ELSEVIER

Journal of Chromatography B, 781 (2002) 207–226

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Methods for homocysteine analysis and biological relevance of the results☆

Véronique Ducros*, Karine Demuth, Marie-Pierre Sauvant, Muriel Quillard, Elisabeth Caussé, Mirande Candito, Marie-Hélène Read, Jocelyne Drai, Isabelle Garcia, Marie-Françoise Gerhardt for the SFBC Working group on homocysteine

Département de Biologie Intégrée, CHU Grenoble, BP 217, 38043 Grenoble, France

Abstract

It is now widely accepted that increased total plasma homocysteine (tHcy) is a risk factor for cardiovascular disease. Hyperhomocysteinemia can be caused by impaired enzyme function as a result of genetic mutation or vitamin B (B_2, B_6, B_9, B_{12}) deficiency. A lot of methods are now available for tHcy determination. High-pressure liquid chromatography (HPLC) with fluorescence detection are at present the most widely used methods but immunoassays, easier to use, begin to supplant in-house laboratory methods. In order to help with the choice of a main relevant homocysteine analytical method, the characteristics, performances and limits of the main current methods are reviewed. One major drawback among all these available methods is the transferability which is not clearly established to date. The impact of both inter-method and inter-laboratory variations on the interpretation of the tHcy results are discussed.

Keywords: Reviews; Homocysteine

Contents

☆This article review has been written within the frame of activity of the SFBC (French Society for Clinical Biology) working group on homocysteine (Chairperson: V. Ducros)

*Corresponding author. Tel.: +33-4-7676-9275; fax: +33-4-7676-5821.
E-mail address: vducros@chu-grenoble.fr (V. Ducros).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00497-X

1. Introduction

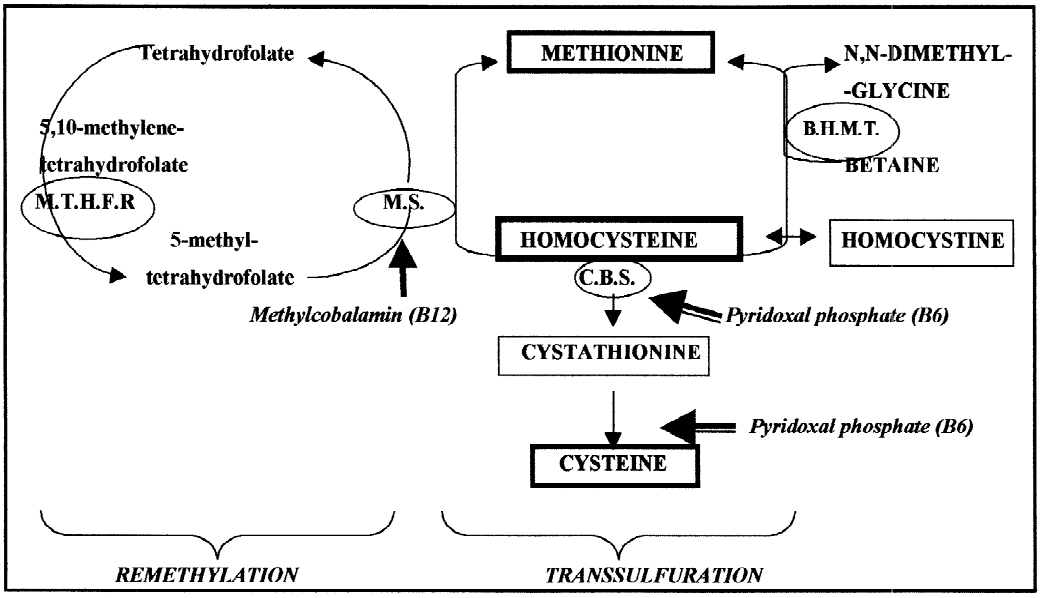
Homocysteine is an endogenous sulfhydryl amino acid, which is generated by the demethylation of methionine. Once formed, homocysteine is either irreversibly catabolized by transsulfuration to cysteine or remethylated to methionine (Fig. 1) [1,2]. These transformations are controlled by enzymatic reactions. Methionine may be regenerated by the remethylation pathway, under the action of the methionine transferase (MS). These reactions are also dependent of the amount of methylenetetrahydrofolate, which is under the control of an other enzyme, the methylenetetrahydrofolate reductase (MTHFR) and the co-factor methylcobalamine (vitamin B12).

In humans, 15–20 mmol of homocysteine are synthesized each day, but most of them are converted to cysteine, under the enzymatic control of the cystathionine β-synthase (CBS), or to methionine. Thus, the plasma homocysteine level is regulated and the normal basal concentration ranks from 5 to 15 μM, with a mean level of about 10 μM [3,4]. For upper levels, hyperhomocysteinemia is described; it is denoted as moderate (15–30 μM), intermediate (30–100 μM) or severe ($>$100 μM) [4].

An elevated plasma homocysteine level may occur as the results of inherited disorders of the main enzymes of its metabolism (mainly, mutations on the MTHFR, MS), but also as the results of nutritional deficiencies of the vitamin co-factors (B6, B12 and mainly folates), of diseases (i.e., chronic renal failure, malignancies, acute lymphoblastic leukemia, anemia, hypothyroidism, diabetes), of physiological factors (i.e., age, sex), of medications (i.e., methotrexate, phenytoin, carbamazepine, nitrous oxide, theophylline, metformin, colestipol, niacin, penicillamine, thiazide diuretics, etc.) or of lifestyle determinants (smoking, coffee consumption, alcoholism, physical activity) [1,4,5].

2. Clinical relevance of total plasma homocysteine

The first abnormality in the homocysteine metabolism was described in 1962 in mentally retarded children and associated to frequent thromboembolic



B.H.M.T. = Betaine Homocysteine Methylene Transferase
C.B.S. = Cystathionine Beta-Synthase
M.S. = Methionine Synthase
M.T.H.F.R. = 5-10, Methylenetetrahydrofolate reductase

Fig. 1. Homocysteine metabolism.

events. A few years later, McCully (1969) described the main feature of this vascular pathology, denoted homocystinuria. Afterwards, Wilcken and Wilcken mentioned, for the first time in 1976, the frequent association between abnormal plasma homocysteine level and coronary disease [4].

Nowadays, an increased attention is given to the homocysteine level in serum or plasma, since epidemiological studies (retrospective, cross-sectional, cohort and case–control studies) have suggested that this biological parameter has been related to several clinical disorders (i.e., neural tube defects, pregnancy complications, mental disorders, psychogeriatric troubles, cancer, hyperinsulinemia) [6,7] and mainly to cardiovascular diseases [2,5,8,9]. Most of the studies described a strong, dose-dependent, biologically plausible and positive association between moderate hyperhomocysteinemia and the risk of cardiovascular diseases. Moreover, homocysteine has been recently described as an independent risk factor for atherosclerotic and atherothrombotic vascular disease; moderate hyperhomocysteinemia occurs in 20–30% of patients with atherosclerosis disease [4].

An increment in fasting plasma homocysteine of 1 μM has been associated with an increase in risk of coronary heart disease of 10–20% [10]. Recent meta-analysis [5,8] showed that an increase of plasma homocysteine of 5 μM is associated to a significant increased risk of coronary heart disease (for men: OR=1.6; $IC_{95\%}$=[1.4;1.7] and for women: OR=1.8; $IC_{95\%}$=[1.3;1.9]) and also of cerebrovascular disease (OR=1.5; $IC_{95\%}$=[1.3;1.9]); for these authors, a reduction of 5 μM of plasma homocysteine level would decrease the cardiovascular risk by 1 third. For Clarke and Armitage [11], a more realistic reduction of cardiovascular risk would be 10–15%, values which are still of great clinical and economic interests; this last point has been strictly estimated by an another group [12].

Concurrently, several experiments and clinical trials have showed that an oral supplementation with folates, vitamin B6 and/or vitamin B12, normalized

the plasma homocysteine level [13–17]. A folate intake of at least 0.4–0.5 mg/day—associated or not with vitamin B6 (16.5 mg daily) and B12 (0.5 mg daily)—is described as necessary to assess an optimal homocysteine level at a population level [18,19]. Furthermore, the definitive response of the benefit of vitamin supplementation for the prevention of cardiovascular disease will be brought only by the results of the on-going randomized controlled trials of homocysteine lowering [5,11,20,21].

In this context, clinicians are more and more frequently asking for homocysteine determination performed for the exploration of cardiovascular abnormalities (mainly, in ischaemic heart disease, stroke, arterial and venous thrombotic disease) [22] and, consequently, the number of analyses of plasma homocysteine levels has increased significantly in biological laboratories. In 1999, Langman and Cole [23] even mentioned homocysteine as the ''cholesterol of the 90s''. Furthermore, the analysis of homocysteine can be performed with various analytical methods, for which the transferability is not established to date. Thus, analytical results must be always cautiously examined and sometimes the affirmation of hyperhomocysteinemia is not easy to state, in view of the few differences between the mean homocysteine levels in controls and in patients, linked to the inter-method and inter-laboratory variability [24].

Several types of methods are now available for homocysteine determination in plasma. Among them, some are nearly obsolete like radioenzymatic determination, whereas chromatographic methods are still used and immunoassays become broadly employed due to full automation. Table 1 summarizes the main characteristics of the current analytical methods. In this review, we describe each type of method and try to highlight the advantages and drawbacks of the methods for routine use (quickness, automation and sample throughput) while considering precision and accuracy of the homocysteine determination.

