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Review

Stable-isotope dilution LC–ESI-MS/MS techniques for the quantification of total homocysteine in human plasma☆

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ABSTRACT

Homocysteine is an endogenous sulphydryl aminoacid irreversibly catabolized by transsulfuration to cysteine or remethylated to methionine. Increased plasma levels of homocysteine are an independent risk factor for atherosclerosis and cardiovascular disease. Accurate and reliable quantification of this amino acid in plasma samples is essential in clinical practice to explore the presence of a hyperhomocysteinemia, for instance after an ischemic event, or to control a possible adjunctive risk factor in patients at higher risk. In this review, LC–ESI-MS/MS methods are discussed and compared with other analytical methods for plasma homocysteine. LC–ESI-MS/MS is a technique combining the physicochemical separation of liquid chromatography with the analysis of mass spectrometry. It is based on stable-isotope dilution and possesses inherent accuracy and precision. Quantitative analysis is achieved by using commercially available homocystine-d_8 as an internal standard. Taking advantage of the high sensitivity and specificity, approaches involving LC–ESI-MS/MS require less laborious sample preparation, no derivatization and produce reliable results.

Contents

1. Introduction

1.1. Homocysteine metabolism and genetic defects

Homocysteine (Hcys, 2-amino-4-mercaptobutyric acid) is a non-essential sulfur-containing amino acid formed during the metabolism of methionine, an essential amino acid present in foods regularly consumed within diet. Hcys is present in plasma in different forms (Fig. 1). The sum of all the forms of Hcys, total Hcys (tHcys), present in plasma includes: Hcys bound to proteins (bHcys) or bound to thiols such as cysteine, GSH, Cys-Gly, and free Hcys (fHcys) [1–3]. Hcys is a product of two major pathways: remethylation and transsulfuration, regulated by 5,10-methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS), respectively, [4] (Fig. 2). Both pathways are coordinated by *S*-adenosylmethionine which is the sole source of methyl groups for all methylation reactions within the cell [5]. The primary remethylation pathway involves remethylation of Hcys to

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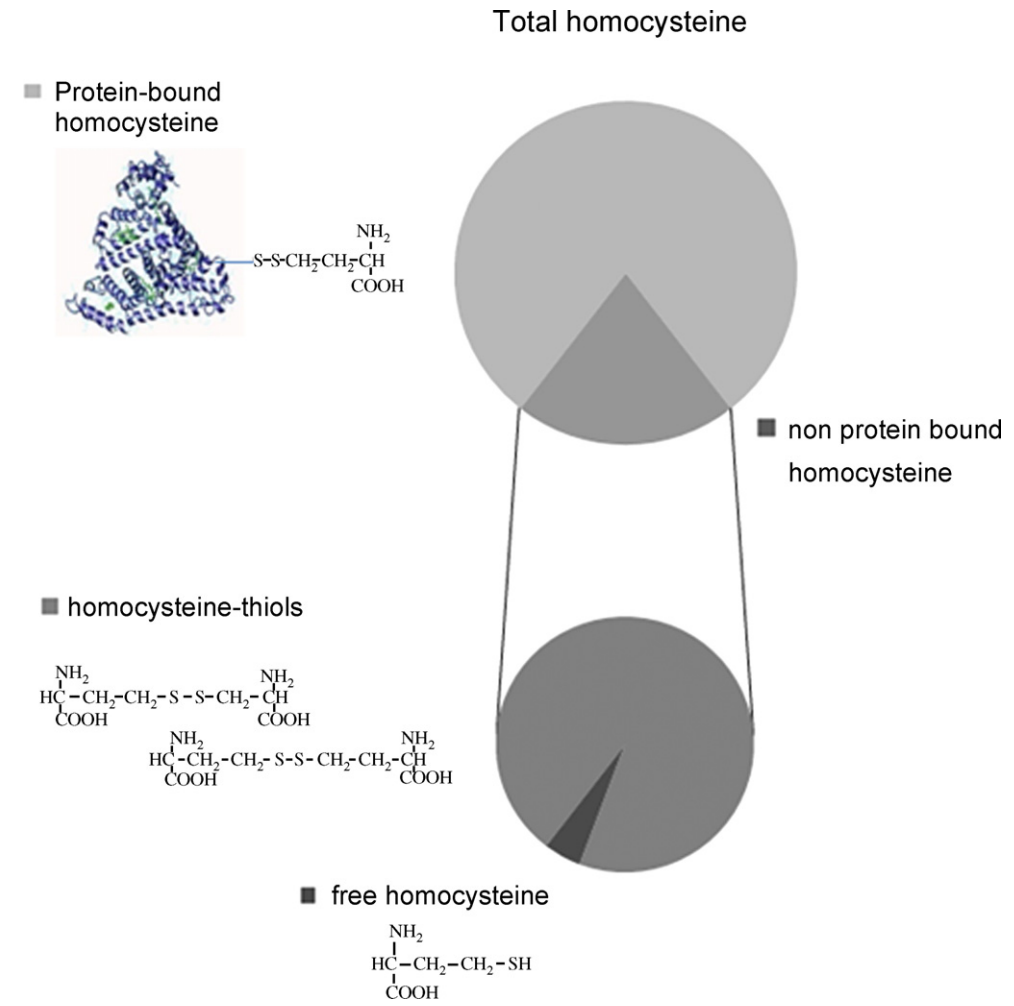

Fig. 1. Distribution of homocysteine forms in human plasma. Homocysteine and mixed disulfides between Hcys and Cys are shown.

