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Aqueous normal phase chromatography improves quantification and qualification of homocysteine, cysteine and methionine by liquid chromatography–tandem mass spectrometry

Christian Hellmuth, Berthold Koletzko*,[1], Wolfgang Peissner[1]

Division of Metabolism and Nutritional Medicine, Dr. von Hauner Children's Hospital, Ludwig-Maximilians-University of Munich, Lindwurmstr. 4, D-80337 Munich, Germany

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ABSTRACT

Elevation of plasma homocysteine concentration is recognized as an independent predictor of cardiovascular disease risk. Therefore, quantification of homocysteine and related sulphur amino acids cysteine and methionine from plasma samples is routinely performed in clinical laboratories. Due to the highly hydrophilic character of these amino acids, previously reported LC–MS methods often suffered from very short chromatographic retention resulting in inadequate separation from matrix background and possible co-eluents. In the present method, aqueous normal phase (ANP) chromatography was introduced to improve chromatographic separation for liquid chromatography–electrospray ionization tandem mass spectrometry. Selective qualification of analytes and internal standards was achieved by qualifier ion monitoring. Using this enhanced selectivity, spurious co-eluents were identified and separated from the analyte signal by optimization of chromatographic conditions. Method validation proved high precision and accuracy (intra-assay reproducibility 1.2–4.3% CV, inter-assay reproducibility 3.4–6.1% CV, accuracy 91.3–105.9%). Total cycle time of 7 min and low costs per sample allow high-throughput application in clinical diagnostics and research trials.

1. Introduction

Aqueous normal phase (ANP) chromatography represents an important new technology for the separation of endogenous metabolites in biological matrices. It enables high-resolution separations of hydrophilic compounds which are not adequately retained and separated on conventionally used stationary phases in reversed-phase operation mode [1–3]. In contrast to hydrophilic interaction chromatography (HILIC) and ion-pair reversed phase chromatography, which have also been proposed for the separation of hydrophilic molecules [1], ANP chromatography relies neither on the use of higher buffer concentrations nor of ion-pair reagents. Eluent systems suitable for ANP chromatography utilize acetonitrile (or acetone) as the weak and water as the strong elution solvent, including minor amounts of volatile buffers or acids as modifiers. The high content of organic solvent in the eluent system is both favorable for compatibility with mass spectrometry detection and also enables straightforward sample preparation protocols using organic solvent protein precipitation without subsequent solvent evaporation [1–5].

The ANP chromatography method presented here is useful for accurate und selective quantitation of the hydrophilic sulphur amino acids homocysteine (Hcy), cysteine (Cys) and methionine (Met). Several observational studies have confirmed that elevated plasma Hcy concentration might be an independent predictor of cardiovascular disease risk [6–8]. There is considerable interest in quantitative methods for the measurement of plasma Hcy both for clinical diagnosis and for epidemiological research. The biochemistry of Hcy is closely linked to Met and Cys. The essential amino acid Met is first activated to S-adenosylmethionine, a universal methyl donor for transmethylation reactions, including synthesis of neurotransmitters and methylation of DNA and RNA [6,9]. After donating its methyl group, S-adenosylmethionine is converted to S-adenosylhomocysteine, which is subsequently hydrolyzed to Hcy and adenosine. Hcy is remethylated to Met or irreversibly converted to Cys by transsulfuration [10]. Hcy can also be converted to homocysteine-thiolactone by methionyl-tRNAse in an error-editing reaction [11] or S-nitroso-homocysteine [12]. Hyperhomocysteinemia has also been related to nutritional deficiency (folate or cobalamin) [13], disease (diabetes) or genetic factors [9,14]. In addition to increased cardiovascular disease risk [6,7], hyperhomocysteinemia is also a risk factor for stroke, tumor [15], Alzheimer's disease and dementia [16].

Interest in Hcy as a diagnostic and predictive biomarker has furthered development of several quantitative analytical methods.

* Corresponding author. Tel.: +49 89 5160 3967; fax: +49 89 5160 7742.
E-mail address: office.koletzko@med.uni-muenchen.de (B. Koletzko).
[1] Contributed equally.