Table 1
Main characteristics of the current analytical methods

Method[a]	Sample pretreatment	Plasma volume (μl)	Upper limit of of linearity (μ*M*)	C.V. inter-assay for mean tHcy level (%)	Throughput	Reagent cost estimation by test[b] (euros)
GC–ID-MS	High workload +derivatization	100	30 [51]–300 [38]	2.6 [38] 5.3 [49] 5.7 [51]	96/day [130]; 160/day [28]	2.3
LC–MS–MS	High workload	100	60 [55]	5.9 [55]	40/h [55]	1.0
HPLC–FD	High workload +derivatization	60–150 50 (Bio-Rad)	50 [71]–300 [78] 100 (Bio-Rad)	3.2 4.8 (Bio-Rad)	90/day 150/day (Bio-Rad)	5.4
HPLC–ED	High workload	60 [92]	100 [92]	5.6 [92]	60/day [92]	
IEC	Medium workload	500	100–1000	7.8 [104]	25–50/day	1.73
FPIA	None	50 [128]	45–50 [128,130]	3.1	20/h Imx [128] 60/h AxSYM [129]	11.5
ICL	None	15	50	3.9	150/h	11.5
EIA	Low workload	25	50 [135]	6.2 [135]	96 tests/2.5 h	12 (test in
Enzymatic method	Low workload None	100 [137] 5 [136]	80 [137] 100 [136]	2.8 [137] 3.7 [136]	45/h [136]	duplicate)
CE-LIF	High workload +derivatization	100	200 [116]	7.8 [116]	100/day [116]	0.76

[a] GC–ID-MS, gas chromatography–mass spectrometry with isotopic dilution; LC–MS–MS, liquid chromatography with tandem mass spectrometry; HPLC-FD, high-pressure liquid chromatography with fluorescence detection; HPLC–ED, high-pressure liquid chromatography with electrochemical detection; IEC, ion-exchange chromatography; FPIA, fluorescence polarization immunoassay; ICL, chemiluminescence immunoassay; EIA, enzyme-linked immunoassay; CE-LIF, capillary electrophoresis with laser-induced fluorescence.

[b] Taking into account neither equipment cost, nor technician salary.

3. General considerations on methodology

At physiological pH, homocysteine exits in trace amounts in reduced form, whereas most homocysteine exists as various disulfide forms. About 70% is bound to albumin (protein-bound homocysteine or homocysteine–albumin mixed disulfide), whereas the remaining 30% exists as mixed disulfides with other thiols, and among them, the cysteine–homocysteine disulfide is the most abundant. Storage of whole plasma or serum causes redistribution of plasma thiols so the protein-bound fraction increases at the expense of the free, acid-soluble fraction. So, reliable determination of free homocysteine requires immediate blocking of free thiols at the sampling time. This sample preparation is not convenient for clinical settings. Therefore, determination of free homocysteine has largely been abandoned and determination of total (free+protein-bound) homocysteine is currently performed, and the term of total homocysteine (tHcy) is used for this entity [25].

Several pre-analytical variables are important to control in order to prevent artificial increase of plasma homocysteine concentrations after blood sampling. Among them, some are linked to the subject and others are linked to the sample handling itself.

3.1. Variables in relation to the subject

3.1.1. Fasting conditions

Several studies [4,26,27] have reported variations in plasma homocysteine concentrations according to high or low protein diet. After a high protein meal, plasma homocysteine concentration goes through a minimum at 4 h then increases to the initial value in 8 h. It is recommended to carry out blood sampling after a fast of 12 h and a light meal the evening before blood collection [28].

3.1.2. Influence of age, sex and ethnicity

Plasma homocysteine concentration is related to age and gender. The age-related increase in plasma homocysteine has been shown to be linked, at least partly, to the decline of glomerular function with age [29] but also to the decrease in vitamin B status with old age. Women have lower plasma homocysteine concentration than men until they reach the age of menopause. The influence of sex hormones on plasma homocysteine is uncertain [30]. However, the sex difference may be explained by the higher muscle mass in men, and so by the higher creatine synthesis in men than in women. Plasma creatinine and homocysteine concentrations are significantly correlated in healthy subjects. Data on ethnic group differences are not consistent [28]. One of the reasons may be that mutations of the encoding enzymes involved in homocysteine metabolism have different allele frequencies among the various ethnic populations; for example, the C677T mutation of methylene tetrahydrofolate reductase (MTHFR) gene, characterized by reduced enzyme activity and thermolability, occurs in about 10% of the Caucasian population, whereas it is almost absent in African and American populations. This polymorphism has been involved in increased plasma homocysteine concentrations under conditions of low folate status.

3.1.3. Effect of posture

As plasma homocysteine is to a large extent bound to albumin, a decrease of plasma homocysteine concentration has been evoked [26] and further checked [31,32] when the subject is in a supine position during venepuncture. This decrease seems to be not so negligible (around 7% of decrease) but wide ranges have been observed in the two studies.

3.1.4. Effect of venous stasis

A 2.8% increase of the plasma homocysteine concentration has been reported after 3 min tourniquet application [32]. This small increase can be generally not taken into consideration in regard to other parameters increasing plasma homocysteine concentrations.

3.1.5. Intra-individual variations

Intra-individual variations of plasma homocysteine concentrations have been reported to range from 7 to 15% [31–34]. Nevertheless, only one determination seems to be sufficient [27,34].

3.2. Variables in relation to the sample handling

3.2.1. Choice of anticoagulant

Almost all determinations of homocysteine in blood are performed on plasma instead of serum.

Because of the continuous production of homocysteine in the erythrocytes, which is released to the extracellular compartment [26], a faster centrifugation is required and sampling on dry tube is avoided. The most used anticoagulant is EDTA. Data from literature show slight variations of plasma homocysteine concentrations according to blood drawn into EDTA, sodium citrate, lithium heparin or dry tube [35–37] (Fig. 2). Other anticoagulants or additives have been recommended to stabilize plasma homocysteine concentration at room temperature such as sodium fluoride plus heparin [38], 3-deazaadenosine [39], and acidic citrate [36]. However the addition of sodium fluoride is not totally accepted as a positive effect [40], whereas the interest in acidic citrate has been recently confirmed for the conservation of total blood at room temperature during 6 h [41]. The advantage of acidic citrate is that suitable tubes are commercially available (Stabilyte®, Biopool, Umeä, Sweden), and thus, these tubes can be used for epidemiological studies when the sample handling cannot be performed quickly enough. As 3-deazaadenosine is a specific inhibitor of the conversion of *S*-adenosyl homocysteine to homocysteine, this additive cannot be used with samples used for immunoassays [42].

3.2.2. Total blood storage

Homocysteine is synthetized in several tissues. Its intracellular synthesis, metabolism, and release in the extracellular compartment settle plasma homocysteine concentration [43]. The intra-erythrocyte concentration in homocysteine has been evaluated at 0.8 μ*M*, a level 10 times less than in plasma [44]. The speed release of homocysteine from blood cells is nearly constant and is independent of plasma homocysteine concentration. After 1 h of total blood storage at room temperature, plasma or serum homocysteine concentrations increase by about 10%. This increase reaches 20–35% after 4 h and 60–75% after 24 h [26,35]. More recently, other authors have confirmed this homocysteine increase but in lower proportions: a significant increase from the fourth hour of storage at room temperature, and this increase reaches more than 25% at the tenth hour of storage [45]. When blood samples are drawn into EDTA tubes and stored on ice, total homocysteine is stable for at least 4 h [35,37].

3.2.3. Plasma storage

After centrifugation and separation of plasma from cells, homocysteine is stable: 4 days at room tem-

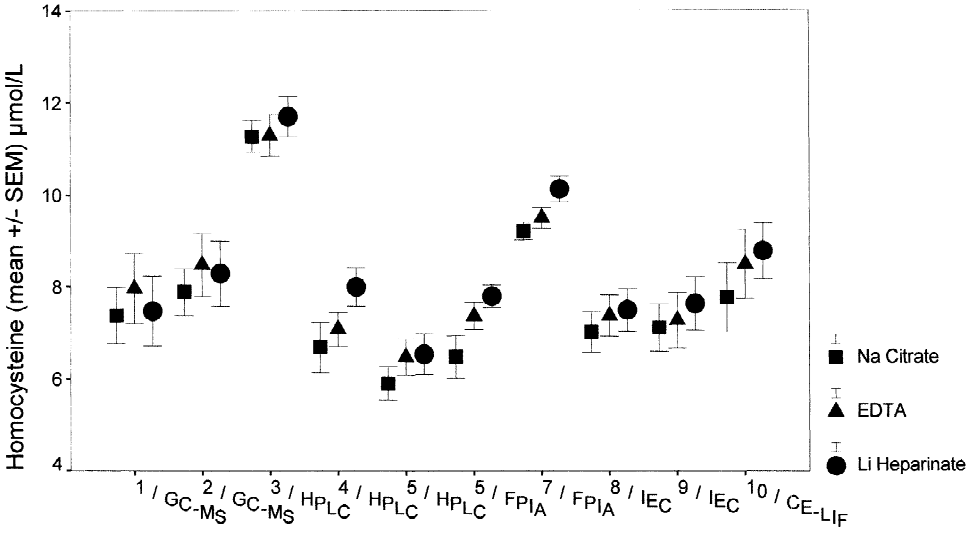


Fig. 2. Influence of the type of anticoagulants on tHcy concentration. Each laboratory analyzed its own plasma samples, so the mean was different from one laboratory to another one. In almost all laboratories, tHcy on Na citrate<tHcy on EDTA<tHcy on Li heparinate. (1,2) GC–ID-MS, (3,5) HPLC SBD-F, (4) HPLC Bio-Rad kit, (5,7) FPIA IMx, (8,9) IEC ion-exchange chromatography, (10) CE-LIF. Laboratory number 5 used two methods. Published with permission of Annales de Biologie Clinique [37].

perature, several weeks at 0–2 °C, and several years at −20 °C [4,26,35,44,46].

3.2.4. Hemolysis interference

Hemolysis does not influence plasma homocysteine concentration, at least hemolysis does not increase plasma homocysteine concentration [26,35]. A determination of homocysteine concentration in total blood sample after cell lysis at the time of blood sampling has been proposed [47].

4. Chromatographic methods

Chromatographic methods have been used extensively for tHcy determinations until the recent introduction of immunoassays. Among chromatographic methods, those coupled to mass spectrometry (GC or LC), high-pressure liquid chromatography (HPLC), and ion-exchange liquid chromatography have been distinguished.

