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Simple plasma work-up for a fast chromatographic analysis of homocysteine, cysteine, methionine and aromatic amino acids

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Abstract

Simplified sample workup obviating protein precipitation and eluent evaporation commonly employed in earlier reports using chloroformate-mediated derivatization of aminothiols prior to mass spectrometric (MS) detection is presented. The reduction of disulfides in plasma is accomplished with dithiothreitol within minutes. A simultaneous derivatization with ethyl chloroformate (ECF) and extraction of derivatives into organic phase takes place within seconds. Along with *S*-amino acids, also aromatic amino acids can be determined during a 5-min run. Gas chromatography with flame ionization detection (GC–FID) proved to be sensitive enough to reach plasma homocysteine levels. A prerequisite for a reliable quantitation was fulfilled under the given conditions. Intra-assay precision was <5%, recoveries from spiked plasma complete (101.2%), detection and quantitation limits for homocysteine came to <1 and 3 μmol/l. Our results were in full agreement with those obtained by liquid chromatography (r=0.999 for homocysteine and 0.987 for cysteine), and were close to two homocysteine immunoassays (r=0.991 and 0.939, respectively).

Keywords: Homocysteine; Cysteine; Methionine; Aromatic amino acids

1. Introduction

The uniquely rapid derivatization of amino acids with alkyl chloroformates in aqueous media [1–3] is becoming increasingly popular also for the determination of plasma homocysteine (Hcy) levels by GC–MS [3–9]. This one-step procedure provides for a simultaneous and instantaneous acylation of amine and thiol groups, and for the esterification of carboxyl groups. The methodology proved to be fairly competitive to the current plasma Hcy assays as reflected in recent reviews [9–11].

However, studies dealing with the chloroformate approach [4–8] fail to agree on the optimal conditions for sample prep and derivatization [12]. Although selective ion monitoring (SIM) MS-detection can ignore impurities present on column and quantitative analysis using deuterium-labeled internal standards can be done with a fair reproducibility [4,7,8], the sample work-up is rather laborious and column lifetime shortened due to the contamination. Some of the drawbacks have been highlighted in a

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recent disputation [12], e.g., co-injection of the precipitating agent and lipids into the separating capillary, duplication of reaction conditions optimized for the class of protein amino acids, redundant evaporation of excessive volumes of extraction solvent, and others.

Our recently published advanced method for amino acid profiling in body fluids [3] eliminates the need for plasma protein precipitation and for eluent evaporation following solid-phase extraction. It also involves a simultaneous phase-transfer derivatization with the concentration of amino acid derivatives into organic layer. This fast method for plasma pretreatment was re-optimized in this study for the quantitation of Hcy, cysteine (Cys) and methionine (Met). Ethionine served as internal standard (I.S.). ECF was preferred as derivatization reagent over larger alkyl chloroformates [3,5,7] with the purpose of suppressing the unnecessary extraction of the more polar low-molecular mass amino acid derivatives. As just the opposite concerns the important class of aromatic amino acids, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), they were involved in the analysis. Moreover, it is shown here that the inexpensive and general-purpose FID detection can render reliable data and similar quantitation limits like the commonly employed MS detection [4–8]. In order to ensure such performance with GC–FID we identified a convenient capillary column able to resolve all analytes of interest and to eliminate interferences with unknown sample components.

2. Experimental

2.1. Plasma samples

All plasma samples used in this study were aliquots of clinical material remaining after routine Hcy (Cys) determination. Part of the samples was delivered from the 1st Faculty of Medicine (Charles University, Praha) following assay of Hcy and Cys by liquid chromatography with fluorimetric detection (LC-FD) [13]. Another portion was supplied by the Hospital Na Homolce (Praha), after assaying Hcy via the automated immunofluorescence method (Abbott IMx assay). Venous blood samples were collected by a standardized procedure into heparin- or EDTA-containing vials, and cooled immediately in ice water. Plasma was separated by centrifugation at 2000 *g* for 15 min at 4 °C within 30 min of collection, and stored at −20 °C until further processing.