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Hcy can be assessed with expensive enzyme- and immunoassays [17]. Various high performance liquid chromatography (HPLC) methods were developed using ultraviolet, fluorescence, or electrochemical detection [18]. Gas chromatography–mass spectrometry methods [19,20] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) were established for the analysis of Hcy or its metabolites, including Hcy-thiolactone [12] and N-homocysteinylation at albumin lysine-525 [21]. LC–MS/MS methods afford several advantages including high specificity by multiple reaction monitoring (MRM), elevated sensitivity and decreased run time. Methods for LC–MS/MS analysis of total homocysteine (tHcy) were published first by Magera in 1999 [22] and subsequently by others [23,24]. More recently, tHcy was measured together with related metabolites including total Cysteine (tCys) [25,26], Met [25] and methylmalonic acid [27]. Previously published LC–MS/MS methods achieved cycle times of about 3–4 min by using C_{18}-, C_8- or CN-columns [23,24,28]. Common to these proposed methods is that very short cycle time was achieved by sacrificing adequate chromatographic retention and separation of analytes, which eluted almost immediately after the column void volume. In LC–MS/MS analysis however, inadequate separation from complex biological matrices may result in ion-suppression effects and spurious signals due to co-eluting isobaric components [29].

The reported application utilizes ANP chromatography to optimize separation of the hydrophilic amino acids Hcy, Cys and Met. Occurrence of co-elution bias was systematically assessed and excluded using qualifier ion monitoring.

2. Experimental

2.1. Chemicals and reagents

Methionine and dithiothreitol were supplied from Sigma (Taufkirchen, Germany). Acetonitrile, water, water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid (all LC–MS grade) were purchased from J.T.Baker (Griesheim, Germany). Homocysteine, cysteine and formic acid (LC–MS grade) were supplied by Fluka (Taufkirchen, Germany). Plasma controls (ClinChek® Plasma Control, Lyophilized, for Amino Acids, Levels I and II; ClinChek® Plasma Control, Lyophilized, for Homocysteine, Levels I and II, ClinCal® Calibrator for Hcy) and ClinMass® d8-Homocystine were obtained from Recipe (Munich, Germany).

2.2. ESI-MS/MS operating conditions

A hybride triple quadrupole mass spectrometer (4000 QTRAP®, AB Sciex, Darmstadt, Germany) with a TurboIon source operating in positive electrospray ionization mode (+ESI) was used. System operation and data acquisition were controlled using Analyst™ software 1.5 (AB Sciex, Darmstadt, Germany). Multiple reaction monitoring (MRM) mode was utilized. Ion spray voltage was set to 5.5 kV and source temperature (TEM) to 700 °C. Collision activated dissociation gas (CAD) was set at 4 psi and nitrogen was used as collision gas. Nebulizer gas (GS1) was set to 80 psi just like auxiliary gas (GS2). Curtain gas (CUR) was set to 50 psi. Compound dependent operating parameters (declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)) were optimized by injecting pure solutions of Hcy, Cys and Met into the ion source with a syringe pump in manual tuning mode. Full product spectra with varying collision energy were recorded and the three highest intensity fragments were used for quantification and qualification (see Section 3.1 and Table 1).

Table 1
Instrument settings. MRM transitions and optimized ion path parameters of analytes and internal standard for the ABSciex 4000Qtrap. Qualifier ions are marked with an asterisk (*).

	MRM transition	DP [V]	EP [V]	CE [V]	CXP [V]
Hcy_01	136.0/90.0	41	10	17	6
Hcy_02*	136.0/56.1	41	10	27	10
Hcy_03*	136.0/118.1	41	10	11	8
Hcy-d4_01	140.1/94.0	41	10	17	6
Hcy-d4_02*	140.1/59.1	41	10	27	10
Hcy-d4_03*	140.1/122.0	41	10	11	8
Met_01	150.1/104.0	41	10	15	6
Met_02*	150.1/133.0	41	10	15	8
Met_03*	150.1/61.0	41	10	31	10
Cys_01	122.0/59.0	41	10	33	8
Cys_02*	122.0/76.0	41	10	20	8
Cys_03*	122.0/105.0	41	10	14	8

2.3. LC–ESI-MS/MS analysis

An Agilent 1200 SL series HPLC system (Waldbronn, Germany) was used, combining a G1379B degasser, a G1312B binary pump, a G1367C autosampler and a G1316B column oven. A silica hydride-based stationary phase (Cogent Diamond Hydride, 15.0 cm × 2.1 mm, 4 μm particle size, MicroSolv Technology Corporation, Eatontown, USA) was used with a Phenomenex® Luna HILIC SecurityGuard® pre-column (4.0 mm × 2.0 mm, Phenomenex®, Aschaffenburg, Germany). Autosampler injection volume was set to 2 μl, eluent flow rate to 500 μl/min. After 3 min of equilibration time, the gradient of the mobile phase started with 10% eluent A (water with 0.1% formic acid) and 90% of eluent B (acetonitrile with 0.1% formic acid), linearly increasing to 70% of eluent A at the end of the run after 4 min.