4.1. GC–MS methods

GC–MS methods have been limited to specific laboratories because these methods are cumbersome and a mass spectrometer is essential. However GC–MS and particularly, GC–MS with isotopic dilution (ID) are reference methods which means that they are valid methods in terms of accuracy and precision. An attractive advantage is, with some modifications, the co-determination of methionine, and other compounds of the transsulfuration pathway [26]. Practically, these methods require the reduction of the disulfide bonds between homocysteine and other thiols or plasma proteins after the addition of the internal standard (Table 2). Among the reducing agents, dithiothreitol (DTT) in alkaline medium is preferable to 2-mercaptoethanol. Nearly all the methods used an ion-exchange step to separate the amino acids. For the derivatization step, two kinds of reagents have been used: *N-tert*.-butyldimethylsilyl and *N*(*O*,*S*)-alkoxycarbonyl alkyl ester derivatives. The advantages of *N*(*O*,*S*)-alkoxycarbonyl alkyl ester derivatives are they are formed at room temperature and in a very short time (2–3 min). However *N-tert*.-butyldimethylsilyl compounds give an intense ion at $m/z = M - 57$ and are more suitable in regards of lifetime of the GC column.

All GC–ID-MS used (3,3,3′,3′,4,4,4′,4′–$^{2}H_{8}$) homocystine as internal standard [48–52]. The deuterated homocystine was used to mimic the losses associated with the reduction, sample preparation, and measurement of the homocysteine in plasma. Therefore, if reduction or recovery of homocysteine is incomplete and/or re-oxidation occurs and/or

Table 2
GC–MS and LC–MS–MS methods

Methods	Internal standard	Sample preparation	Derivation	Main advantages	References
LC–MS–MS with ID	D8 homocystine	Reduction + Protein precipitation	No derivation	Very high specificity Definitive method	[55]
GC–MS with ID	D8 homocystine	Reduction + (Protein precipitation) + Ion exchange chromatography	Silylation	High specificity Reference method	[48,49] [38,51]
	D8 homocystine	Reduction + Protein precipitation + Ion exchange chromatography	Chloroformate	High specificity Reference method	[50,52]
GC–MS	Aminoethylcysteine	Reduction + Protein precipitation	Chloroformate	Medium specificity	[53]
	None	Reduction + Protein precipitation + SPME	Chloroformate	Medium specificity	[54]

SPME, solid-phase microextraction.

derivatization is not fully complete, the homocysteine measurement will not be affected because the deuterated internal standard will also undergo losses to the same extent as the natural homocysteine. The original method was reported by Stabler et al. in 1987 [48]. Some modifications such as decreasing the amount of the deuterated internal standard [38] or using a curve calibration with the deuterated internal standard [51] have been done to achieve minimal imprecision and inaccuracy. A within-run and between-run C.V. of approximately 5% is commonly attainable. The detection limit is low and currently less than 1 μ*M*. Using a quadruple GC–MS system equipped with an autosampler, we are able to prepare and to analyze about 70 samples per day.

Few GC–MS methods do not use an internal labeled standard [53,54]. The use of any internal standard other than a labeled homocystine will not necessarily account for the same losses and so not provide the same accuracy of measurement. We believe that there is a real advantage to use GC–IDMS. If the cost of the internal deuterated standard is a little bit expensive ($780 for 0.1 g), nearly 100 000 samples can be analyzed with this amount and the accuracy of the method, which is its greater quality, is preserved.

4.2. LC–MS–MS method

Recently a new method has been proposed that takes advantage of the analytical specificity and sensitivity due to the combination of stable isotope dilution and liquid chromatography–electrospray ionization (ESI) tandem mass spectrometry (LC–MS–MS) [55]. An adaptation of this method to the determination of homocysteine in blood spot has been performed [56].

This method requires the same first steps than GC–MS methods (Table 2). Samples were mixed up with the internal deuterated standard (3,3,3′,3′,4,4,4′,4′–$^{2}H_{8}$) homocystine, and the conversion of the disulfide forms into reduced homocysteine was obtained by the addition of dithiothreitol. Proteins were precipitated by the addition of formic and trifluoroacetic acid in acetonitrile. The supernatant was loaded into a liquid chromatograph and the amino acids were eluted with an acetonitrile–formic acid mobile phase. The mass spectral analysis of the eluate was performed by a LC–MS–MS benchtop triple quadrupole mass spectrometer operating in positive ion mode. Ionization was achieved with a turbo ion spray.

The calibration curves were obtained with serum overloaded with known amounts of D,L-homocysteine. The linearity was tested from 2.5 to 60 μ*M*. The intraassay precision was 4, 5.3 and 3.6% and the interassay precision was 5.9, 3.6 and 2.9% at, respectively, 3.9, 22.7 and 52.8 μ*M* of homocysteine. The correlation between the LC–MS–MS method and the HPLC assay was $y = 1.097x - 1.377$, and between LC–MS–MS and FPIA assay was $y = 1.039x + 0.025$. These data showed identical homocysteine results over the concentration range encountered in routine specimen analysis.

Except for the need for the expensive tandem mass spectrometer, this new method presents the characteristics of the GC–MS method (the use of internal standard allowed the control of the extraction step, the potentially interfering substances were removed by chromatography, the reagents used are inexpensive), and also a higher specificity due to the use of tandem mass spectrometry. But one of its main advantages is the sample preparation based on a simplified quick method which avoids the time-consuming step of derivatization (40 samples are prepared in less than 1 h including incubation time, and total instrument acquisition cycle time was 3 min per sample).

4.3. HPLC methods

Analytical procedures which use HPLC to separate homocysteine are, by far, the most commonly used ones. Unfortunately, a wide variability in the manipulation of samples, chromatographic conditions, and sample detection and quantification has made standardization impossible to date. Indeed, although all HPLC procedures for tHcy quantification require the reduction of the disulfide bond between homocysteine and other thiols or protein, followed by protein precipitation, they differ with regard to the detection system used after HPLC separation. As the detection system determines the necessity or not of doing a derivatization of homocysteine, as well as the selection of reducing agent, HPLC procedures for tHcy quantification are numerous. They can be

classified as those which make use of pre- or post-column derivatization followed by fluorescence or ultraviolet detection, and those which identify non-derivatized homocysteine by electrochemical detection (Table 3). A major advantage of all HPLC methods is that they allow the joint determination of different plasma thiol compounds.

4.3.1. With photometric detection

HPLC with photometric detection involves HPLC separation followed by post-column derivatization and spectrophotometric detection. A method for plasma tHcy that also measures cysteine, cysteinylglycine, glutathione and glutamylglycine has been described [57]. The assay involves reduction of sample with dithiothreitol, separation of the thiols by HPLC, and post-column derivatization with the 4,4′-dithiodipyridine thiol-specific labeling agent and subsequent detection of the products by ultraviolet absorption at 324 nm. The method is characterized by high precision (1.5% intra-assay; 2.5% inter-assay) and sensitivity (less than 50 n*M*). However, the method is laborious and throughput is modest. It is not the preferred method in the majority of laboratories publishing their data, but it may be useful in laboratories that do not possess a fluorescence detector.

4.3.2. With fluorescence detection

HPLC with fluorimetric detection is the most widely HPLC procedure to determine plasma tHcy concentrations. The different methods described involve precolumn derivatization with fluorogenic reagents for thiols followed by HPLC and fluorescence detection. All of them present similar analytical performances (mean within-assay C.V., 1.0%; mean between-assay C.V., 3.2%), and they can be classified on the basis of the fluorogenic reagent used. The ideal reagent should be nonfluorescent, contain no fluorescence impurities, and react rapidly and specifically with homocysteine and other thiols to form stable products. No reagent meets all these requirements, but among the fluorogenic reagents available, monobromobimane (mBrB), halogenosul-

Table 3
Available HPLC methods to measure plasma tHcy concentrations

Detection method	Advantages	Disadvantages	References
Photometric	High sensitivity High precision (1.5%; 2.5%)	-Laborious method -Modest throughput	[57]
Fluorimetric	High sensitivity High precision (1.0%; 3.2%)		
mBrB (excitation 380 nm, emission 470 nm)	Derivatization at room temperature: Full automation possible	-Fluorescent degradation products: Gradient elution chromatography	[59,61–65]
SBD-F and ABD-F (excitation 380 nm, emission 510 nm)	SBD-F and ABD-F non-fluorescents: Isocratic separation	-Derivatization at 50 or 60 °C: Full automation difficult -SBD-F adducts light sensitive	[66–68,70–78,80–84]
OPA (excitation 340 nm, emission 450 nm)	Possible determination of other amino acids	-Several manual steps: Not suitable for full automation -OPA non thiol-specific	[88–91]
Electrochemical	High sensitivity, High specificity No derivatization Full automation possible	-Precision < Fluorimetric and photometric methods (3.9%; 5.6%) -Detector maintenance	
Amperometric detector Coulometric detector Pulsed integrated amperometry			[93–95] [92,98,99] [100,102]

fonylbenzofurazans (SBD-F and ABD-F), and *o*-phthaldialdehyde (OPA) have been found practical.

Monobromobimane couples rapidly with thiols at pH 8.0 at room temperature to yield a highly fluorescent thiother [58], which makes full automation feasible [59]. However, the reagent itself, the hydrolysis products, and the impurities are fluorescent [60], giving rise to peaks that may interfere with homocysteine determination. Thus, derivatization with mBrB initially necessitated complex chromatography, such as a gradient elution program, to obtain satisfactory separation between the compounds of interest and interferences [61,62]. Then, some improved procedures have been described, in which the formation of hydrolysis products was minimized by using a shorter reaction and processing time [63] or in which the pH dependence of the elution of interfering material was exploited [65]. Finally, a method based on derivatization with mBrB followed by isocratic separation and fluorimetric detection has been recently described [66]. The most commonly used reducing agent for these methods involving derivatization with mBrB is sodium borohydride.