2.2. Materials and stock solutions

Standards of sulfur-containing amino acids (Cys, Hcy, Met, ethionine, cystine, homocystine and cysteinylglycine), aromatic amino acids (Phe, Tyr, and Trp) and various reagents such as ECF, dithiothreitol (DTT), pyridine, ethanol, chloroform, isooctane, potassium oxalate monohydrate and sodium metabisulfite were obtained from Sigma–Aldrich (Praha, Czech Republic). Phenomenex (Torrance, CA, USA) supplied 50-μl sorbent tips for physiological amino acid profiling or protein hydrolysates (doubled capacity). PD (positive displacement) tips of 0.5 and 1.25 ml volume were obtained from Brand (Wertheim, Germany). Adjustable 100-μl transferpettor pipettes with glass capillary was supplied by Merck (Praha), while 200-μl microcapillary round tips (No. T 1906) by Sigma–Aldrich (Praha). Tapered polypropylene (PP) 1.1-ml reaction vials came from Continental Laboratories (San Diego, CA, USA), flat-bottom glass vials 8×40 mm from Kimble/Kontes (Vineland, NJ, USA). The vortex mixer (50–2400 rpm) was supplied by P-Lab (Praha).

Amino acid calibrators were prepared either in equimolar concentration (50 μ*M* each, without cystine and homocystine), or in concentrations similar to plasma levels (10, 20 and 200 μ*M* of Hcy, Met and Cys, and 50 μ*M* of each aromatic amino acid). Individual solutions of cystine and homocystine were prepared at a concentration of 500 and 50 μ*M*, respectively. Aqueous HCl (50 m*M*) was used as solvent for all calibrators. The concentration of ethionine in the I.S. solution was 20 μ*M*.

Working solutions for the reduction and derivatization steps were prepared as follows:

The reducing solution, i.e., 50 m*M* DTT (0.8%) plus 10 m*M* (about 0.2%) potassium oxalate hydrate, was mixed daily with I.S. solution in the ratio of 99:1 and used within 2 weeks while refrigerated.

The eluting medium was a mixture of 1% aqueous NaCl, ethanol and pyridine in ratio of 75:40:10, the reactive medium consisted of isooctane, chloroform and ECF in ratio of 12:4:1.

2.3. Sample preparation

One hundred microliters of the reducing solution (mixed with I.S. solution as specified above) and 100 μl of plasma (or serum) were added into a tapered 1.1-ml PP vial (or an 8×40-mm glass vial). The content was gently mixed and let to react for 2–3 min, or anytime longer. A sorbent tip was attached to a 1.25-ml PD tip and after immersing the tapered end into the fluid the content was sucked slowly through the exchanger bed by moving the piston in the PD tip a few millimeters at a time. After passing the fluid through the sorbent completely, 150 μl of water–ethanol (2:1) were added (while keeping the tip possibly in the vial) and passed through the sorbent bed, more rapidly than in the loading step. The liquid was completely drained from the sorbent tip. The PD tip was removed and its contents discarded. Next a 0.5-ml PD tip with its piston pulled back half way was attached to the sorbent tip. After adding 150 μl of the eluting medium into the same working vial, the fluid was sucked into the resin bed till it reached the filter or slightly above it. By pushing the piston down the resin is being displaced into the vial (repeated if the tip not emptied completely). Subsequently, 150 μl of the reactive medium (2×75 μl with the transferpettor) was added into the vial and the content mixed by vortexing unstoppered for about 15 s, until the upper organic layer became clear. Following the pyridine-scavenge step accomplished by addition of 100 μl 1 mol/l HCl and brief vortexing for 3–5 s, an aliquot of the upper organic phase was transferred into an autosampler vial using a microcapillary tip.

For calibration and reproducibility studies plasma samples were replaced with standard solutions of amino acids added to the reducing solution. For blank runs the reducing solution co-mixed with physiological (about 1% aqueous NaCl) solution was passed through the procedure.

2.4. GC–FID analysis

GC analysis employed GC-17A apparatus with FID and AOC-20i Autoinjector, both from Shimadzu (Kyoto, Japan). The separation was carried out on a 10-m×0.25-mm ZB-AAA fused-silica capillary column (Phenomenex) run with a temperature program of 150–330 °C at 30 °C/min under a constant velocity mode. Injector and FID temperatures were 240 and 330 °C, respectively. Hydrogen served as carrier gas under head pressure of 30 kPa. Split injection (2.5 μl) was performed at an increased initial head pressure of 50 kPa applied for 0.2 min (approximate split-ratio 1:10). A Siltek-deactivated split liner (I.D. 4 mm) with Siltek wool supplied by Restek (Bellefonte, PA, USA) was employed. CSW software version 1.7 (DataApex, Praha) was applied for a computer-assisted processing of the chromatographic data.