The HPLC system was coupled via a 2-position Valco switching valve (VICI AG International, Schenkon, Switzerland) to a 4000 QTrap mass spectrometer operating in triple quadrupole mode with settings detailed in Section 2.2. Analytes and internal standard (IS), d_4-homocysteine (d_4-Hcy), were detected with MRM mass transitions shown in Table 1.

2.4. Collection and handling of plasma samples

For method development and evaluation, 227 plasma samples from volunteers examined for their folic acid status were used. The samples were collected into Li-Heparin containing tubes, centrifuged and the plasma stored at −80 °C. The vials were unfrozen at room temperature and sample preparation was started immediately after thawing. Additional plasma samples of two in-house volunteers were collected in the same way and used as quality control plasma for the assessment of inter-batch reproducibility. Lyophilized RECIPE® control plasma samples were dissolved in 3.0 ml water according to the manufacturer's instructions.

2.5. Preparation of aqueous standards

Aqueous calibration samples were freshly prepared before sample preparation. The stock solution contained 9.72 mM Hcy, 9.96 mM Met, 39.7 mM Cys and dithiothreitol (77 mg/ml) in water. The stock solution was diluted with water to yield a series of 10 standard samples with approximate ranges of 0.1–100 μM (Hcy, Met) or 0.4–400 μM (Cys), covering the range of physiological concentrations.

2.6. Sample preparation

Sample preparation was done by a liquid handler GILSON® GX-271 (Middleton, WI, USA). 20 μl of plasma or aqueous calibration

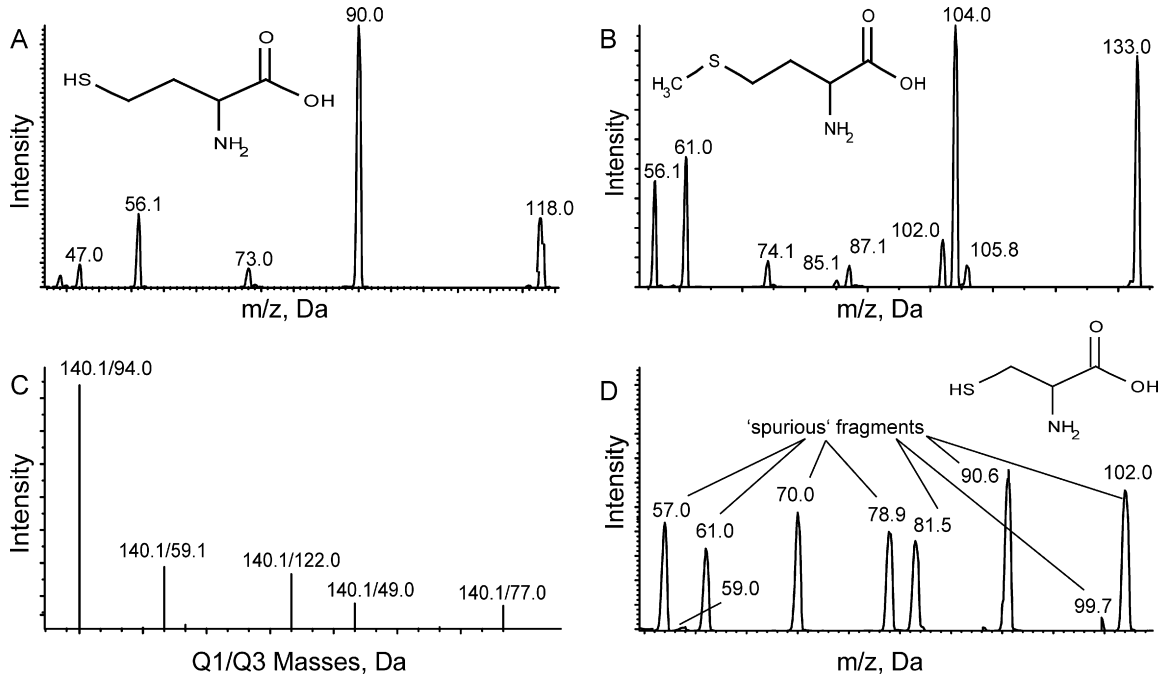

Fig. 1. Product scans. (A) Final products scan of Hcy after compound optimization. (B) Final products scan of Met after compound optimization. (C) MRM transitions of d_4-Hcy. (D) Final products scan of Cys after compound optimization with spurious fragments.