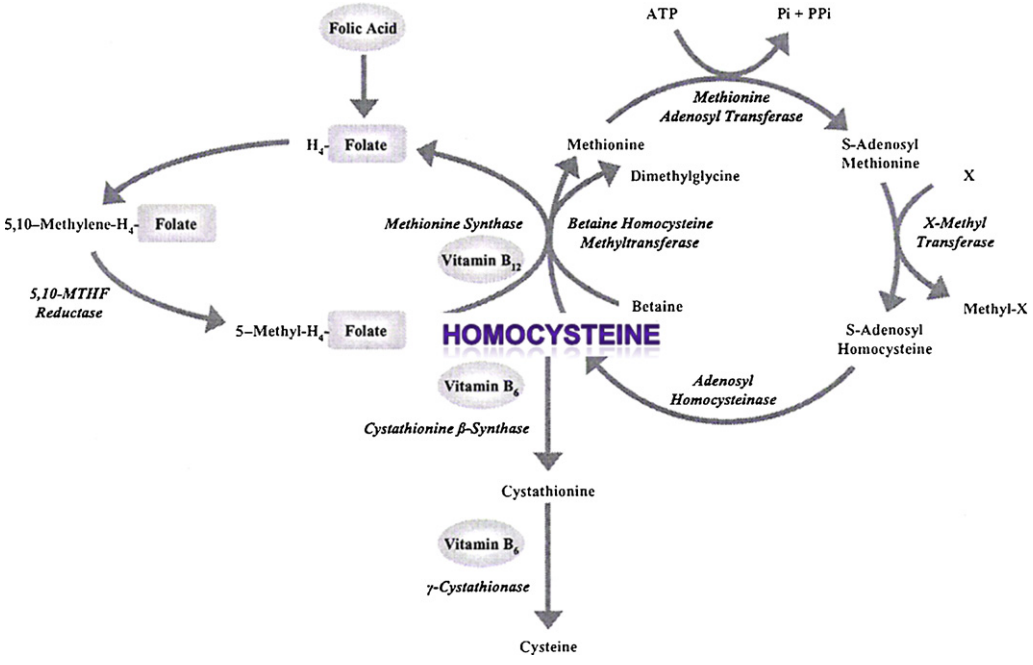

Fig. 2. Hcys metabolism including the anabolic remethylation pathway and the catabolic transsulfuration pathway.

methionine and is catalyzed by the vitamin B12-dependent enzyme methionine synthase. This reaction utilizes the folate-containing methyl donor 5-methylenetetrahydrofolate which is generated by MTHFR. However, when methionine plasma levels are low, Hcys is produced by means of the remethylation to methionine. In the presence of an excess of methionine, Hcys is produced as a product of the transsulfuration pathway, an irreversible process that involves two vitamin B6-dependent enzymes (CBS and γ-cystathionase) resulting in the production of cystathionine and, in turn cysteine and other sulfur-containing compounds, including glutathione.

Under normal conditions, the plasma concentration of tHcys is maintained at a low constant level; the normal basal plasma concentration ranges between 5 and 15 μM, with a mean level of about 10 μM [6]. However, diverging tHcys values have been reported in literature [7,8]. Mean values of plasma tHcys concentration are significantly higher in males than in females [9]. However, several hereditary and acquired conditions can alter the normal state of Hcys metabolism [10,11]. Genetic defects in the enzymes involved in Hcys metabolism markedly increase tHcys levels, thus determining hyperhomocysteinemia (defined as plasma tHcys levels greater than the 90th or 95th percentile of levels in general population) [12] and is defined as moderate (15–30 μM), intermediate (30–100 μM) or severe (>100 μM) hyperhomocysteinemia [6].

Mutations in the CBS (in homozygous or compounds heterozygous mutation) are associated with severe hyperhomocysteinemia and an excess rate of premature thrombotic events, including stroke and venous thrombosis [13]. This alteration is inherited as an autosomal recessive disorder that results in the accumulation of Hcys and methionine in tissues and blood. At variance with the most rare mutations in the CBS, the common C677T substitution in the MTHFR gene (18% in the homozygous state), producing a thermolabile enzyme with reduced activity (valine substitution by alanine) can determine, in the presence of low folate levels, moderate or intermediate tHcys plasma levels [14]. This abnormality decreases Hcys remethylation to methionine and causes the inactivation of methionine synthase and, in turn, an accumulation of Hcys.

1.2. Hyperhomocysteinemia and atherothrombosis: epidemiological evidence

Hcys was first described in 1932 by Butz and Vigneaud [15]. The clinical relevance of Hcys was proposed about 30 years later, when McCully described the first abnormality in the Hcys metabolism in a child with mental retardation, dislocated ocular lenses, accelerated growth, osteoporosis, and a tendency to thrombosis [16]. This child had a rare inherited enzymatic defect that was caused by deficiency of CBS [17]. In 1969, a link between atherothrombotic changes and levels of homocysteinemia in severe hyperhomocysteinemic and homocystinuric patients with different genetic defects was noted and it was hypothesized that increased tHcys plasma levels may contribute to atherosclerosis [8].