3. Results and discussion

3.1. Reduction step

In retrospection, there is no consensus in the literature published during the last decade regarding the conditions for the reduction step, i.e., the release of Hcy and Cys from plasma proteins, and the cleavage of the disulfides. Large differences appear especially regarding the amount of DTT or DTE (dithioerythritol) employed, and the reaction time and temperature recommended [14–21]. Although rapid kinetics for the reduction process (<1 min) were reported as early as 1985 [14], but confirmed only in the late 1990s [15], incubation times of 10–60 min and temperatures ranging from 4 to 40 °C were often reported. Moreover, the recommended concentration range for the reducing agent extended over two orders of magnitude, i.e., from <0.01 [16] to >1% [15]. One would expect at least an inverse relationship between the concentration of the reducing agent and reaction time, i.e., a higher concentration should require a shorter reaction time. Nevertheless, one report shows plasma treated under extremely low DTT concentrations (0.008%) at 4 °C for 30 min [16], while another uses the same time for treating plasma with 0.5% DTT at 40 °C [17]. At the same time, a tendency for gel formation in plasma was noticed at DTT concentrations >0.15% associated with longer reaction times [18]. This lack of agreement in reduction conditions suggests that this step of plasma pretreatment is most delicate.

With another reducing reagent, the recently introduced [22], and increasingly popular TCEP (tris-

carboxyethylphosphine), some variations in reduction time have been also reported, ranging from a few minutes [23] to 15–60 min [13]. In the latter case the reduction efficiency of TCEP and that of TBP (tributylphosphine) was comprehensively investigated as a function of concentration, reaction time and temperature and the more advantageous use of TCEP highlighted. DTT/DTE reagents could not be used, e.g., in LC–FD methods involving SBD-F (ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate) for aminothiol derivatization since also the excess reducing reagent would react besides the analytes.

The afore-mentioned reducing agents were also tested in our experiments. Such reagents should meet the following expectations: should not precipitate plasma proteins, should not prevent amino acid uptake onto the SPE sorbent, and should not give side-products which could interfere in the GC analysis. A possible cross-reaction with the derivatizing reagent was markedly suppressed by washing the reducing agent away during the solid-phase extraction. Based on preliminary tests, phosphines were readily abandoned as reducing reagents because they caused multiple interference. On the other hand, DTT was preferred over DTE because the latter reagent caused a small baseline elevation in the region of Hcy elution.

Lacking consensus in previous reports brought up a further question: should DTT be used by itself, or with an additive? As a matter of fact, plasma samples contained already anticoagulant additives like EDTA (ethylenediaminotetraacetic acid), or heparin with fluoride. Alternative additives were tested as Hcy preservatives, e.g., acidic citrate, oxalic acid, sodium metabisulfite, etc. Our tests showed that addition of 1–2% of citrate, EDTA or fluoride to DTT (0.5%) had a rather negative impact on sample processing and total yields. We suspected that the culprit was the limited capacity of the SPE tips for plasma samples treated with cations in excess [3]. Taking into account the plasma-inherent sodium cations competing with amino acids during the loading step, only 100–150 μl of plasma sample could be applied while excessive addition of salts would cause amino acid losses. To overcome this problem, SPE tips with higher loading capacity had to be used.

The reduction kinetics was followed by adding decreasing concentrations of DTT (from 100 down to 1 m*M*) to pooled, or pooled and spiked plasma samples, and also to calibration samples (with added cystine and homocystine) at room temperature, within a time span of 1–45 min. The addition of potassium oxalate and sodium metabisulfite was found to be useful, surprisingly to a different way. By adding disulfides to plasma together with DTT (0.8−1.0%), or with DTT and oxalate (0.8+0.2%), the reduction to Hcy and Cys took place almost instantaneously, with yields differing less than 5% within 1–15 min reaction time. No further improvement was observed upon using longer times and higher DDT concentrations. A decreased DDT concentration down to 0.5 and 0.2% required prolongation of the reduction time to about 10 and 20 min. Despite this, recovery of Hcy and Cys of only 90% was obtained in the latter case. An oxalate concentration of 0.2% was found to be sufficient to prevent clogging of plasma and to promote reduction kinetics slightly.