samples, respectively, was mixed with 20 μl of an aqueous IS solution containing 5 μg/ml d_8-homocystine (d_4-homocysteine after reduction) in a Riplate® 1.2 ml 96-deepwell plate (Ritter, Schwabmuenchen, Germany). Thereafter, 20 μl of the reduction reagent dithiothreitol (77 mg/ml) [10] was added and the plate was mixed for 5 min on a neoLab® thermo shaker (Heidelberg, Germany) with 600 rpm at room temperature. After incubation for 15 min at room temperature, 100 μl of the precipitation reagent (0.1% formic acid in acetonitrile) was added. The solution was mixed again for 5 min and then incubated for 30 min at 4 °C. After centrifugation for 10 min at 500 × *g* at room temperature, 2 μl of the supernatant was injected into the HPLC–MS/MS system, effectively loading an equivalent volume of 0.25 μl plasma. Blank and zero samples were prepared in the same way using water instead of plasma.

As an optional variation of the aforesaid protocol, sample clean-up using filter plates was tested. To this end, 100 μl of supernatants from the centrifugation step in Section 2.6 was transferred to solvent-resistent 96-well filter plates (MultiScreen® Solvinert, 0.45 μm pore size, Millipore, Billerica, MA, USA) with hydrophilic or hydrophobic membranes, respectively. The filtrate was collected in standard 96-wellplates after centrifugation at 500 × g for 5 min at room temperature. This filtration procedure was not found to be necessary and was therefore omitted in the final sample preparation protocol (see Section 3.3).

2.7. Method comparison

The developed LC–MS/MS method for the determination of Hcy was compared with an ADVIA Centaur® XP Immunoassay System (Siemens, Eschborn, Germany), a fully automated competitive chemiluminescence immunoassay for Hcy. 20 EDTA plasma samples from healthy male volunteers were analyzed in comparison. For determining the Hcy concentration using the immunoassay, the blood samples were analyzed shortly after collection. Aliquots of blood samples were transferred to Eppendorf vials, centrifuged at 2500 rpm for 5 min and the plasma stored at −80 °C until determination using the reported LC–MS/MS method.

3. Results and discussion

3.1. ESI-MS/MS condition optimization

High signal intensity was observed when the pure solutions of Hcy and Met were analyzed in positive ESI mode. The $+Q_1$ full scan mass spectra showed predominately protonated molecular ions at *m*/*z* 136.0 for Hcy and *m*/*z* 150.1 for Met. Major product ion of Hcy was *m*/*z* 90.0 (loss of 46 Da). Further Hcy fragments 56.1 and 110.1 showed similar intensity after optimization of ion path conditions (Fig. 1). Met was fragmented into product ions *m*/*z* 104.0, 133.0 and 61.0 with descending order of intensity (Fig. 1). During continuous infusion analysis, we noticed 'spurious' fragments in the product ion scan for Cys, which were not reported in previously published work and suppressed the primary product ions of Cys (Fig. 1). We decided to scan for the three major product ions of Cys (*m*/*z* 59.0, 76.0 and 105.0) found in previously published articles [25,26] and publicly available databases [30,31] and to optimize compound dependent parameters in manual tuning mode. All collected mass transitions were subsequently validated by chromatographically separating the analytes from isobaric solvent interferences. As mentioned above, this procedure was especially necessary in the case of Cys for sorting out 'spurious' product ions probably resulting from solvent impurities.

For each analyte the highest intensity product ion was utilized for quantification and the next two as qualifier ions. Qualifier ions permit accurate identification since the ratio between product ions of a given precursor is known to be a consistent property of the analyte molecule in the absence of co-eluents. Observed qualifier ratios were approximately normally distributed with standard deviation of 2% of the mean (Fig. 2). Based on the precision of the qualifier ion ratio determined from aqueous standards, we used cut-off limits outside of 91–109% of the normalized qualifier ion ratio for the exclusion of suspect samples. To our knowledge qualifier ions were not used for Hcy, Met and Cys analysis before. Using this approach, reliable analyte qualification in complex biological matrices is easily achieved (see also Section 3.2).

