATION

Prior to the LC-MS/MS analysis a sample preparation is performed. The samples are spiked with the internal standard, the bound homocysteine is released (reduced) and the sample matrix removed.
In details, 50 µL of Internal Standard and 50 µL of a reduction Reagent are added to 50 µL of the sample (calibrator, control, patient), mixed for 30 sec and then incubated for 5 min at room temperature.
Homocystine-d8 is used as the internal standard, but it is reduced during sample preparation and therefore is detected as homocysteine-d4 for the quantification.
Then 200 µL of a precipitation Reagent B is added to the reduced sample, mixed for 30 sec on a vortex mixer and incubate for 5 min at 4 ° C and finally centrifuged for 5 min at 10000 x g.
One microliter (1µL) of the supernatant is injected into the LC-MS/MS system.

HPLC-MS/MS

The chromatographic separation is performed by isocratic elution on a Transcend II system operated in UHPLC mode using mobile phases and analytical column provided with the kit. Detection was performed by SRM on a TSQ Endura triple quadrupole mass spectrometer with a heated electrospray ionization operated in positive mode. Two SRM transitions were included in the acquisition method for quantification and confirmation, respectively.
The MS conditions used and the mass transitions for the analyte and the internal standard are reported in Table 1 and Table 2, respectively.

Table 1. MS conditions

Source type	Heated electrospray ionization (HESI) in positive mode
Vaporizer temp	440 ° C
Ion Transfer Tube 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 (FWHM)	0.7
Q3 (FWHM)	0.7

Source conditions and scan parameters

Table 2. SRM transitions for Homocysteine and Internal Standard

Substance	Precursor (m/z)	Product (m/z)	Collision Energy (V)
Homocysteine (Quantifier)	136.18	90.25	24
Homocysteine (Qualifier)	136.18	56.15	34
Homocysteine-d4 (Quantifier)	140.25	94.23	20
Homocysteine-d4 (Qualifier)	140.25	60.25	24

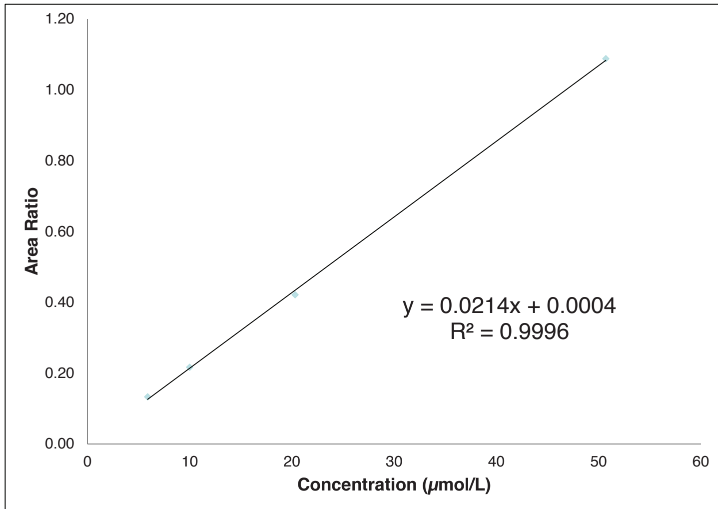
Two transitions (one Quantifier and one Qualifier) have been selected for homocysteine and for the Internal Standard, homocysteine-d4

RESULTS

A full analytical validation was performed; the assay proved to be linear in the calibration range covered by the kit, with a limit of quantification (LOQ) of 0.117 μmol/L, an upper limit (ULOQ) of 50.70 μmol/L and a correlation factor (R^2) above 0.999.
For the linearity test 5 samples of each level were prepared and analyzed in one batch and for sensitivity test 5 samples of each dilution were prepared and analyzed in one batch.

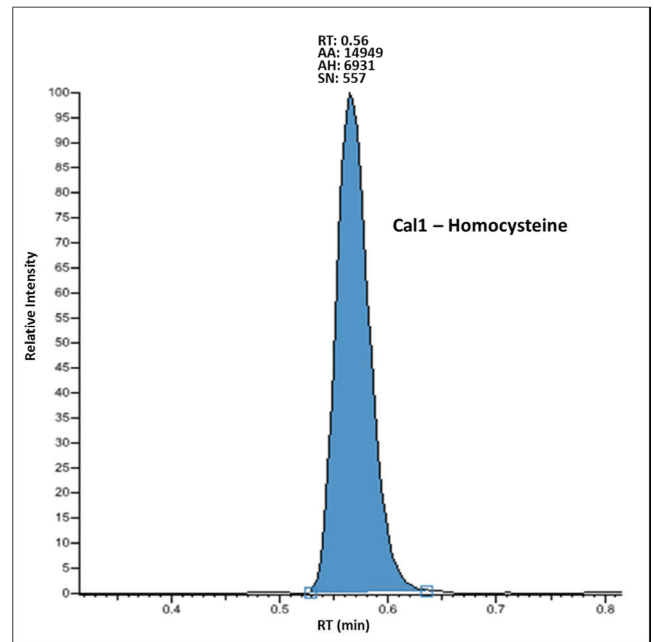
Calibration curve and results on linearity range test and on the analytical sensitivity of the assay are reported in Figure 2, Table 3 and Table 4, respectively.