The clinical significance of tHcys has expanded enormously in recent years after its recognition as an indicator of nutritional cofactor deficiency (folate and cobalamin) [18,19], a risk factor of cardiovascular disease (CVD) (atherosclerosis, heart disease, and thromboembolism) [20], and as a contributing factor to the pathogenesis of neural tube defects [21]. Elevated tHcys plasma levels have been also described to be associated with pregnancy complications [22] and psychiatric disorders [23].

Several studies (retrospective, cross-sectional, cohort and case-control studies) have described a strong association between moderate hyperhomocysteinemia and the risk of CVD. A recent meta-analysis [24] of 27 observational studies showed that an increase by 5 μM tHcys plasma levels is associated with a higher risk of coronary heart disease (CHD) [1.8 fold (95% CI 1.3–1.9) in women and 1.6-fold (95% CI 1.4–1.7) in men. Following, observational studies have also provided consistent support for an association between hyperhomocystinemia and atherosclerotic vascular disease [25,26]. However, the results from prospective studies are less consistent [25–29]. Some of them reported a statistically significant association between elevated tHcys and CHD [25,26] or stroke [27]. In contrast, other studies failed to demonstrate a significant association between tHcys plasma levels and CVD [28,29]. Metanalyses of prospective observational studies of first events demonstrated an association between hyperhomocysteinemia and increased risk of CVD [30,31]. Several studies documented an association between mortality in patients with pre-existing coronary [32,33] or peripheral vascular disease and tHcys plasma levels, independent of traditional risk factors [34,35], or with cerebrovascular events in patients with significant stenosis of the carotid artery [36], suggesting that tHcys may promote acute thrombotic events leading to cardiac death or stroke.

Given the functional nature of MTHFR C677T single nucleotide polymorphism and its relationship to tHcys plasma levels [37,38], it is possible to perform Mendelian randomization analyses of cohort studies. Wald et al. [39], comparing the high risk TT genotype to other genotypes, reported a 21% (95% CI 6–39%) increased risk of CHD and a non significant 31% (95% CI −20% to +215%) increased risk of stroke.

In this context, clinicians frequently request tHcys determination to explore the presence of a hyperhomocysteinemia after an ischemic event, or to control a possible adjunctive risk factor in patients at higher risk [40]. Thus, in last years, the number of tHcys tests is significantly increased.

2. Pre-analytical phase – sample collection, processing and storage

A time- and temperature-dependent release of fHcys from erytrocytes, leading to an artificial increase in the plasma of tHcys, is observed if blood is not centrifuged after venopuncture [41,42]. Thus, after blood drawing, it is necessary to eliminate erythrocytes as soon as possible. Placing the sample tubes on ice delays the release of fHcys by erythrocytes for approximately 1 h [43]. Once erythrocytes have been removed, tHcys is stable in plasma for 4 days at room temperature and is stable at −20 °C and −70 °C for a long time, with repeated freeze/thaw cycles having no effect [44,45]. Furthermore, storage of whole plasma or serum may cause a different distribution of plasma thiols, increases in bHcys but decreases in fHcys levels. Thus, reliable determination of fHcys requires immediate blocking of free thiols at the sampling time [46]. This sample preparation is not convenient for clinical settings. Therefore, given the instability of its reduced form and the diversity of its oxidized forms, the determination of fHcys is not used anymore. Currently, determination of tHcys is performed [46].

As previously mentioned, at physiological pH the most abundant fraction is that present as various disulfide forms. For this reason, one of the most critical steps in the sample processing procedure is the reduction of disulfide bonds before quantification. The selection of reducing agent depends on the separation and detection system used. Sodium or potassium borohydride are potent reducing agents, but their use in the reduction procedures is very labor-intensive [47]. Furthermore, formation of gas during the reduction with borohydride may add further pre-analytical issues. Tri-*n*-butylphosphine does not cause the previously mentioned problems; however, this compound is irritant and poorly soluble in water, so it must be dissolved in dimethylformamide before use. TCEP (tris(2-carboxylethyl) phosphine) is frequently used in substitution of other phosphine containing molecules [48]. TCEP is nonvolatile, stable, and soluble in aqueous solution and thus is more suitable for routine use [48]. Also sulfhydryl reagents, such as dithiothreitol (DTT) or 2-mercaptoethanol, have been used

as reductants. DTT seems to be the most suitable reducing agent. Many methods for the determination of plasma tHcys utilize DTT as reducing agent, thus stabilizing Hcys monomers once they are formed. The optimal DTT incubation time was determined by time-course experiments that demonstrated complete and instantaneous reduction of bHcys [49].