Halogenosulfonylbenzofurazans (SBD-F and ABD-F) are not fluorescent, their thiol adducts are stable, and no fluorescent hydrolysis products are formed. Thus, their use allows isocratic separation, resulting in clean chromatograms with no reagent peaks [66] even if the first described method was based on a gradient elution program [67]. In addition, like mBrB, SBD-F and ABD-F are thiol-specific. However, contrary to mBrB, they do not react with thiols at room temperature. Indeed, ABD-F reacts quantitatively with thiols at 50 °C at pH 8.0–9.5 for 5–10 min [68], and the less-reactive SBD-F requires more drastic conditions (pH 9.5 and 60 °C for 1 h) [69]. Thus, due to the low reactivity of SBD-F, assays based on this agent may be difficult to fully automatize. Despite this disadvantage, detection of the fluorescent SBD-F derivative after HPLC is currently the most widely used HPLC assay for plasma tHcy, at least in part because SBD-F adducts show a markedly lower retention time than ABD-F adducts [70]. Another advantage of halogenosulfonylbenzofurazans is that the stability of the adducts (for 8 h at 0 or 25 °C in the dark) allows autoinjection. Since the work of Araki and Sako [67] and Ubbink et al. [66], many modifications of these methods have appeared in the literature. Some of them include the introduction of an internal standard, such as cysteamine [71,72], mercaptopropionylglycine [73,74], and *N*-acetylcysteine [75,76]. Although the use of internal standard normally improves the recovery and precision of chromatographic methods, the extent to which the internal standard fulfils this role in tHcy analysis has been controversial [77]. The different methods described also vary with regard to the reducing agents used, which can be classified in three groups. Sulfhydryl agents, such as dithiothreitol, dithioerythreitol or 2-mercaptoethanol have been used [78], but they can form adducts with thiol-specific reagents and therefore may consume derivatization reagent [79]. Sodium or potassium borohydride are potent reductants which have also been used [74], but they can form gas and foam during the reduction procedure, rendering it a very labor-intensive step and constituting a substantial source of error [78]. Phosphine agents, such as tri-*n*-butylphosphine (TBP) and tris-(2-carboxyl-ethyl)-phosphine (TCEP) do not cause the problems mentioned for sulfhydryl agents or borohydrides. However, TBP, which is the most commonly used reducing agent [66,67,70,71,75,77,80–83], is an irritant with an unpleasant odor and is poorly soluble in water, so that it must be dissolved in dimethylformamide for use. Thus, it was recently proposed to replace TBP by TCEP, which is nonvolatile, stable, and soluble in aqueous solutions [84].

Some manufacturers have developed HPLC kits in order to optimize HPLC separation and to make this assay less laborious and also to improve sample throughput. Among them, the Bio-Rad HPLC kit (Bio-Rad Laboratories, Ivry sur Seine, France) was first developed by using ABD-F as derivatizing agent [85]. Plasma (50 μl) and internal standard were mixed with 50 μl of trialkylphosphine (reducing agent) and 100 μl of ABD-F. Samples were then incubated at 50 °C for 5 min and then at 4 °C for 5 min, followed by trichloroacetic acid precipitation of plasma proteins. The supernatant was removed for HPLC analysis after centrifugation for 5 min at 10 000 *g*. The chromatographic conditions were standardized as follows: a Bio-Rad analytical reversed-phase C_{18} column (70×3.2 mm I.D.), the temperature of the column oven was set at 45 °C, Bio-Rad mobile phase, isocratic flow-rate of 0.7 ml/min, and a spectrofluorimeter detector with the excitation and emission wavelengths of 385 and 515

nm, respectively, for detection of ABD-F thiols. Results were calculated using a Bio-Rad calibrator set at 18 μ*M*. The time retention of homocysteine was around 3 min after injection. The between-run C.V.s ($n=22$) for the Bio-Rad HPLC were 4.8 and 3.8% at tHcy concentrations of 8.4 and 32.9 μ*M*, respectively (Grenoble's laboratory data, personal communication). The limit of linearity given by the manufacturer was linear up to 100 μ*M*. The Bio-Rad HPLC method has been found to overestimate tHcy at low concentrations and underestimate tHcy at higher concentrations when compared to the in-house HPLC method, a modified Fortin and Genest HPLC method [86]. Zhang et al. [87] evaluated the DS30 tHcy system manufactured by Drew Scientific. This small HPLC system provided a 5-cm reversed-phase column and a complete reagent set for the determination of tHcy with TCEP as reducing agent and SBDF for derivatization. Analytical performance was sufficient and there was a good correlation with the HPLC method commonly used in the Center for Disease Control in Atlanta. However, the authors pointed out that peak recognition depends on cysteine and cysteinyl–glycine concentrations, that could lead to misidentification in diluted sample.

O-Phthaldialdehyde (OPA) is a fluorogenic reagent, non-thiol-specific since it reacts with all primary amino groups to form highly fluorescent isoindoles. Thus, its use requires high-resolution chromatography to obtain satisfactory separation of homocysteine from all the other amino acids present in plasma. In addition, derivatization with OPA requires previous carboxymethylation with iodoacetate, i.e., an additional manual step rendering the assay not suitable for full automation. Despite these disadvantages, different assays using OPA have been published since the possible determination of other amino acids is an attractive feature of these methods [88]. The major difference between methods is the reducing agent used. When 2-mercaptoethanol is used [89,90], it reacts with iodoacetate which must be added in excess. However, when sodium borohydride is used [91], it might increase the complexity of the sample handling of these assays, particularly due to the generation of excessive foaming.

4.3.3. With electrochemical detection

HPLC with electrochemical detection is also often used and has the major advantage that no derivatization of thiols is required prior to detection. The different methods described offers several attractive features, including increased sensitivity, high specificity, shortened run time (approximately half that for fluorescence detection), simple sample processing and autoinjection. However, the precision of these methods is inferior to that reported for photometric and fluorimetric detection, with means intra- and inter-assay C.V.s of 3.9 and 5.6%, respectively, [92]. The electrochemical assays, which have not emerged as a popular choice of methodology for plasma tHcy, can be classified on the basis of the detector used.

The reference electrochemical detection method for tHcy is the assay described by Malinow et al. [93] who modified the method of Smolin and Schneider [94]. The assay involves reduction of sample with sodium borohydride, separation of the thiols by HPLC, and detection of non-derivatized thiols with dual mercury and gold (Hg/Au) amalgam electrodes, which affords great specificity towards sulfhydryl components [95]. However, a major weakness with this assay is a possible contamination of the flow cell and electrode fouling leading to its deterioration. A gold electrode has also been used successfully [96]. The gold electrode requires less preparation and maintenance, exhibits good selectivity for thiols, and do not require the use of toxic mercury. A platinum electrode has also been tested in the BAS (BAS Technicol, Cheshire, UK) kit [97].

Coulometric detectors, which use porous carbon electrodes [92,98,99], and pulsed integrated amperometry [100] have next been developed to replace amperometric detectors and then offer a potential solution to contamination problems. However, it has been reported that the porous carbon electrode is less selective than the Hg/Au amalgam electrode, and that interference with other electroactive components in plasma such as uric and ascorbic acid may present a problem [101]. Recently, the first method based on pulsed integrated amperometry has nonetheless been modified and simplified, allowing the simultaneous determination of homocysteine and methionine [102].

4.4. Ion-exchange chromatography

Ion-exchange chromatography seems to have been

used for total homocysteine determination mainly when an amino acid analyzer was available in a laboratory. Cation-exchange chromatography was obtained with a sulfonic column. A post-column ninhydrin reaction led to a colorimetric detection at 440 and 570 nm. The determination of tHcy with a conventional amino acid analyzer requires conversion of the disulfide forms into reduced homocysteine. 2-Mercaptoethanol and dithiothreitol have been used as reducing agents. Homocysteine is protected against reoxidation by the sulfosalicylic acid used for protein precipitation, by the presence of reducing agent in the sample and by the low pH of the mobile phase [26].

Several optimizations of this ninhydrin-based determination have been published. In 1989, Andersson et al. described the elution of tHcy and methionine in 35 min using a LC5000® (Biotronic) [103]. In 1997, Candito et al. reported a short program on a Beckman 2300® analyzer, achieving separation of tHcy in 36 min, without interference of methionine, even after a methionine load [104]. In 1998, Briddon reported a complete chromatography of amino acids, including homocysteine in his reduced form [105]. Complete aminogram being achieved in about 2 h, the sample capacity, in this case, is very low. Parvy et al., using a Jeol JLC 500 AminoTac®, and our personal experience (Caen's laboratory data, personal communication), using an Hitachi L-8500A®, achieved separation of homocysteine, methionine and nor-leucine as internal standard, in 13 min, leading to a sample capacity of 50 samples/24 h [106,107].

Analytical performance (sensitivity limit ~1 μ*M*, linearity from 100 μ*M* or 1 m*M*, reproducibility ~5%) were sufficient and there is a good correlation with other HPLC or FPIA methods [106,107]. The co-determination of methionine is useful for the interpretation of severe hyperhomocysteinemia (together with determination of cysteine and cystathionine in longer programs). The sample preparation is simple and a high degree of automatization is advantageous, but the sample capacity is low (50/24 h) when compared to HPLC or immunoassays. The choice of this method is governed by practical reason in a laboratory experimented on amino acid analyzer until the sample capacity is compatible.

5. Capillary electrophoresis

In the recent years, this powerful separation method became increasingly popular. Several procedures for an accurate determination of homocysteine by capillary electrophoresis (CE) have been recently published [108–116]. Most of them used laser fluorescence as detection, but UV detection has also been proposed. Compared to HPLC, CE offers the advantages of a very small analyte volume required for the assay, an excellent resolving power, a short time analysis, no solvent used and ease of automation.