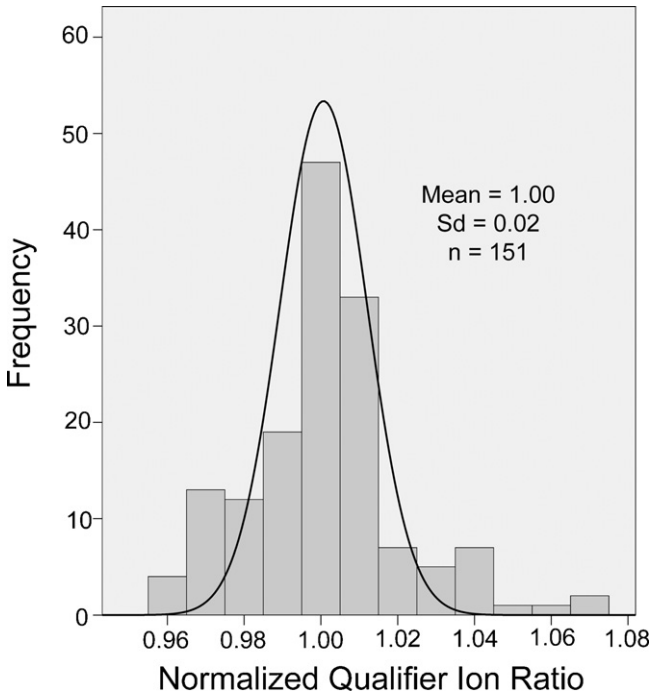

Fig. 2. Qualifier Ions. Histogram with density curve for the ratio of the first qualifier ion for Hcy (136.0/56.1) to the main Hcy transition (136.0/90.0). 151 plasma samples were analyzed during method development.

3.2. Chromatography

As mentioned above, the major aim of this method development was to achieve better chromatographic separation for simultaneously quantification of Hcy, Met and Cys. Best peak shape and resolution were observed using the silica hydride column in aqueous normal phase (ANP) mode. ANP is a chromatographic method using aprotic organic solvents (mostly acetonitrile, or sometimes acetone) at concentrations above 50–70% as the weak and water (optionally together with methanol) as the strong elution solvent. The surface of silica hydride columns is composed primarily of Si-H moieties with low-carbon content [3,5]. Retention mechanism of ANP chromatography on silica hydride stationary phases is currently not completely understood [1,5]. It is presumed that, in addition to partitioning effects similar to HILIC chromatography, analytes may also interact directly with the stationary phase surface [5,32]. The water layer on the stationary phase seems to be weaker and less dense in comparison to HILIC, permitting rapid equilibration [4,5]. In contrast to HILIC, high buffer concentrations, which often result in significant ion suppression during mass spectrometric detection, are not necessary to obtain good peak resolution. Therefore, ANP chromatography with minor amounts of volatile acidic modifiers provides very MS-friendly conditions for selective and sensitive quantitation of positively charged polar compounds.

Utilizing these advantages of ANP chromatography, separation of Hcy, Met and Cys from matrix components was considerably improved compared to previous methods [25–28]. Retention times (RT) of Hcy (3.3 min), Cys (3.35 min) and Met (3.3 min) were acceptably short, with good separation from the column void volume (1.1 min). In favor of improved retention, chromatographic run time was prolonged (4 min gradient time plus 3 min equilibration time) compared to previously published methods which used run times of only 2–4 min. In these methods, sufficient separation of analytes from matrix components was sacrificed for very short cycle time. Elution of Hcy directly following the column void volume was however potentially not sufficient to separate analytes and IS from interfering matrix constituents.

The need for chromatographic separation was underscored in this work by analyzing 227 plasma samples from healthy male volunteers participating in a clinical trial with folic acid supplementation. Co-eluents with transitions m/z 140.1/122.0 (d_4-Hcy_03) and m/z 150.1/133.0 (Met_02) were found, see Fig. 3. These co-eluents only emerged in plasma of some of the subjects studied. To our knowledge these co-eluents were not described in previous studies nor appeared in published chromatograms. Interfering signals (Fig. 3) of co-eluents would lead to biased ratios of qualifier ions and therewith to an imprecise qualification when separated insufficiently from the internal standard or analyte. This case demonstrates impressively the advantages of proper chromatographic separation, especially when dealing with complex biological matrices whose composition is supposed to change between samples. Co-eluents may also influence the quantification ion and therefore bias the calculated quantitation result. The use of adequate chromatographic separation together with continuous qualifier ion monitoring therefore represents an important improvement towards accurate and unbiased quantification of endogenous metabolites by LC–MS/MS.