3. Analytical phase

tHcys can be measured by means of different methods. Results obtained with different methods are often not very comparable each other because of considerable inter-method and inter-laboratory variability [25]. Reported approaches for the measurement of plasma tHcys include: ion-exchange chromatography [50], immunoassays [51–53] (fluorescence polarization immunoassay, FPIA, or chemiluminescence immunoassay, ICL, or enzyme-linked immunoassay, EIA), HPLC [54–56] (with photometric, fluorescence or electrochemical detection), capillary electrophoresis [57] (with photometric or laser fluorescence detection), GC–MS [58–60], and LC–ESI-MS/MS [61]. Many of them have significant disadvantages, including derivatization protocols, are expensive and time-consuming. Compared with the above mentioned, LC–ESI-MS/MS seems to be the most suitable method because of its inherent accuracy, high sensitivity, specificity and high throughput for tHcys analysis [61].

3.1. Liquid chromatography with electrospray ionization tandem mass spectrometry

LC–ESI-MS/MS is a relatively new technique that combines the advantages of physicochemical separation by liquid chromatography with the unique mass analysis by mass spectrometry. Analytical methods based on stable-isotope dilution and LC–ESI-MS/MS are valid in terms of accuracy and precision. Liquid chromatography is a powerful separation method that is compatible with virtually any mass spectrometer and ionization technique including electrospray ionization (ESI). The development of ESI tandem mass spectrometry has considerably improved the measurement of plasma tHcys. tHcys was first measured by stable-isotope dilution and electrospray MS/MS by Magera et al. [61], followed by other groups [67–69]. Later, the method of Magera et al. was adapted to analyze other thiols including homocysteine [70] widely used in the clinical diagnosis of metabolic disorders.

Kuhn et al. [62] performed chromatographic separation on phenomenex C8 precolumn eluted with aqueous solution containing MeOH–H_2O–FA (FA = formic acid) (50:50:0.001,v/v). They showed that a short C8 column allows a quick chromatography, reduces fouling of the sample cone of the mass spectrometer in comparison to the methods without any column, increases sensitivity, suggesting that this column removes ion suppressive components. Furthermore, this method provides reliable results and can be applied to small samples volumes, as usually obtained from pediatric patients.

Rafii et al. [9] performed liquid chromatography on a Symmetry C8 column and MeOH–H_2O–FA (30:70:0.1, v/v) as mobile phase. Li et al. [63] utilized a Hypersil aquasil C18 column using a mobile phase of MeOH–H_2O–FA (10:90:0.02, v/v). Polar C18 phase gives greater retention for polar compounds and shows high stability.

The analytical process of tHcys analysis by LC–ESI-MS/MS is shown in Fig. 3. With respect to the ESI process the reader is referred to the literature [64].

Maximum sensitivity for Hcys is achieved by measuring product ions from the fragmentation of the protonated molecule cation $[M+H]^+$. Full scan mass spectra show predominate protonated molecular ion $[M+H]^+$ at m/z 136 for Hcys and m/z 140 for internal standard d_4-Hcys, IS*). The protonated molecular ions selected by the first quadrupole (Q1) dissociated in the collision chamber to product ions which are selected by the third quadrupole (Q3). Neutral loss of a HCOOH (46 amu) from $[M+H]^+$ yields the product ion at m/z 90 for Hcys and m/z 94 d_4-Hcys (Figs. 4 and 5). Fig. 6 shows a typical LC–ESI-MS/MS chromatogram from the analysis of tHcys in a human plasma sample. The LC-MS/MS process provides high sensitivity due to enhanced S/N ratio and increases speed of analysis due to decreased time for sample cleanup and short analysis time.

In the LC-MS/MS measurement of Hcys, ion suppression is observed both in plasma and urine samples. Analysis of Hcys in

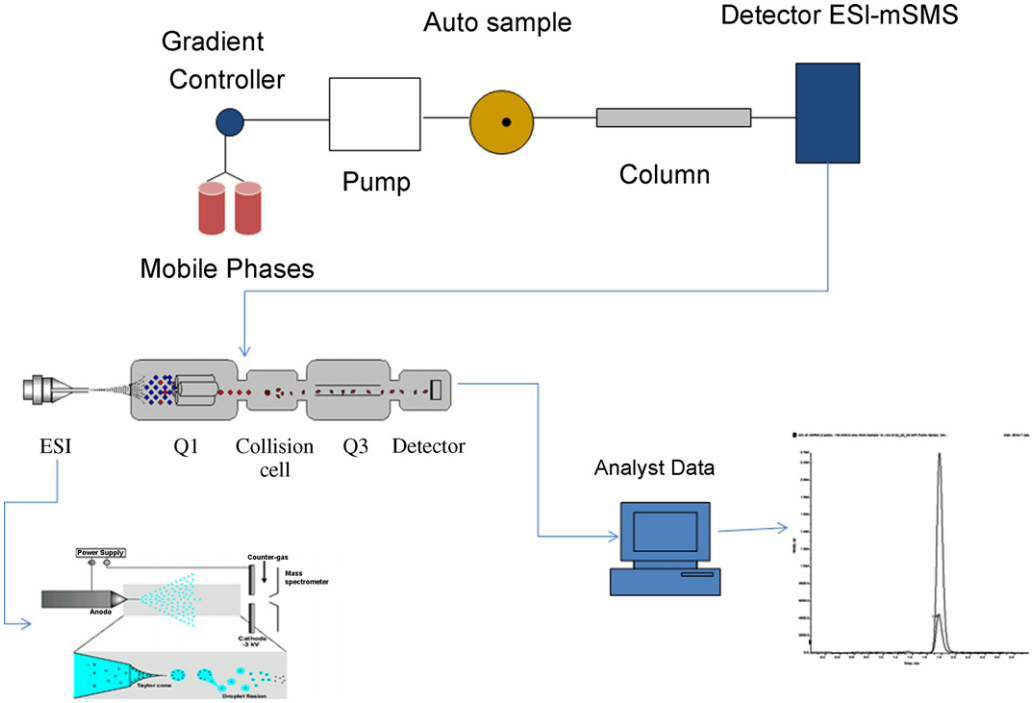

Fig. 3. Schematic representation of Hcys analysis using LC–ESI-MS/MS.