5.1. With photometric detection

Analysis of thiols and disulfides using CE has been first limited to endogenous compounds [117] or to the thiol forms [118,119]. In these procedures, thiols were underivatized [108] or derivatized with various reagents [109,113] (Table 4). For derivatization, pH and temperature conditions must be respected. Russel and Rabenstein [108] described the first quantitation of underivatized thiols and disulfides by CE with UV detection. However, the determination of underivatized thiols and disulfides presented an inherent lack of sensitivity, due to their UV absorbance characteristics. So, thiols were derivatized with the 5,5′-dithio-bis-2-nitrobenzoic acid or Ellman's reagent that improved results (shorter analysis time, lower detection limit). Plasma has been more recently considered [109,113,114]. First, reduction of disulfides with phosphine and deproteinization with sulfosalicylic or trichloracetic acid were necessary. The derivatization of reduced aminothiols (tHcy, Cys, GSH) with mBrB or ABD-F was executed before the separation of mB-thiols or ABD-thiol conjugates by CE with photometric detection (234 or 250 nm and 220 nm for mB-thiols and ABD-thiols, respectively). Kim et al. [120] demonstrated the chiral separation of D,L-homocysteine by CE with UV detection by complexing the ABD-F derivatized homocysteine forms with a γ-cyclodextrine added in buffer.

5.2. With laser fluorescence detection

Fluoresceine isothiocyanate (FITC) was first used

Table 4
Characteristics of CE methods

Detection method	Internal standard	Specimen sample preparation	Derivatization	Main advantages	References
Photometric					
200 nm	None	Aqueous standards	Underivatized		[108]
220 nm		Plasma	ABD-F	Automation possible	[109]
234 or 250 nm		Reduction + Protein precipitation	mBrB	High precision	[113,114]
Fluorimetric					
Amine function					
FMOC Ar^+		Serum or urine			[121,124]
(351 nm)		Reduction + Protein precipitation			
FITC Ar^+	Homocysteic acid	Serum		Derivatization at room	[115]
(488 nm)		Reduction + Protein precipitation		temperature	
Sulfhydryl function				*Thiol specificity*	
				High sensitivity and precision	
				Detection (pmol)	
SBD-F; ABD-F		Standard thiols		ABD-F derivatized thiols faster versus SBD-F, good stability	
				Full automation possible	[112]
6-IAF Ar^+	*N*-Acetylcysteine	Serum or plasma		Possible determination of	
(488 nm)		Reduction + Protein precipitation		other thiols	[125,126]
				Analysis by CE in 10 min	
FM Ar^+	Cysteamine	Plasma			
(488 nm)		Reduction + Protein precipitation			[111]
5-BMF Ar^+	*n*-(2-Mercapto	Plasma			
(488 nm)	propionyl)-glycine	Reduction + Protein precipitation			[110]

mBrB, monobromobimane; FMOC, 9-fluorenyl-methylchloroformate; FITC, fluoresceine isothiocyanate; SBD-F, ammonium 7-fluoro-2,1,3-benzoxadiazole 4-sulfonate; ABD-F, 4(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; 6-IAF, 6-iodoacetamidofluoresceine; FM, fluoresceine-5-maleimide; 5-BMF, 5-bromomethylfluoresceine.

[115,122–124] because it forms efficient derivatives of amino acids and was compatible with the 488-nm argon ion laser. However, a large number of FITC-buffer by-products are made and interfere with the electropherogram. Recently, thiols in plasma samples were quantified for routine determination [110,111,125,126]. Various thiol-specific fluorogenic reagents: 6-iodoacetamidofluoresceine (6-IAF) [116], 5-bromomethylfluoresceine (5-BMF) [110], or fluoresceine-5-maleimide (FM) [111] were used allowing different thiol derivatives. 5-BMF reacts slowly with sulfur of peptides and proteins and the authors proposed this analytical reagent for sulfur-containing compounds, particularly for methionine [110,127]. Methionine is not evaluated with other fluorescent reagents. tHcy and glutathione were determined with these three derivatized procedures but future studies seem necessary for the other thiols. However, all these laboratories have a CE system with an argon-ion laser (488/520 nm) for thiol detection. They concluded that capillary electrophoresis can be a simple, fast, accurate and very sensitive method, suitable for routine determinations of plasma homocysteine in clinical studies.

6. Immunoassays

The increasing clinical interest for measuring homocysteine in the 1990s called for rapid, automated methods with high sample throughput more suitable for routine use. Measurement of homocysteine in plasma by an immunoassay appeared to have become the preferred analytical approach.

Protein-bound homocysteine is reduced to free homocysteine and enzymatically converted to *S*-adenosyl-L-homocysteine (SAH) by the action of *S*-adenosyl-L-homocysteine hydrolase (SAHase). The

enzymatic conversion of homocysteine to *S*-adenosyl-L-homocysteine by SAH hydrolase is the basis of the immunoassay first described by Shipchandler and Moore in 1995 [128]. This principle is common to all the immunoassays and, depending of the detection mode, three groups of methods are now available (Table 5). All these methods have a limited dynamic range set at 50 μ*M* and only L-homocysteine can be analyzed. By contrast with many other analytes, all homocysteine immunoassays share, to date, a single commercially available antibody (raised against the SAH compound), and the SAH hydrolase, licensed from Axis (Axis Biochemicals, Grünerløkka, Norway). Indeed, the SAH antibody is the same for all immunoassays. This is an advantage for homogeneity of the results obtained with these different methods, as immunoassays are usually known to cause serious problems of standardization. However, this advantage becomes a main drawback in terms of cost. Despite their low labor cost, immunoassays have a high reagent cost compared to chromatographic methods.

6.1. With fluorescence polarization immunoassay (FPIA)

The SAH compound can be quantified, in a fully automated manner, by using reagents specifically developed for this assay, an anti-SAH antibody and a fluoresceinated tracer compound. The recording of changes in fluorescence polarization resulting from competitive binding of SAH and tracer at the antibody binding site allows for highly precise estimation of tHcy in blood. These reagents are developed on the IMx® and AxSYM® automated analyzers (Abbott Laboratories, Abbott Park, IL). The methodology of the AxSYM assay is very closed to those of IMx assay, but is less time-consuming (for example, AxSYM can process up to 60 samples in 1 h compared to only 20 on IMx) [129].

IMx assay is a fast method (less than 7 min per sample), in which a manual pretreatment step is not required. Calibration, for each lot of kit reactives, uses *S*-adenosyl-homocysteine: seven points, from 0 to 50 μ*M*. Internal quality control uses three levels: 7, 12.5 and 25 μ*M*. The intralaboratory coefficient of variation (C.V.) is given as $<5\%$, but with a 2 to 3% bias when compared with two HPLC and GC methods [130], while no difference was detected from GC in another study [131], which observed higher C.V.. In a study, compared with an HPLC method and an enzyme-linked immunoassay, FPIA showed the lowest within-run and between run C.V. [132].

The mean recovery of homocysteine added (from 6 to 26 μ*M*) to two plasma samples: respectively, 7.3–16.2 μ*M* was 97.1 and 99.9% intralaboratory [128]. There was a correct correlation of results with an HPLC method: $r^2=0.82$ (fluorescent reagent: ABD-F), while a good correlation with GC–MS was found: $r^2=0.95$, method considered as a reference method [83,133]. This last study considers that FPIA may become a method of choice in routine homocysteine assay. It is also reported as giving the best

Table 5
Main characteristics of immunoassays for the quantitative determination of plasma homocysteine

Name	FPIA	ICL	EIA
Principle		Competitive immunoassays	
No. of antibodies	One	One	Two
Antibody label	None (capture antibody)	Enzymatic label: ALP	Enzymatic label: HRP
Detected signal	Fluorescence	Luminescence	Colorimetry
Target of immunoreaction	SAH	SAH	SAH
Dynamic range	2–50 μ*M*	0.5–50 μ*M*	2–50 μ*M*
Sample volume required	50 μl	15 μl	25 μl
Assay duration	60 min	70 min	150 min
Adjustment interval	≥ 2 weeks	2 weeks	Each assay

ALP, alkaline phosphatase; EIA, enzyme immunoassay; FPIA, fluorescence polarized immunoassay; HRP, horseradish peroxidase; ICL, immuno-chemiluminescence; SAH, *S*-adenosyl-homocysteine.

performance [134]. No cross-reactivity is observed with L-cysteine, nor with methionine (negligible for this last at higher than physiological concentrations) and, actually, few pharmacological interferences are known, but it is necessary to control the blank values: in the case of high values, with alarm, as the homocysteine concentration is wrong.

6.2. With chemiluminescence immunoassay (ICL)

The SAH compound of the sample can be determined by chemiluminescence on the Immulite 2000 analyzer (DPC, Los Angeles, CA), using reagents specifically developed for this assay by DPC. These reagents are, firstly, SAH bound to polystyrene beads, acting as a competitor, and secondly, anti-SAH antibody labeled with alkaline phosphatase (ALP), generating the chemiluminescent signal. After incubation of reagents with the sample SAH, elimination of [antibody–sample SAH] complexes is achieved by on-board centrifugation of the reaction vials, and the residual antibody (bound to SAH beads) is quantified by enzymatic transformation (ALP) of a chemiluminescent substrate. Light is detected by a luminometer and the absorbance is inversely related to the concentration of tHcy. All these reaction steps, as well as plasma reduction and enzymatic transformation of homocysteine into SAH, are fully automatized.

Calibration occurs twice a month and uses *S*-adenosyl-homocysteine in aqueous–proteic solution. Internal quality control uses three levels with respective values near 7, 12 and 25 μ*M*. The within run variation for tHcy values of 4.2, 13.9, and 27.7 μ*M* in 20 parallel determinations were, respectively, 9.9, 7.0 and 5.4% (Rouen's laboratory data, personal communication). The between run variation for tHcy levels of 4.2, 13.9 and 27.7 μ*M* in 20 parallel determinations realized on 5 days were, respectively, 8.2, 3.9 and 4.3% (Rouen's laboratory data, personal communication). Among immunoassays, ICL is the more recent. This system presents the same advantages as FPIA: fully automatized method, low quantity of plasma needed, fast method (results delivered 70 min after automate loading). Actually, there is a very good agreement between results obtained with FPIA or ICL methods on patient samples (Rouen's laboratory data, personal communication).

6.3. With enzyme-linked immunoassay (EIA)

The solid-phase immunoassay is based on competition between SAH in the sample and the immobilizad SAH bound to the microtiter plate for binding sites on a monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labelled with the enzyme horseradish peroxidase is added. The reaction is stopped by adding sulfuric acid. The peroxidase activity is measured spectrophotometrically after addition of substrate, tetramethylbenzidine; the absorbance is inversely related to the concentration of tHcy. This method is now commercially available from Axis Biochemicals.