Retention times of d_4-Hcy, Hcy, Met and Cys were very similar, thus we assumed that ion suppression affecting the intensity of each analyte and the IS were also comparable. In this case it is possible to use only one IS (d_4-Hcy) for all analytes to improve the cost-effectiveness of the assay. Chromatographic RT was very stable over at least 88 samples of one batch with coefficients of variation of 0.34% (Hcy), 0.51% (Met) and 0.35% (Cys). Variation of retention times between batches was 3.08–3.49 min (Hcy), 3.07–3.31 min (Met) and 3.15–3.37 min (Cys) with about 300 plasma samples analyzed in total. All qualifier ion ratios were determined within defined acceptance limits (see Section 3.1), so that further co-eluents could be excluded (Fig. 2).

3.3. Sample preparation

For protein removal different methods were tested. Initially, filters with a hydrophobic polytetrafluoroethylene (PTFE)/polypropylene (PP) membrane were used. It was observed that in several wells a liquid residue remained in the filter plate after centrifugation and the filtered amount of liquid phase exhibited unacceptably high variation. This unwanted effect is probably associated with the size of the aqueous fraction in the mixture. If the hydrophilic fraction forms a layer between the hydrophobic membrane and the liquid supernatant, the repellent interactions between polar and non-polar phases will not allow the fluid phase to pass through the filter. To avoid this problem, hydrophilic filters can be used or the hydrophobic filters have to be pre-wet with organic solvent.

As pre-treatment of filter plates adds an additional working step and enhances the total analysis time, we compared a preparation method utilizing hydrophilic filter with modified PTFE/PP membrane and preparation without any filter plate (see Section 2.6) using 33 plasma samples. Measured Hcy, Met and Cys concentrations showed very low deviation between the two preparation methods (Table 2). Both reproducibility and accuracy exhibited no significant differences between protocols using hydrophilic filter plates or no filtration at all (intra-day reproducibility 6.0% CV vs. 4.1% CV, accuracy 100.41% vs. 101.04%). Since both methods gave nearly identical results, sample preparation without any filter was clearly the more cost-effective and straightforward alternative and was therefore used in the final protocol.