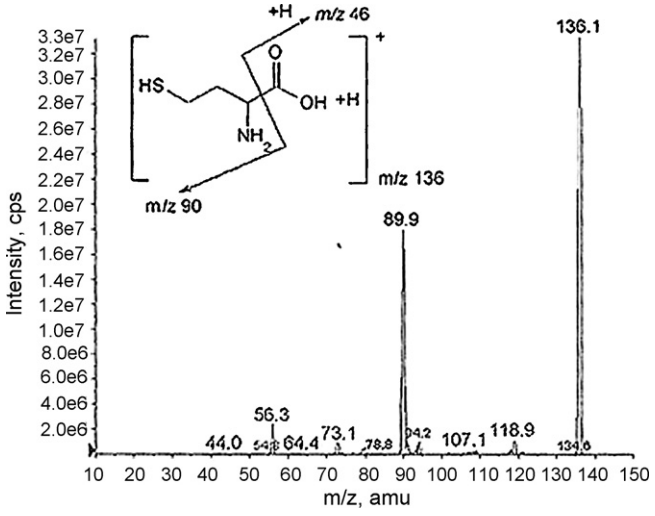

Fig. 4. ESI mass spectrum of Hcys.

water and physiological fluids such as plasma and urine give different results due to matrix effects. To eliminate matrix interferences Rafii et al. [9] prepared plasma and urine calibrators in order to match the correct matrix of the samples. Matrix effects seem to be more pronounced in urine than in plasma, perhaps due to a high salt content [65] that causes ion suppression.

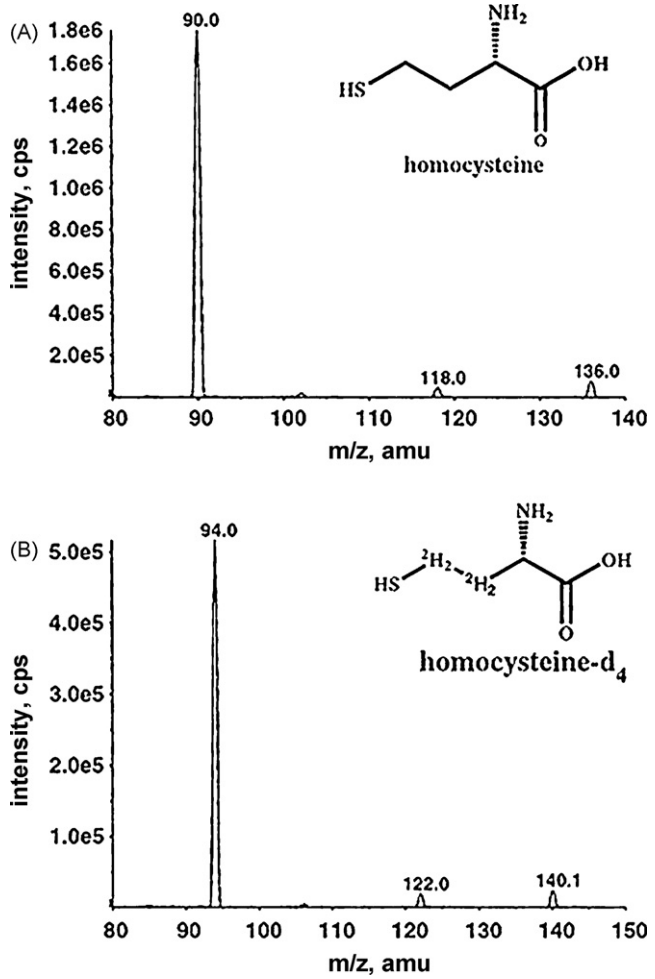

Fig. 5. Product ion ESI mass spectra of Hcys (A) and d_4-Hcys (B) generated from the parent ions $[M+H]^+$ at m/z 136 for Hcys and m/z 140 for d_4-Hcys.

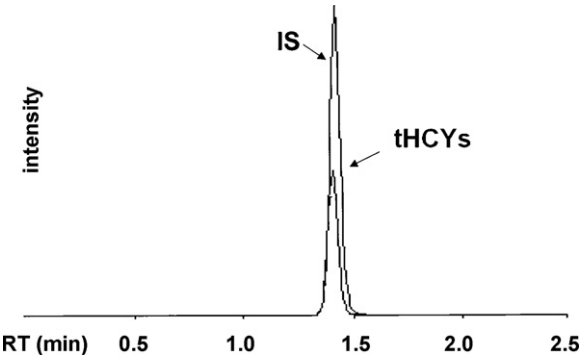

Fig. 6. LC–ESI-MS/MS chromatograms of tHcys and the IS obtained from plasma analysis (m/z 90 for Hcys and m/z 94 for d_4-Hcys).