3.2. Pitfall in the reduction step

Unlike with plasma samples, DTT alone, or in combination with oxalate, was rather ineffective in reducing the parent analytes in standard solution samples (pH close to 8 due to the present oxalate). The reduced forms were generated only at very low yields (<10% Hcy, about 30% Cys) even after reaction times as long as 45 min. In this case, however, metabisulfite proved to be a more effective reductant. After combining it with DTT (0.5+0.5%), about 30% of homocystine and 70% of cystine were transformed into their reduced forms within 10 min. Even more effective was its combination with oxalate (0.5+0.5%, no DTT added) leading to a nearly complete reduction of cystine and about 60% of homocystine after only 10 min. At the same time, the latter mixture was rather ineffective in treating plasma, yielding only 40–50% conversion rates. However, addition of metabisulfite to the DTT-oxalate solutions did not aid to the kinetics any more and was, therefore, omitted.

Furthermore, the same inability to reduce disulfides effectively and smoothly was experienced also in human urine samples, in this case even with both

the reducing agents. These surprising results of a different efficacy of the DTT reductant for treating disulfides in plasma and/or cerebrospinal fluid (CSF) and in some other media has not been reported previously to our knowledge. The findings suggest the existence of an unidentified factor in plasma (and CSF) that promotes the reduction process. This hypothesis may explain the wide variety of conditions recommended throughout the literature. The unexpected phenomenon is certainly worth further investigation.

3.3. Outcome of the validation studies

Proper selectivity of the present approach towards Hcy analysis by GC–FID was a prerequisite for generating any validation data. Special attention was given to ensure single-peak elution for the least abundant plasma amino acids of interest, i.e., Hcy and Met, and also for the internal standard. The ZB-AAA capillary column, employed for GC amino acid analysis in the corresponding kit [3], was found to be suitable also for this tailored application. Blank and plasma samples processed without the reducing step did not show any baseline elevation in the region of Hcy and Met elution. On the contrary, replacing DTT by DTE resulted in a small baseline elevation in the critical region of Hcy elution, equivalent to about 1 μmol/l of this amino acid. This was the only reason why DTT was preferred over DTE, an otherwise insignificant issue with GC–MS analysis.

Linearity studies were carried out using five different concentrations within the range 0–150 μmol/l for Hcy, Tyr and Trp, and 0–1000 μmol/l for Cys, Met and Phe. An increased range for the two latter amino acids was justified by Met-loading tests with some subjects, and a possible occurrence of hyperphenylalaninemia in some plasma samples. The analyses of calibration samples produced linear curves with $r^2 > 0.99$ for all the amino acids considered in this study.

Detection limits for the determination of Hcy, Met, Phe and Tyr in plasma was slightly below or around 1 μmol/l, while those for Cys and Trp close to 2 μmol/l. The limit of quantitation, defined as a 5:1 signal-to-noise ratio, was 3 μmol/l for the former group, and 5 μmol/l for the latter group of amino acids. Compared to GC–MS measurements, our detection limit came in between the one reported for Hcy when analyzed as EOC ethyl ester (0.2 μmol/l) [4] or as isobutyloxycarbonyl ethyl ester [7], and the value reported by Sigit et al. for the former case (5 μmol/l) [8]. Kataoka et al. [24] measured amino thiols as isopropyloxycarbonyl methyl esters in ether-extracted plasma by GC with the selective flame photometric detection. The sensitivity reported by these authors was 2 μmol/l for Hcy, i.e., nearly equal to the one achieved by us with a common, non-specific detector (FID).

The intra-assay (within-batch) precision of the method was determined by replicate analyses ($n=8$) of the calibration sample and of pooled plasma samples with normal and elevated Hcy/Cys levels. The same plasma samples were subjected to inter-assay (between-day) variation studies within a period of 10 weeks (eight replicate measurements spread over the interval). Intra-assay values were less than 5% C.V. (coefficient of variation), while inter-assay values over a 10-week period exceeded 5% C.V. for Hcy (control group), Trp and Tyr (Table 1). In this respect, it is worth mentioning here the unique observation made by Gautier et al. [25], who found Hcy stable even after exposing plasma to room temperature for over 2 days.

A measurement of analytical recovery targeted particularly Hcy and Cys. The efficiency of the reducing step was evaluated simultaneously by spiking pooled plasma samples with known amounts of the oxidized forms, homocystine and cystine. Added amounts were in the range 0–50 μmol/l for homocystine, and 0–200 μmol/l for cystine. The mean recovery was found to be 101.2% for Hcy, and 102.5% for Cys, respectively (Table 2), proving a high degree of reproducibility for this procedure.