Calibrators were prepared by dissolving SAH in assay buffer. The calibration curve is made by fitting the concentration–absorbance data with a four-parameter logistic function. The quantification limit of the Axis homocysteine EIA is <0.5 μ*M* with a C.V.$<20\%$. Mean recoveries of known amounts of SAH and crystalline L-homocystine added to plasma samples were, respectively, 101 and 92%. The within assay variation was estimated after analyzing three samples containing 8.1, 13.6, and 27.3 μ*M* tHcy in 21 parallel determinations and the C.V.% were, respectively, 5.0, 4.3 and 5.5%. The between assay variation is based on 21 succeeding analytical set-ups performed over 3 days and the C.V.% was, respectively, of 5.4, 6.2 and 8.2% [135]. The cross-reactivity of the monoclonal SAH antibody against interfering compounds was tested by adding the compounds to the calibrators or to the samples. The compounds tested were cysteine, *S*-adenosyl-L-methionine (SAM), cystathionine, methionine, glutathione thiolactone and adenosine. SAM was the only compound to substantially affect positively the performance of the assay; when present in sample at >10 μ*M*. The fact that only SAM affects the determination of homocysteine is explained by the structural resemblance to SAH and the corresponding recognition by the anti-SAH antibody.

The microtiter plate version of the EIA has a very high potential capacity with no manual deproteinization or cumbersome chromatography step. Multiple plates can be analyzed simultaneously and the method can be partially or fully automated with inexpensive apparatus available in many laboratories.

7. Enzymatic assays

In order to avoid the need for a specialized equipment, enzymatic colorimetric assays have been developed more recently. Tan et al. [136]. have developed, on a 96-well microtiter plate, a single enzyme tHcy assay based on a highly specific homocysteine α,γ-lyase (rHcyase). After reduction of all species containing homocysteine, rHCYase specifically converts in a first step, homocysteine to α-ketobutyrate, ammonia, and H_2S and in a second step, the H_2S combines with *N*,*N*-dibutylphenylene diamine to form 3,7-bis(dibutyl amino)phenothiazine-5-ium chloride, which is highly fluorescent. Matsuyama et al. [137] have developed another enzymatic reaction by using homocysteine methyltransferase. This enzyme transfers the methyl group of D-methionine methylsulfonium to Hcy, leading to the generation of L-methionine and D-methionine. In a second step, D-amino acid oxidase oxidizes D-methionine with the simultaneous production of hydrogen peroxide, followed by oxidation of 10-(carboxymethyl-aminocarbonyl)-3,7-bis(dimethylamino) phenothiazine to yield methylene blue with an absorbance maximum at 660 nm. The capture of the remaining dithiothreitol is performed by addition of *N*-ethylmaleimide in the second step, enabling the oxidation of the redox indicator and the generation of the colored product. This assay is applicable to all colorimetric-based clinical chemistry analyzers.

These two methods have a higher linearity than the immunoassays, 100 and 80 μ*M*, respectively. Less steps than for EIA are needed, three and two, respectively, but manual pretreatment of the samples is required for the second method as well as the background determination of D-amino acids. The size of blood samples (5 μl) is very low for the enzymatic fluorescent assay and allowed assay of plasma derived from a finger prick.

8. Methods evaluation

As previously described, a lot of methods are now available for tHcy determination. Commercial kits using immunoassays, easier to use, begin to supplant in-house laboratory methods. A few more or less large studies have already evaluated the method differences in terms of imprecision and accuracy [85,130,131,133,138] but none has evaluated the impact of analytical variability on the clinical interpretation of biological results. The lack of standardization for tHcy measurements is harmful for the determination of a biological cut-off of hyperhomocysteinemia. After reviewing the main criteria for establishing analytical performances between methodologies, we discuss this point.

8.1. Definitive, reference and field methods

Until now, the GC–MS with ID (use of deuterated internal standard) was defined as the definitive method and often chosen in comparison studies as the reference method [107,130,131,133]. However, from the arrival of MS–MS benchtop instruments in different laboratories with the development of protein research, LC–MS–MS with ID has become the candidate definitive method. So, in the area of standardization, LC–MS–MS with ID should be used as the definitive method because this method presents the highest degree of accuracy and precision, and GC–MS with ID should be a reference method. All other types of methods (HPLC, immunoassays, ion-exchange chromatography) should be considered as field methods if they are accurate, robust and practical.

8.2. Accuracy

As tHcy is considered as a risk factor for cardiovascular disease, we need to use methods with high accuracy and high precision to evaluate this risk factor. A few reports have shown some comparison studies between a large number of methods used for homocysteine measurements [107,130,131,133]. All these studies for FPIA and almost all for EIA agree on a negative bias when these immunoassays are compared to the results given by GC–MS with ID. One cause of this inaccuracy can be the lack of one accepted reference substance to calibrate homocysteine determinations. However, as immunoassays have their own calibrants and these calibrants are not detected by the other methods of homocysteine

determination, a great difficulty is induced to explore the bias between the different types of methods. We recently compared six different methods (GC–ID-MS, HPLC (TBP/SBD-F or TCEP/ABD-F), FPIA, EIA, EC-LIF and ion-exchange chromatography) used by nine laboratories to determine plasma homocysteine in 41 human samples [107]. One GC–ID-MS was chosen as a reference method on the basis of its analytical performances. Under these conditions, the requirement for minimum performance (bias< 12.8%) as defined in Ref. [131] was not met by two laboratories (one using an FPIA method, and the other the CE-LIF method); but even GC–ID-MS is a reference method, we need to perform tHcy method comparison studies against a highly accurate reference method (LC–MS–MS with ID) before reaching a final conclusion. Moreover, as some methods were used by only one laboratory, it is difficult to conclude whether the observed differences between the methods are method-specific or part of the among-laboratory variation, as was seen with the FPIA [107].

8.3. Imprecision

The requirement for minimum performance in terms of analytical imprecision is 5.3% [131]. The analytical imprecision of each method used to determine tHcy is already close to the limits of the minimum performance and all the studies for tHcy comparison methods [107,130,131,133] showed that the evaluation of among-laboratory imprecision does not reach a such cut-off; currently a 9% among-laboratory imprecision was obtained. So the inter-method and intra-laboratory variations in tHcy determination are often too high to be acceptable. Among inter-laboratory studies, several studies are in agreement that between-laboratory variations are too high. One study [138] has indicated that the variability between laboratories contributed largely to the bias between laboratories; another study [131] has indicated that between-laboratory variations within one method can exceed between-method variations. So the variability among assays makes the interpretation of the tHcy results difficult in terms of cardiovascular risk for the patient associated with hyperhomocysteinemia.

8.4. Impact of inter-method variation on the interpretation of the biological result

The inter-method variation on tHcy determination has also be found too high to be able to show the differences between patients with coronary atherosclerosis and controls [86]. In our interlaboratory study, we have underlined this problem by two arguments [107]. First, no tested method is able to recover small added amounts of L-homocystine (+1 μM) with precision. Secondly, considering plasma tHcy values arranged in two groups according to the upper reference limit for plasma tHcy value commonly set at 15 μM, in the tHcy group values found less or equal to 15 μM by GC–ID-MS, a maximum 25% of the values are found higher with the other methods and in the group of tHcy values found superior to 15 μM by GC–ID-MS, a maximum 30% of the values are found lower with the other methods. So we point out that the inter-method and inter-laboratory standardization is a pre-requirement to the establishment of a biological cut-off to interpret the results. As others, we are fully aware of the need for a definitive reference method and the introduction of standard reference material, as well as an external quality assessment program, to make progress in the standardization of tHcy determinations.

8.5. Cost

The measurement of plasma tHcy is still a relatively expensive analysis. In-house chromatographic methods have low reagent cost but high labor cost. Inversely, reagents for automated immunoassays are more expensive, but labor costs are lower.

9. Conclusion

Several comparison method studies [130–132,139–141] are in agreement in claiming that FPIA methods give comparable results to HPLC or GC–MS methods. EIA methods present a higher imprecision than FPIA, in particular when manual sampling is used [107,130]. ICL methods are too new to have been tested enough, but seem promising. So among methods, immunoassays are the more

convenient especially for large screening programs of hyperhomocysteinemia detection. But knowing their limitations, high values need to be checked by using reference methods in a specialized laboratory. Chromatographic methods allowing co-determination of other amino acids are still useful for diagnosis of cases caused by enzyme defects. However, the establishment of a primary reference material for homocysteine standardization will be beneficial to harmonize tHcy determination across laboratories.