In all reports regression equations of calibrator curves reveal good linearity. A calibration curve is constructed by plotting the Hcys/IS peak area ratio against Hcys concentration. In experiments performed by Kuhn et al. [62], the calibration curve of the Hcys assay was linear over the calibration range up to 61.6 μM; the limit of detection (LOD) and the lower limit of quantification (LOQ) were 1 and 1.8 μM, respectively. Magera et al. [61] reported an intra-assay and inter-assay precision of 3.6–5.3% and 2.9–5.9% for Hcys mean values of 3.9, 22.7 and 52.8 μM, respectively. Li et al. [63] reported intra- and inter-assay precision of less than 3.24% and 4.04%, respectively.

Several authors compared the analysis of plasma tHcys by means of LC–ESI-MS/MS with the analysis by means of other established methods [61,63,64,66]. In experiments by Magera et al. [61], the correlation between the LC–ESI-MS/MS and the HPLC assay was close ($r = 0.975$) and similar to that obtained by Rafii et al. [9] (Fig. 7). Magera et al. compared LC–ESI-MS/MS with FPIA (IMx) and observed a close correlation ($r = 0.969$) for HCys (Fig. 8) [61].

After the reduction and deproteinization steps, Hcys is separated from other sample components by rapid liquid chromatography followed by mass spectrometry [9,61,71]. Chromatography allows a separation of analyte and removes potentially interfering substances. Hcys is a highly polar amino acid, and this makes difficult to retain non-derivatized Hcys reversed-phase materials. Therefore, liquid chromatographic conditions are important for adequate retention and separation and require optimization. In Table 1 the main characteristics of the LC–ESI-MS/MS analytical methods are summarized. Magera et al. [61] used a mobile phase composed of (ACN: acetonytrile) ACN–H_2O–FA (60:40:0.001, v/v) and performed chromatographic separation on LC-CN. Also, Tuschl et al. [72] used ACN–H_2O–FA (1:1:0.0005, v/v) as mobile phase. This method is rapid and sensitive and avoids the need for derivatization and pre-analytical chromatography. The gain in sensitivity attributable to chromatographic efficiency might be evaluated by comparing S/N ratios, i.e. the S/N ratio of a real sample injected into the column with the S/N injected directly to source (FIA). However, FIA provides reliable information only when analyzing relatively pure samples; therefore, this method is generally unacceptable for the majority of complex samples [73].

Some authors contemporarily quantified tHcys and folate and developed a reversed-phase procedure utilizing C18 SPE cartridges [59]. Analytes are eluted from the cartridges and subsequently chromatographed on a supercosil LC-CN cyano analytical column with gradient elution using of HCOOH in MeOH.

3.2. Isotope dilution mass spectrometry (IDMS)

The IDMS technique involves the addition of a known amount of the isotopic analogue of the analyte of interest to a known

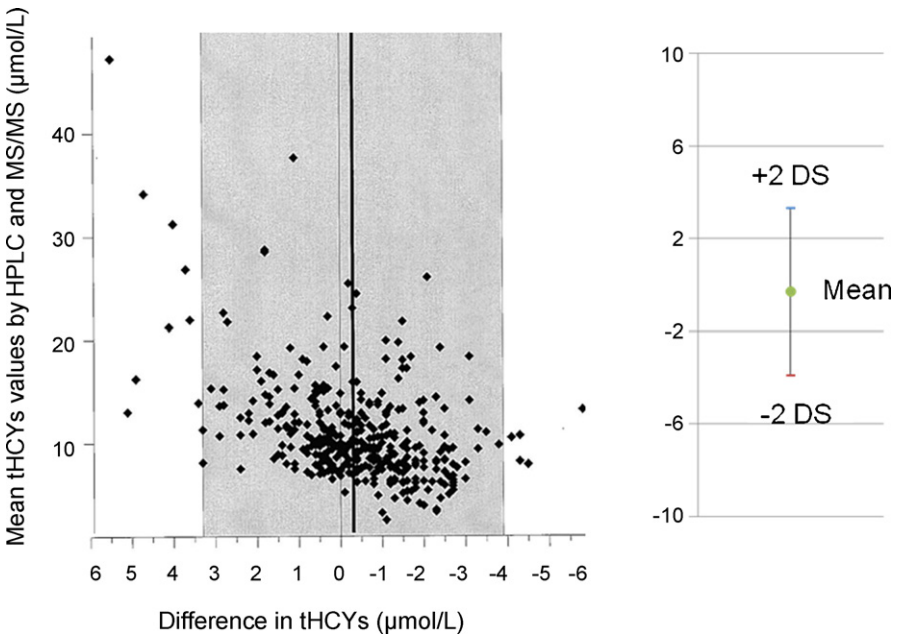

Fig. 7. Bland–Altman plot of the difference between Hcys levels by the LC–ESI-MS/MS and their previous HPLC method. Figure adapted by Magera et al. [61] with permission from Highwire Press.

volume of the sample, which is used as IS in quantitative analyses. The peak area ratio for analyte and IS measured in the sample allows calculation of the analyte concentration [74].