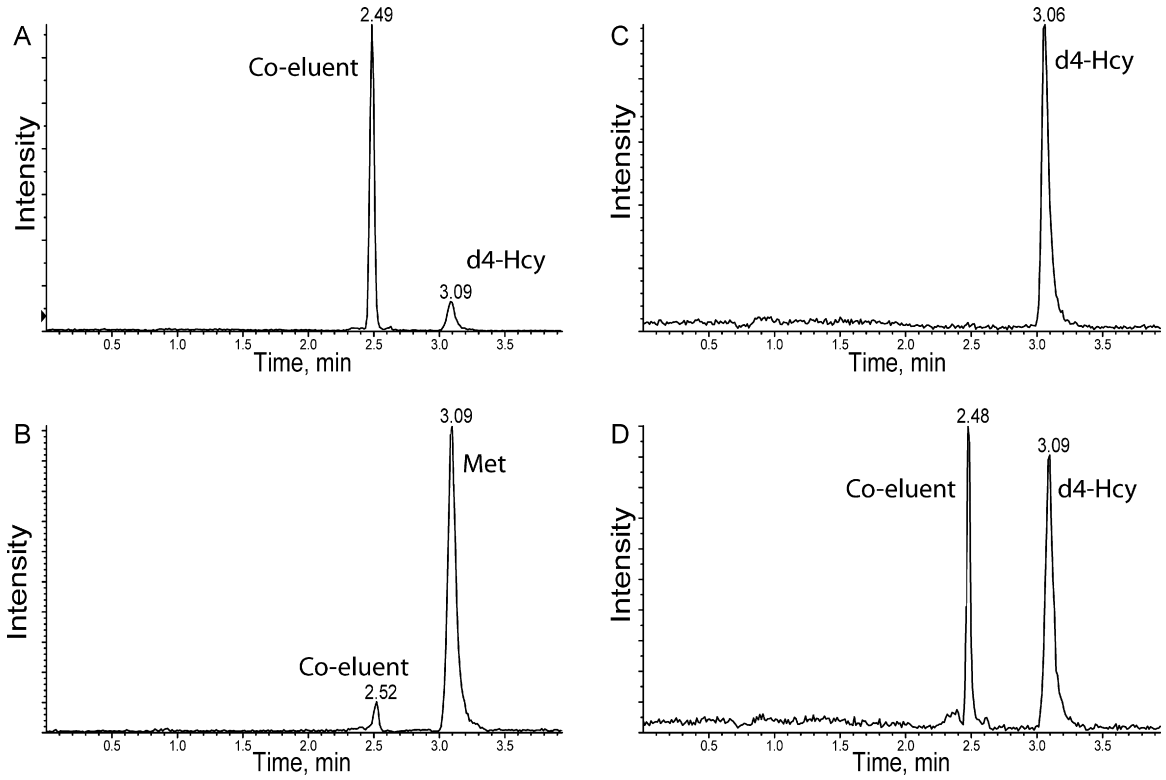

Fig. 3. Separation of interfering co-elutions. (A), (C), (D) Extracted chromatograms of ion transition 140.1/122.0 (d4-Hcy) in plasma samples of 3 different subjects. (B) Extracted chromatogram of ion transition 150.1/133.0 (Met) in plasma.

Table 2
Comparison of sample preparation with and without hydrophilic protein filtration. Measured plasma concentration and deviations of Hcy, Cys and Met of 33 plasma samples.

	Mean without filter [μM]	Mean with hydrophilic filter [μM]	Deviation [μM]	Rel. deviation [%]
Hcy	12.4	12.3	−0.1	−1.0%
Met	32.7	33.0	0.3	1.0%
Cys	244.6	240.3	−4.3	−1.7%

3.4. Linearity, accuracy, recovery and precision

For the determination of limits of detection (LOD) signal-to-noise ratio (S/N) had to be >3, for lower limits of quantification (LLOQ) S/N > 10 was necessary [33]. To identify these limits, aqueous standard samples were used (Table 3). As the standard solution with the lowest concentration was below the LLOQ of Hcy, Met and Cys, this standard was excluded from calibration. The remaining 9 aqueous standards showed good linearity for Hcy, Met and Cys. Fig. 4 illustrates the linearity of Hcy calibration. Calibration equations were $y = 0.0317x + 0.0084$ ($R^2 = 0.9992$, Hcy), $y = 0.0267x + 0.0139$ ($R^2 = 0.9991$, Met) and $y = 0.0059x + 0.0145$ ($R^2 = 0.9993$, Cys). As the calibration equation was created with water samples, the accuracy for plasma samples had to be proven. Accuracy of quantification in plasma samples for Hcy and Met was checked using commercially available lyophilized control plasma. Results are summarized in Table 4. No control plasma was available for Cys, so recovery of Cys was tested by spiking 3 different plasma samples with low and high concentration aqueous solutions (Table 5). Recovery was determined by subtracting the calculated concentration of the unspiked sample from those of the spiked sample. The result of the precision research is shown in Table 3. Three different plasma samples were prepared and analyzed at 5 different dates for the evaluation of inter-batch reproducibility. Accuracy, recovery, precision and LODs/LLOQs were similar to previously reported results [10,14].

Table 3
Precision. Average precision of 4 replicates of 3 plasma samples each. LOD and LLOQ for each analyte.

Analyte	Average intra-batch CV [%]	Inter-batch CV [%]	LOD [μM]	LLOQ [μM]
Hcy	1.66%	4.89%	0.1	1
Met	2.46%	4.80%	0.1	1
Cys	1.97%	5.05%	0.4	4

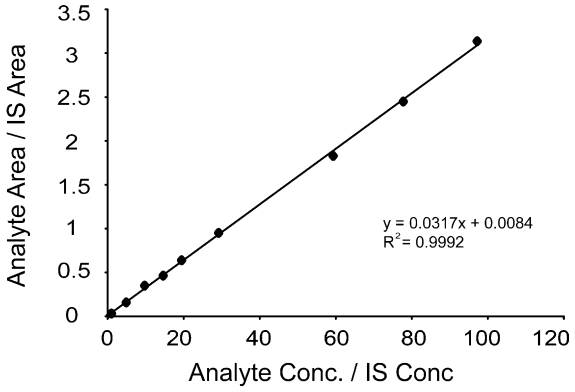

Fig. 4. Calibration curve. Peak area ratio vs. concentration ratio for Hcy with d_4-Hcy as IS.

Table 4
Accuracy and precision of Met and Hcy determination in control plasma. Accuracy is calculated as the quotient of detected and expected concentration. Precision is given as the CV. Each plasma sample was independently processed 5 times.