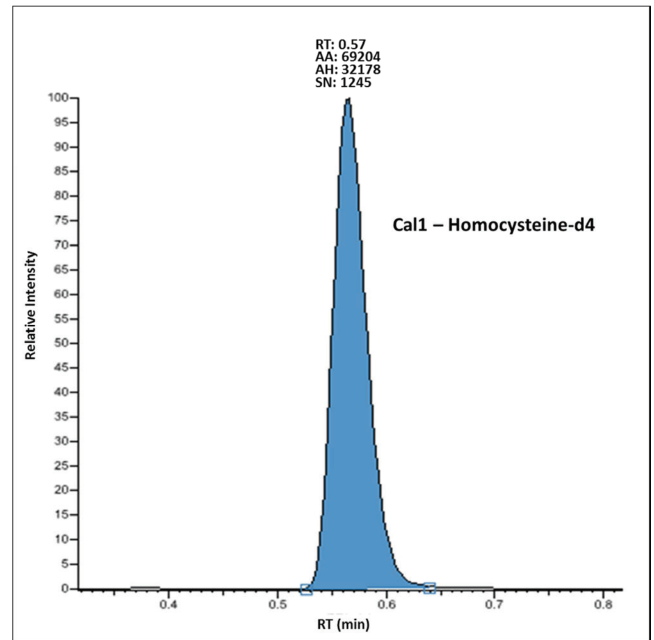
Figure 2. Calibration curve for homocysteine



Matrix matched calibration curve for homocysteine in serum obtained analyzing serum Calibrators and using homocysteine-d4 as Internal Standard

Figure 3. Representative Chromatograms for homocysteine and homocysteine-d4





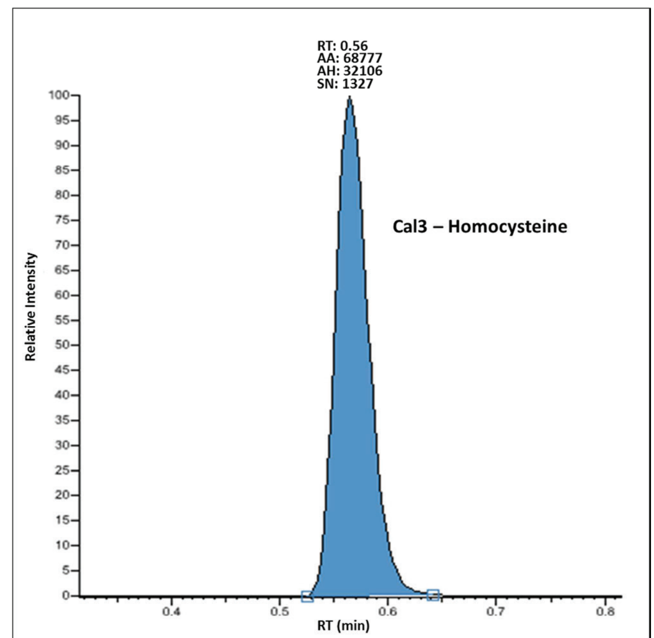


Table 3. Linearity range of the method

Analyte	R^2	Linearity Range (µmol/L)	ULOQ (µmol/L)
Homocysteine	0.9991	0.117 - 50.70	50.70

Coefficient of determination (R^2), Linearity range and Upper Limit of Quantification (ULOQ) for the matrix matched calibration curve obtained for homocysteine in serum

Table 4. Sensitivity of the method

Analyte	Target value (µmol/L)	Mean value LLOQ (µmol/L)	CV	Bias	LOD (µmol/L)
Homocysteine	0.117	0.117	4.28%	-0.29%	0.0389

LLOQ was set to the lowest level that could be determined with a CV < 20%. LOD is considered 1/3 of the LLOQ value.

Chromatographic peaks for homocysteine and homocysteine-d4 at two different level of Serum Calibrators.
Homocysteine concentration in Cal1: 9.99 μmol/L
Homocysteine concentration in Cal 3: 50.7 μmol/L

Accuracy for the assay was evaluated in terms of trueness of measurement using Standard Reference Material® 1955 from NIST prepared and analyzed on 5 different days in single runs each day; the percentage bias between nominal and average back-calculated concentration for these control samples are reported in Table 5.

Table 5. Results of Accuracy test

Sample	n	Target value (μmol/L)	Measured value (μmol/L)	CV	Bias
Level I	5	3.98	4.15	4.59%	4.27%
Level II	5	8.85	9.31	3.20%	5.24%
Level III	5	17.7	18.4	2.89%	3.70%

Accuracy expressed in terms of trueness of measurement for samples Standard Reference Material® 1955 from NIST at 3 Levels of concentration, prepared and analyzed singly in five different days

Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the kit controls at both levels in replicates of eight (n=8) analysed 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.
The %CV for intra-assay precision and for inter-assay precision for the lower and upper control are reported in Table 6.

Table 6. Precision test results

Test	n	QC1 value (μmol/L)	QC1 CV	QC2 value (μmol/L)	QC2 CV
Intraday precision	8	9.07	1.96%	26.5	1.44%
Interassay precision	15	9.25	3.11%	26.5	3.33%

Repeatability (intraday precision) and Reproducibility (interassay precision) of the method. For the intraday precison test 8 samples of each level were prepared and analysed in one batch and for the interassay precision 3 samples of each level were prepared and analysed on 5 different days

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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 system connected to a TSQ Endura triple quadrupole mass spectrometer.
This analytical method meets research laboratory requirements, sensitivity and linearity of response covering analyte concentration ranges provided by the kit.

REFERENCES

1. W. Herrmann, R. Obeid: Homocystein. In: L. Thomas (Hrsg), Labor und Diagnose: Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik, 8. Auflage, TH-Books Verlagsgesellschaft, Frankfurt/Main 2012, p. 705-711.

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