The IDMS technique provides sensitive analysis and yields precise and accurate results. Complete recovery of the analyte is not required because the determined value is based on measuring the ratio between the analyte and the stable-isotope labeled IS which behaves chromatographically identically with the analyte. To ensure the same expected behavior during the analytical procedure, it is particularly important that full equilibration between the analyte and its isotopic analogue is achieved in the matrix. IDMS avoids shortcomings that may occur during sample processing, chromatographic separation and MS detection [75]. IDMS is considered as a definitive method for quantitative analysis of very low concentrations.

3.3. Matrix effects in LC–ESI-MS/MS

Matrix effects may be important limitations of the LC-MS/MS method. Matrix component that co-elute with Hcys can interfere with the ionization process [76], causing ionization suppression or enhancement [77,78]. This phenomenon is generally not reproducible or repeatable between various sample batches and may compromise analytical reliability of LC-MS/MS methods. Thus, matrix effects may influence precision and accuracy of assay: the degree of ion suppression for an analyte and an IS may be different depending on the biological matrix analyzed. Ion suppression is a

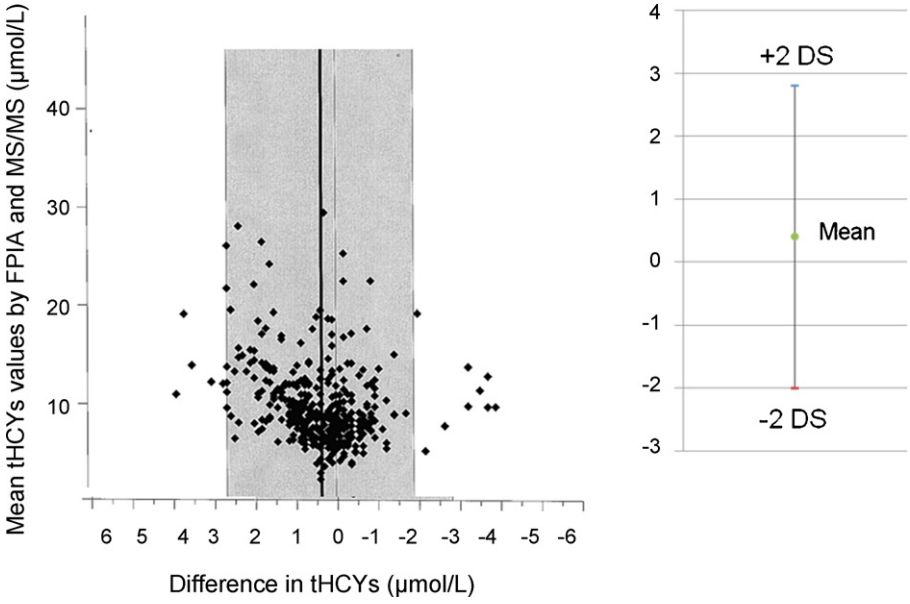

Fig. 8. Bland–Altman plot of the difference between Hcys levels by the LC–ESI-MS/MS and Abbott IMx FPIA. Figure adapted by Magera et al. [61], with permission from Highwire Press.

Table 1
Main characteristics of LC–ESI-MS/MS analytical methods for measuring plasma homocysteine.

HPLC column	Mobile phase	Mass spectrometer	MS/MS conditions	Precision, accuracy, recovery	Range	LOD	LOQ	Ref.
Waters symmetry C8 column (100 × 2.1 mm, 3.5 μm)	MeOH–H_2O–FA (30:70:0.1, v/v) flow rate: 0.25 ml/min	API 4000 (Applied Biosystems)	DP: 42 (Hcys)–43 (IS); EP: 3; CE: 16; CXP: 6	Intra-assay CV: 2.9–6.1% (n = 3); inter-assay CV: 4.8–6.4% (n = 9); accuracy: 100%; recovery: 94–100%	9.44 ± 1.20 μM (males); 7.99 ± 1.68 μM (females)	N.R.	N.R.	[9]
Hypersil aquasil C18 column (50 × 2.1 mm, 5 μm)	MeOH–H_2O–FA (10:90:0.02, v/v) flow rate: 0.25 ml/min	API 3000 (Applied Biosystems)	DP: /; N.R.; EP: /; N.R.; CE: 20; CXP: / N.R.	Intra-assay CV: ≤3.24% (n = 6); inter-assay CV: ≤4.04% (n = 36); accuracy: 90.2–104.0%; recovery: 96.4% (Hcys), 98.3 (IS)	13 ± 3.4 μM (healthy controls)	0.5 μM	5 μM	[63]
LC-CN (30 × 4.6 mm) 3 μm	ACN–H_2O–FA(60:40:0.001, v/v)flow rate: 1 ml/min	API 2000 (PerkinElmer Sciex)	DP: /; N.R.; EP: /; N.R.; CE: 17; CXP: / N.R.	Intra-assay CV: 3.6–5.3%;Inter-assay CV: 2.9–5.9%; accuracy: /; N.R.; recovery: 94.2% (20 μM–97.8% (50 μM)	N.R.	N.R.	0.8 μM	[61]
N.R.	ACN–H_2O–FA (1:1:0.0005, v/v) flow rate: 0.15 ml/min	API 2000 (PerkinElmer Sciex)	CE: 19	Intra-assay CV: 10.2% (n = 10); inter-assay CV: 11.5% (n = 5); accuracy:/recovery: 49.2% (2 μM); 92.8% (10 μM); 97.4% (20 μM)	N.R.	1 μM	4 μM	[72]
C8 column (4.0 mm × 3.0 mm) 5 μm	MeOH–H_2O–FA (50:50:0.001, v/v) Flow rate: 0.1 ml/min	Quattro (Micromass)	DP: / N.R.; EP: / N.R.; CE: 12(IS)-13(Hcys); CXP: / N.R.	Intra-assay CV: /; inter-assay CV: /; accuracy: /; recovery 94.7 ± 6.3%	13.6 ± 3.6 μM (healthy controls)	1 μM	1.8 μM	[62]