	Plasma level	Set value [μM]	Mean [μM]	SD [μM]	CV	Accuracy [μM]
Hcy	Low	10.1	9.86	0.72	7.26%	97.65%
	Medium	14.4	15.25	0.40	2.65%	105.90%
	High	23.5	23.40	0.72	3.06%	99.57%
Met	Low	39	38.60	1.13	2.93%	98.97%
	High	68.2	62.25	0.78	1.25%	91.28%

Table 5
Recovery of Cys in spiked plasma ($n = 3$). Recovery is the quotient of the measured difference to unspiked plasma and spiked amount.

Spike level	Spike [μM]	Mean [μM]	Mean recovery
Plasma	0	241.71	–
Low	21	265.21	112%
High	63	307.83	105%

3.5. Method comparison

The developed method was compared with a chemiluminescence immunoassay, a standard method for Hcy determination routinely used in the clinical laboratory. Hcy concentrations of 20 plasma samples analyzed with both methods showed good correlation ($y = 0.9703$, $x - 0.7953$, $R^2 = 0.9881$, y = LC–MS/MS, x = immunoassay) (Fig. 5). In addition, both methods showed favorable distribution of concentration-dependent deviation from the mean of the two determinations, as shown by a Bland–Altman plot (Fig. 5). Even with high concentrations of Hcy, the difference of measured values was in the limits of agreement of the Bland–Altman plot (±1.96 SD). Hcy concentrations determined by LC–MS/MS tended to be little lower compared to the immunoassay. Mean difference was −1.12 μM, which may be explained by differences in sample storage, since the samples for the immunoassay were longer stored at room temperature. Kuhn et al. found an increase of Hcy in serum samples compared to plasma Hcy, accusing the additional time until coagulation as the reason for release of Hcy from erythrocytes [24]. Therefore it seems advisable to remove plasma from erythrocytes as soon as possible after sample collection [14].

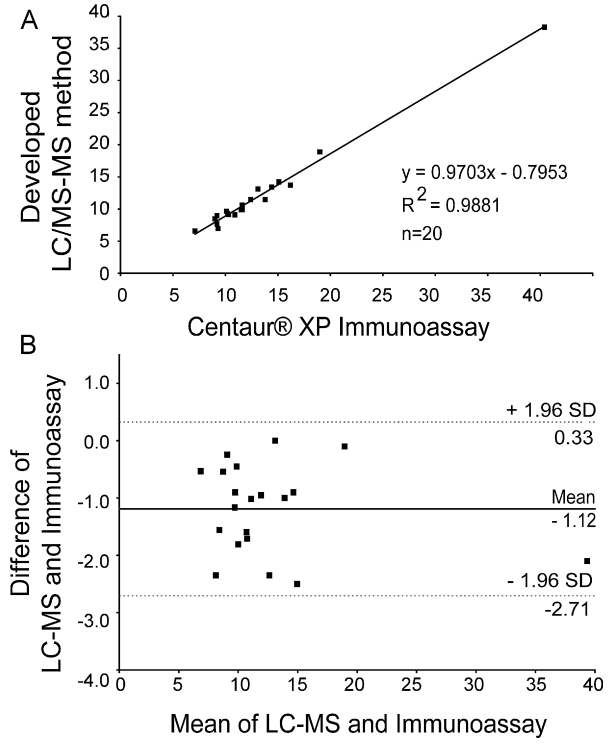

Fig. 5. Method comparison. (A) Linear regression to the comparison of the LC–MS/MS method and Centaur® XP Immunoassay. (B) Bland–Altman plot of the difference vs. the mean value of the LC–MS/MS method and Centaur® XP Immunoassay for 20 plasma samples.

4. Conclusions

The developed LC–MS/MS method permits efficient quantification of Hcy, Cys and Met. Reproducibility and accuracy of the method are comparable to previously published protocols. Improved chromatographic separation and continuous qualifier ion monitoring assure unbiased determination. The benefits of using ANP chromatography pre-separation for LC–MS/MS coupling were exemplified in this report using an expensive 4000Qtrap mass spectrometry system (as this was available in the authors' laboratory). The relatively high concentrations of Hcy, Met and Cys in plasma samples, however, permit seamless implementation of the proposed method using standard entry-level LC–MS/MS equipment. Straightforward sample preparation, short run time and low costs per sample allow analysis of large numbers of sample, e.g. from epidemiological studies.

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