Addendum

Members of SFBC working group on homocysteine (in alphabetic order):

- Mirande Candito, Laboratoire de Biochimie, Hôpital Pasteur, Nice
- Elisabeth Caussé, Laboratoire de Biochimie, CHU Rangueil, Toulouse
- Rémy Couderc, Laboratoire de Biochimie, Hôpital Armand Trousseau, Paris
- Karine Demuth, Laboratoire de Biochimie, Hôpital Européen Georges Pompidou, Paris
- Jocelyne Drai/Isabelle Garcia, Laboratoire de Biochimie, CH Lyon-Sud
- Véronique Ducros, Département de Biologie Intégrée, CHU Grenoble
- Anne-Marie Gachon, Laboratoire de Biochimie, Hôtel-Dieu, Clermont-Ferrand
- Marie-Françoise Gerhardt, Laboratoire de Biochimie, Hôpital Saint-Joseph, Paris
- Muriel Quillard, Laboratoire de Biochimie Médicale, Hôpital Charles Nicolle, Rouen
- Marie-Hélène Read, Laboratoire de Biochimie Pédiatrique, Département Génétique et Reproduction, CHU Caen
- Marie-Pierre Sauvant, Laboratoire Environnement et Santé Publique, Faculté de Pharmacie, Clermont Ferrand

References

[1] V. Fonseca, S.C. Guba, L.M. Fink, Endocrine Rev. 20 (1999) 738.
[2] G.J. Hankey, J.W. Eikelboom, Lancet 354 (1999) 407.
[3] D.A.J. Brouwer, H.T.M.E. Welten, D.J. Reijngoud, J.J. van Doormaal, F.A.J. Muskiet, Clin. Chem. 44 (1998) 1545.
[4] H. Refsum, P.M. Ueland, O. Nygard, S.E. Vollet, Annu. Rev. Med. 49 (1998) 31.
[5] J.W. Eikelboom, E. Lonn, J. Genest, G. Hankey, S. Yusuf, Ann. Intern. Med. 131 (1999) 363.
[6] Y.I. Kim, Nutr. Rev. 57 (1999) 314.
[7] H. Bar-On, M. Kidron, Y. Friedlander, A. Ben-Yehuda, J. Selhub, I.H. Rosenberg, G. Friedman, J. Intern. Med. 247 (2000) 287.
[8] C.J. Boushey, S.A.A. Beresford, G.S. Omenn, A.G. Motulsky, J. Am. Med. Assoc. 274 (1995) 1049.
[9] G.H.J. Boers, Thromb. Haemost. 78 (1997) 520.
[10] P. Verhoef, M.J. Stampfer, E.B. Rimm, Curr. Opin. Lipid. 9 (1998) 17.
[11] R. Clarke, J. Armitage, Semin. Thromb. Hemost. 26 (2000) 341.
[12] J. Hornberger, Am. J. Public Health 88 (1998) 61.
[13] L.J. Appel, E.R. Miller, S.H. Jee, R. Stolzenberg-Solomon, P.H. Lin, T. Erlinger, M.R. Nadeau, J. Selhub, Circulation 102 (2000) 852.
[14] E.G.J. Vermeulen, C.D.A. Stehouwer, J.W.R. Twisk, M. van den Berg, S.C. de Jong, A.J.C. Mackaay, C.M.C. van Campen, F.C. Visser, C.A.J.M. Jakobs, E.J. Bulterijs, J.A. Rauwerda, Lancet 355 (2000) 517.
[15] D.W. Jacobsen, Clin. Chem. 44 (1998) 1833.
[16] M.R. Malinow, P.B. Duell, D.L. Hess, P.H. Anderson, W.D. Kruger, B.E. Phillipson, R.A. Gluckman, P.C. Block, B.M. Upson, New Engl. J. Med. 338 (1998) 1009.
[17] E. Brunner, I. White, M. Thorogood, A. Bristow, D. Curle, M. Marmot, Am. J. Public Health 87 (1997) 1415.
[18] Homocysteine Lowering Trialists' Collaboration, Br. Med. J. 316 (1998) 894.
[19] M.R. Malinow, A.G. Bostom, R.M. Krauss, Circulation 99 (1999) 178.
[20] R. Clarke, J. Armitage, J Cardiolvasc. Risk 5 (1998) 249.
[21] G. Sunder-Plassmann, W.C. Winkelmayer, M. Fodinger, Exp. Opin. Invest. Drugs 9 (2000) 2637.
[22] W.G. Christen, U.A. Ajani, R.J. Glynn, C.H. Hennekens, Arch. Intern. Med. 160 (2000) 422.
[23] L.J. Langman, D.E.C. Cole, Clin. Chim. Acta 286 (1999) 63.
[24] E. Arnesen, H. Refsum, K.H. Bonaa, P.M. Ueland, O.H. Forde, J.E. Nordrehaug, Int. J. Epidemiol. 24 (1995) 704.
[25] P.M. Ueland, Clin. Chem. 41 (1995) 340.
[26] P.M. Ueland, H. Refsum, S.P. Stabler, M.R. Malinow, A. Andersson, R.H. Allen, Clin. Chem. 39 (1993) 1764.
[27] J.H. Graeme, J.W. Eikelboom, Lancet 354 (1999) 407.
[28] K. Rasmussen, J. Moller, Ann. Clin. Biochem. 37 (2000) 627.
[29] L. Norlund, A. Grubb, G. Fex, H. Leksell, J.E. Nilsson, H. Schenck, B. Hultberg, Clin. Chem. Lab. Med. 36 (1998) 175.
[30] M.G. Wouters, M.T. Moorrees, M.J. van der Mooren, H.J. Blom, G.H. Boers, L.A. Schellekens, C.M. Thomas, T.K. Eskes, Eur. J. Clin. Invest. 25 (1995) 801.
[31] P. Thirup, S. Ekelund, Clin. Chem. 45 (1999) 1280.
[32] K. Rasmussen, J. Moller, M. Lyngbak, Clin. Chem. 45 (1999) 1850.
[33] R. Clarke, P. Woodhouse, A. Ulvik, C. Frost, P. Sherliker, H. Refsum, P.M. Ueland, K.T. Khaw, Clin. Chem. 44 (1998) 102.

[34] U.C. Garg, Z.J. Zheng, A.R. Folsom, Y.S. Moyer, M.Y. Tsai, P. McGovern, J.H. Eckfeldt, Clin. Chem. 43 (1997) 141.
[35] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[36] H.P.J. Willems, G.M.J. Bos, W.B.J. Gerrits, M. den Heijer, S. Vloet, H.J. Blom, Clin. Chem. 44 (1998) 342.
[37] V. Ducros, M. Candito, E. Caussé, R. Couderc, K. Demuth, M.E. Diop, J. Drai, A.M. Gachon, I. Garcia, M.F. Gerhardt, C. Philippe-Bourgeois, M.H. Read, M.P. Sauvant, Ann. Biol. Clin. 59 (2001) 33.
[38] J. Moller, K. Rasmussen, Clin. Chem. 41 (1995) 758.
[39] F. Al-Khafaji, A. Bowron, A.P. Day, J. Scott, D. Stansbie, Ann. Clin. Biochem. 35 (1998) 780.
[40] M.P. Hughes, T.H. Carlson, M.K. McLaughlin, D.D. Bankson, Clin. Chem. 44 (1998) 2204.
[41] J.F. Salazar, B. Herbeth, G. Siest, P. Leroy, Clin. Chem. 45 (1999) 2016.
[42] W.W. Woltersdorf, A. Bowron, A.P. Day, J. Scott, D. Stransbie, Ann. Clin. Biochem. 36 (1999) 533.
[43] A. Andersson, A. Isaksson, B. Hultberg, Clin. Chem. 38 (1992) 1311.
[44] J.B. Ubbink, W.J.H. Vermaak, A. Van den Merwe, P.J. Becker, Clin. Chim. Acta 207 (1992) 119.
[45] P. Houzé, M. Dussaucy, Y. Courties, B. Bousquet, Ann. Biol. Clin. 57 (1999) 611.
[46] B. Israelsson, L. Brattström, H. Refsum, Scand. J. Clin. Lab. Invest. 53 (1993) 465.
[47] R. Probst, R. Brandl, M. Blümke, D. Neumeier, Clin. Chem. 44 (1998) 1567.
[48] S.P. Stabler, P.D. Marcell, E.R. Podell, R.H. Allen, Anal. Biochem. 162 (1987) 185.
[49] S.P. Stabler, J. Lindenbaum, D.G. Savage, R.H. Allen, Blood 81 (1993) 3404.
[50] J. Pietzsch, U. Julius, M. Hanefeld, Clin. Chem. 43 (1997) 2001.
[51] V. Ducros, D. Schmitt, G. Pernod, H. Faure, B. Polack, A. Favier, J. Chromatogr. B 729 (1999) 333.
[52] Y. Shinohara, H. Hasegawa, K. Tagoku, T. Hashimoto, J. Chromatogr. B 758 (2001) 283.
[53] J.O. Sass, W. Endres, J. Chromatogr. A 776 (1997) 342.
[54] S.W. Myung, M. Kim, H.K. Min, E.A. Yoo, K.R. Kim, J. Chromatogr. B 727 (1999) 1.
[55] M.J. Magera, J.M. Lacey, B. Casetta, P. Rinaldo, Clin. Chem. 45 (1999) 1517.
[56] K. Gempel, K.D. Gerbitz, B. Casetta, M.F. Bauer, Clin. Chem. 46 (2000) 122.
[57] A. Andersson, A. Isaksson, L. Brattström, B. Hultberg, Clin. Chem. 39 (1993) 1590.
[58] R.C. Fahey, G.L. Newton, R. Dorian, E.M. Kosower, Anal. Biochem. 111 (1981) 357.
[59] A. Pastore, R. Massoud, C. Motti, A. Lo Russo, G. Fucci, C. Cortese, G. Federici, Clin. Chem. 44 (1998) 825.
[60] W. Baeyens, G. van der Weken, B. Lin Ling, P. de Moerloose, Anal. Lett. 21 (1988) 741.
[61] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35 (1989) 1921.
[62] D.W. Jacobsen, V.J. Gatautis, R. Green, Anal. Biochem. 178 (1989) 208.
[63] D.W. Jacobsen, V.J. Gatautis, R. Green, K. Robinson, S.R. Savon, M. Secic, J. Ji, J.M. Otto, L.M. Jr Taylor, Clin. Chem. 40 (1994) 873.
[64] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[65] A.R. Ivanov, I.V. Nazimov, L. Baratova, A.P. Lobazov, G.B. Popkovich, J. Chromatogr. 913 (2001) 315.
[66] J.B. Ubbink, W.J.H. Vermaak, S. Bissbort, J. Chromatogr. 565 (1991) 441.
[67] A. Araki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[68] B.L. Ling, C. Dewaele, W.R.G. Baeyens, J. Chromatogr. 553 (1991) 433.
[69] K. Imai, T. Toyo'oka, Y. Watanabe, Anal. Biochem. 128 (1983) 471.
[70] A. Feussner, B. Rolinski, N. Weiss, T. Deufel, G. Wolfram, A.A. Rosche, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 687.
[71] K. Kuo, R. Still, S. Cale, I. McDowell, Clin. Chem. 43 (1997) 1653.
[72] C.M. Pfeiffer, D.L. Huff, E.W. Gunter, Clin. Chem. 45 (1999) 290–292.
[73] B. Vester, K. Rasmussen, Eur. J. Clin. Chem. Clin. Biochem. 29 (1991) 549.
[74] I. Fermo, C. Arcelloni, G. Mazzola, A. D'Angelo, R. Paroni, J. Chromatogr. B 719 (1998) 31.
[75] N.P.B. Dudman, X.W. Guo, R. Crooks, L. Xie, J.S. Silberberg, Clin. Chem. 42 (1996) 2028.
[76] P. Durand, L.J. Fortin, S. Lucier-Cacan, J. Davignon, D. Blache, Clin. Chim. Acta 252 (1996) 83.
[77] R. Accinni, J. Campolo, S. Bartesaghi, G. De Leo, V. Lucarelli, C.F. Cursano, O. Parodi, J. Chromatogr. 828 (1998) 397.
[78] V. Rizzo, L. Montalbetti, M. Valli, T. Bosoni, E. Scoglio, R. Moratti, J. Chromatogr. B 706 (1998) 209.
[79] W. Baeyens, G. van der Weken, P. de Moerloose, Chromatographia 23 (1987) 717.
[80] L.J. Fortin, J. Genest, Clin. Biochem. 28 (1995) 155.
[81] N. Jacob, L. Guillaume, L. Garçon, M.J. Foglietti, Ann. Biol. Clin. 55 (1997) 583.
[82] G. Minniti, A. Piana, U. Armani, R. Cerone, J. Chromatogr. 828 (1998) 401.
[83] F. Ceppa, I. Drouillard, D. Chianea, P. Burnat, F. Perrier, C. Vaillant, Y. El Jahiri, Ann. Biol. Clin. 57 (1999) 474.
[84] B.M. Gilfix, D.W. Blank, D.S. Rosenblatt, Clin. Chem. 43 (1997) 687.
[85] V.C. Dias, F.J. Bamforth, M. Tesanovic, M.E. Hyndman, H.G. Parsons, G.S. Cembrowski, Clin. Chem. 44 (1998) 2199.
[86] H.H. Yu, R. Joubran, M. Asmi, T. Law, A. Spencer, M. Jouma, N. Rifai, Clin. Chem. 46 (2000) 258.
[87] M. Zhang, E. Gunter, C. Pfeiffer, Clin. Chem. 47 (2001) 966.
[88] Y.V. Tcherkas, A.D. Denisenko, J. Chromatogr. A 913 (2001) 309.
[89] K. Hyland, T. Bottiglieri, J. Chromatogr. 579 (1992) 55.
[90] C. Carducci, M. Birarelli, M. Nola, I. Antonozzi, J. Chromatogr. A 1846 (1999) 93.
[91] I. Fermo, C. Arcelloni, E. Devecchi, S. Vigano, R. Paroni, J. Chromatogr. 593 (1992) 171.