N.R., not reported.

common problem in APCI and ESI [65,79,80], the most currently used techniques for API ionization. Ion suppression occurs in the early phase of the ionization process in the LC-MS interface, when a component eluted from the HPLC column influences the ionization of a co-eluted analyte. In LC–ESI-MS/MS the ionization process is crucial [81]. Matrix effects are minimized by using stable-isotope labeled analogues of the analyte at concentrations similar to those expected in the biological sample.

Different mechanisms of ion suppression have been proposed. When the concentration of the analyte exceeds 10 μM, ESI response is often not linear. This might be due to a limited amount of excess charge available on ESI droplets or to saturation of the ESI droplets with analyte at their surfaces, inhibiting ejection of ions trapped inside the droplets [82,83]. Suppression of signal indicates a competition for either space or charge. The efficiency of analytes ionization is determined by its characteristics and concentration. Surface activity and basicity of compounds influence its outcompetition with analytes for the limited charges on the droplet surface [82,83]. In addition, the increase in viscosity and surface tension of the droplets caused by interfering compounds, reducing solvent evaporation and by the ability of the analyte to reach the gas phase, have been evaluated as causes for ion suppression in ESI [82]. Ion suppression in ESI could be influenced by the ability of nonvolatile materials to decrease the efficiency of droplets formation through co-precipitation of the analyte or to prevent droplets from reaching their critical radius required for the emission of gas phase ions [65,82,83].

Several strategies are available to minimize or eliminate matrix interferences. Matrix effects may be reduced by using an IS, preferably a stable-isotope labeled analogue of the analyte, to compensate for the alteration in signal, by injecting smaller volumes or by diluting the biological sample. Arndt et al. [71] minimize the matrix effect by diluting 17–80-fold plasma samples and by injecting 2 μl containing about 0.008 μl of the native plasma sample (∼37-fold smaller injection volume). A further plasma dilution does not give an increase in the tHcys signal.

It has also been shown that the use of lower flow rates and flow splitting may reduce ion suppression. Another possibility to reduce or eliminate matrix effects is to optimize the sample preparation or chromatography parameters so that the analyte peak does not elute in region of suppression [77,84,85]. The solvent front (area of the chromatographic elution in which unretained compounds are eluted) and the end of the elution gradient (area of the chromatographic elution in which strongly retained compounds are eluted) are most affected by interferences. Thus, it is recommendable that the analyte elutes between these two regions, i.e. the solvent front and the end of the elution gradient, in order to avoid the ion suppression.

Recently, Hempen et al. [86] examined two different sample cleanup procedures: (1) reduction step followed by ultrafiltration, (2) reduction step followed by protein precipitation with ACN. In this report the peak area of the IS is 30% lower using the ACN precipitation, thus the second procedure appears to be the one mostly minimizing the matrix effect.

In other reports [9,61,62] the endogenous concentration of tHcys is calculated from an unspiked sample, and subtracted from the calibrators when a standard curve is constructed. To avoid interference from endogenous background, Li et al. [63] describe a new sample preparation to produce zero blank, “free of Hcys”: calibrator standards are achieved by diluting the pooled plasma samples three times with water. However, the calibrators are not comparable to physiological samples because undiluted plasma samples are quantified against the diluted calibrator standards.

Water or physiological samples (plasma or urine used as calibrators) give different precision, as a consequence of a different matrix effect. To improve inter-laboratory and inter-method results, Satter-

field et al. [87] developed a new standard reference matrix (SRM) for serum tHcys and folic acid determined by IDMS.

4. Conclusions

The clinical significance of tHcys has expanded in recent years after its recognition as an important predictor of CVD. A large number of methods are now available for tHcys determination. HPLC methods require derivatization which may be time-consuming. Immunoassays are expensive because of antibodies required. LC–ESI-MS/MS offers a great potential for high-throughput analysis paired with excellent sensitivity, specificity, and accuracy, that appears to be close to most of HPLC based methods with fluorimetric detection. In spite of the relatively high initial investment needed to acquire an LC–ESI-MS/MS apparatus, the analytical characteristics of the LC–ESI-MS/MS methodology are superior and make it reasonable to expect that this technology will be increasingly used in clinical chemistry laboratories for the analysis of HCys and other biomarkers in plasma and other relevant biological samples.

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