[92] J.L. D'Eramo, A.E. Finkelstein, F.O. Boccazzi, O. Fridman, J. Chromatogr. 720 (1998) 205.
[93] M.R. Malinow, S.S. Kang, L.M. Taylor, P.W. Wong, B. Coull, T. Inahara, D. Mukerjee, G. Sexton, B. Upson, Circulation 79 (1989) 1180.
[94] L.A. Smolin, J.A. Schneider, Anal. Biochem. 168 (1988) 374.
[95] D.L. Rabenstein, G.T. Yamashita, Anal. Biochem. 180 (1989) 259.
[96] L.L. Wu, J. Wu, S.C. Hunt, B.C. James, G.M. Vincent, R.R. Williams, P.N. Hopkins, Clin. Chem. 40 (1994) 552.
[97] B.P. Solomon, C. Duda, A.C. Mann, Biochem. Soc. Trans. 26 (1998) S21.
[98] S.C. Martin, I. Tsakas-Ampatzis, W.A. Bartlett, A.F. Jones, Clin. Chem. 45 (1999) 150.
[99] R. Accinni, S. Bartesaghi, G. De Leo, C.F. Cursano, G. Achilli, A. Loaldi, C. Cellerino, O. Parodi, J. Chromatogr. A 896 (2000) 183.
[100] J. Evrovski, M. Callaghan, D.E.C. Cole, Clin. Chem. 41 (1995) 757.
[101] J.B. Ubbink, Semin. Thromb. Hemost. 26 (2000) 233.
[102] D.E.C. Cole, D.C. Lehotay, J. Evrovski, Clin. Chem. 44 (1998) 188.
[103] A. Andersson, L. Brattstrom, A. Isaksson, B. Israelsson, B. Hultberg, Scand. J. Clin. Lab. Invest. 49 (1989) 445.
[104] M. Candito, P. Bedoucha, M.H. Mahagne, G. Scavini, M. Chatel, J. Chromatogr. B 692 (1997) 213.
[105] A. Briddon, Amino Acids 15 (1998) 235.
[106] P. Parvy, J. Bardet, Y. Ohta, D. Rabier, J. Aupetit, P. Kamoun, Ann. Biol. Clin. 58 (2000) 212.
[107] V. Ducros, M. Candito, E. Caussé, R. Couderc, K. Demuth, M.E. Diop, J. Drai, M.F. Gerhardt, M. Quillard, M.H. Read, M.P. Sauvant, Ann. Biol. Clin. 60 (2002) 421.
[108] J. Russell, D.L. Rabenstein, Anal. Biochem. 242 (1996) 136.
[109] S.H. Kang, J.W. Kim, D.S. Chung, J. Pharm. Biomed. Anal. 15 (1997) 1435.
[110] G. Vecchione, M. Margaglione, E. Grandone, D. Colaizzo, G. Cappucci, I. Fermo, A. D'Angelo, G. Di Minno, Electrophoresis 20 (1999) 569.
[111] C. Chassaing, J. Gonin, C.S. Wilcox, I.W. Wainer, J. Chromatogr. B 735 (1999) 219.
[112] S.H. Kang, W. Wei, E.S. Yeung, J. Chromatogr. B 744 (2000) 149.
[113] A.R. Ivanov, I.V. Nazimov, L.A. Baratova, J. Chromatogr. A 895 (2000) 157.
[114] A.R. Ivanov, I.V. Nazimov, L.A. Baratova, J. Chromatogr A 895 (2000) 167.
[115] E. Caussé, R. Terrier, S. Champagne, M. Nertz, P. Valdiguié, R. Salvayre, F. Couderc, J Chromatogr. A 817 (1998) 181.
[116] E. Caussé, N. Siri, H. Bellet, S. Champagne, C. Bayle, P. Valdiguié, R. Salvayre, F. Couderc, Clin. Chem 45 (1999) 412.
[117] J.S. Stamler, J. Localzo, Anal Chem. 64 (1992) 779.
[118] B.L. Ling, W.R.G. Baeyens, Anal. Chim. Acta 255 (1991) 283.
[119] B.L. Hogan, E.S. Yeung, Anal. Chem. 64 (1992) 2841.
[120] I.J. Kim, S.J. Park, H.J. Kim, J. Chromatogr. A 877 (2000) 217.
[121] E. Jellum, A.K. Thorsrud, E. Time, J. Chromatogr. 559 (1991) 455.
[122] E. Jellum, J. Capillary Electrophor. 1 (1994) 97.
[123] Y.F. Cheng, N. Dovichi, Science 242 (1988) 562.
[124] Z. Deyl, F. Tagliaro, I. Miksik, J. Chromatogr. B 656 (1994) 3.
[125] E. Caussé, P. Malatray, R. Calaf, Ph. Charpiot, M. Candito, C. Bayle, P. Valdiguié, R. Salvayre, F. Couderc, Electrophoresis 21 (2000) 2074.
[126] E. Caussé, C. Issac, P. Malatray, C. Bayle, P. Valdiguié, R. Salvayre, F. Couderc, J. Chromatogr. A 895 (2000) 173.
[127] P.S. Mukherjee, H.T. Kames, Biomed. Chromatogr. 10 (1996) 193.
[128] M.T. Shipchandler, E.G. Moore, Clin. Chem. 41 (1995) 991.
[129] P. Pernet, E. Lasnier, M. Vaubourdolle, Clin. Chem. 46 (2000) 1440.
[130] E. Nexo, F. Engbaek, P.M. Ueland, C. Westby, P. O'Gorman, C. Johnston et al., Clin. Chem. 46 (2000) 1150.
[131] C.M. Pfeiffer, D.L. Huff, S.J. Smith, D.T. Miller, E.W. Gunter, Clin. Chem. 45 (1999) 1261.
[132] T. Brunelli, G. Pepe, R. Marcucci, D. Prisco, R. Abbate, S. Fedi, Clin. Lab. 47 (2001) 393.
[133] J.B. Ubbink, R. Delport, R. Riezler, W.J.H. Vermaak, Clin. Chem. 45 (1999) 670.
[134] H.J. Powers, S.J. Moat, Curr. Opin. Clin. Nutr. Care 3 (2000) 367.
[135] F. Frantzen, A.L. Faaren, I. Alfheim, A.K. Nordhei, Clin. Chem. 44 (1998) 311.
[136] Y. Tan, L. Tang, X. Sun, N. Zhang, Q. Han, M. Xu, E. Baranov, X. Tan, X. Tan, B. Rashidi, Z. An, A.W. Perry, R.M. Hoffman, Clin. Chem. 46 (2000) 1686.
[137] N. Matsuyama, M. Yamaguchi, M. Toyosato, M. Takayama, K. Mizunok, Clin. Chem. 47 (2001) 2155.
[138] S.C. Eliason, D. Ritter, H.D. Chung, M. Creer, Clin. Chem. 45 (1999) 315.
[139] C.M. Pfeiffer, D. Twite, J. Shih, S. Holets-McCormack, E.W. Gunter, Clin. Chem. 45 (1999) 152.
[140] I. Fermo, G. Mazzola, A. D'Angelo, R. Paroni, Thromb. Haemost. 83 (2000) 968.
[141] A. Tripodi, V. Chantarangkul, R. Lombardi, A. Lecchi, P.M. Mannucci, M. Cattaneo, Thromb. Haemost. 85 (2001) 291.