Accuracy of the method was checked by comparison to measurements of the same plasma samples with three other established current methods. The parallel measurements were performed at three different clinics. Determination of Hcy and Cys by LC-FD following TCEP reduction and SBD-F derivatization was done at the 1st Faculty of Medicine according to the published protocol [13]. The same plasma samples ($n=27$) were assayed for Hcy using EIA/chemiluminescence detection with Immulite Analyzer (DPC, Los Angeles, CA, USA) at the

Table 1
Reproducibility of amino acid determination in solution of standards[a] and in pooled plasma samples[b] by GC–FID (eight replicate measurement each): ethionine (I.S.) added in amounts of 200 μmol/l

Amino acid	Standards	Controls			Patients		
	Intra-assay C.V. (%)	Mean	Intra-assay C.V. (%)	Inter-assay C.V. (%)	Mean	Intra-assay C.V.(%)	Inter-assay C.V.(%)
S-containing							
Met	3.4	18.7	2.4	4.2	23.0	2.3	4.5
Cys	2.2	240.1	2.7	5.3	390.7	2.4	5.3
Hcy	3.7	7.4	3.2	6.9	17.6	2.1	4.9
Aromatic							
Phe	1.6	51.5	1.8	3.0	66.3	2.2	4.2
Tyr	2.8	50.4	3.7	5.5	59.3	3.2	4.8
Trp	3.3	42.9	4.8	7.6	40.9	4.9	6.9

[a] Plasma-like solutions of standards with Hcy, Met, Cys (10, 20 and 200 μmol/l) and the aromatic amino acids (50 μmol/l each).
[b] Pooled samples prepared by mixing plasma of 15 healthy subjects or 10 patients, respectively. Mean concentration in μmol/l.

Institute of Clinical Biochemistry (Faculty Hospital, Ostrava). Finally, Hcy was assayed using the fluorescence polarization immunoassay (Abbott IMx assay) in another set of plasma samples ($n=52$) at the Hospital Na Homolce (Praha).

With the LC–FD [13] assay besides Hcy, also Cys could be measured and compared with our results. A Passing-Bablok regression [26] showed outstanding agreement between the LC and GC methods for Hcy ($r=0.999$), and a good agreement for Cys ($r=0.987$), as shown in Figs. 1 and 2. Excellent agreement was also achieved between our values and the Immulite assay ($r=0.991$, Fig. 3). On the other hand, a wider scatter was revealed in comparison with the Abbot IMx assay ($r=0.939$, Fig. 4). Employing the Bland-Altman plot [27], a positive bias was found between our GC-based method and each of the comparative assays, the lowest (0.17) being in relation to the LC assay, the highest (2.23) to the Immulite assay. The latter gave obviously lowered values, especially at Hcy levels <7 μmol/l. A chromatographic record of the GC–FID analysis of an equimolar solution of standards and corresponding plasma amino acids is on Fig. 5.

Table 2
Recovery of Cys and Hcy from plasma spiked with cystine and homocystine ($n=3$ for each amount)

Compound	Amount added	Amount found (mean)[a]	C.V. (%)	Recovery (%)
Cys	0	230.6	3.8	0.0
	20	270.1	4.3	98.8
	50	337.2	4.9	106.6
	100	441.9	4.4	105.7
	200	628.7	4.1	99.5
Hcy	0	8.2	1.9	0.0
	2	12.3	3.2	102.5
	5	18.6	3.9	104.2
	20	46.8	2.4	96.5
	50	109.9	2.9	101.7

[a] After recalculation to reduced forms (μmol/l), i.e., doubled amounts expected.

3.4. Concluding remarks

The approach presented here markedly simplifies sample work-up and overcomes shortcomings of earlier procedures based on chloroformate-mediated amino acid derivatization [4–6]. Despite of a recent methodical advancement, eliminating elution of captured amino acids with ammonia [7], current plasma processing protocols are still time-consuming [3,12]. By combining derivatization and the derivative extraction into one single step, as recently described for amino acids [3,28], and for a urinary dipeptide [29], a further improvement was made in the chloroformate methodology [2]. The uniquely rapid process of extracting analytes of interest from a complex plasma matrix into a very pure organic extract is accompanied with additional advantages, i.e., with the possibility of analyzing the same sample by LC–MS [3,30].

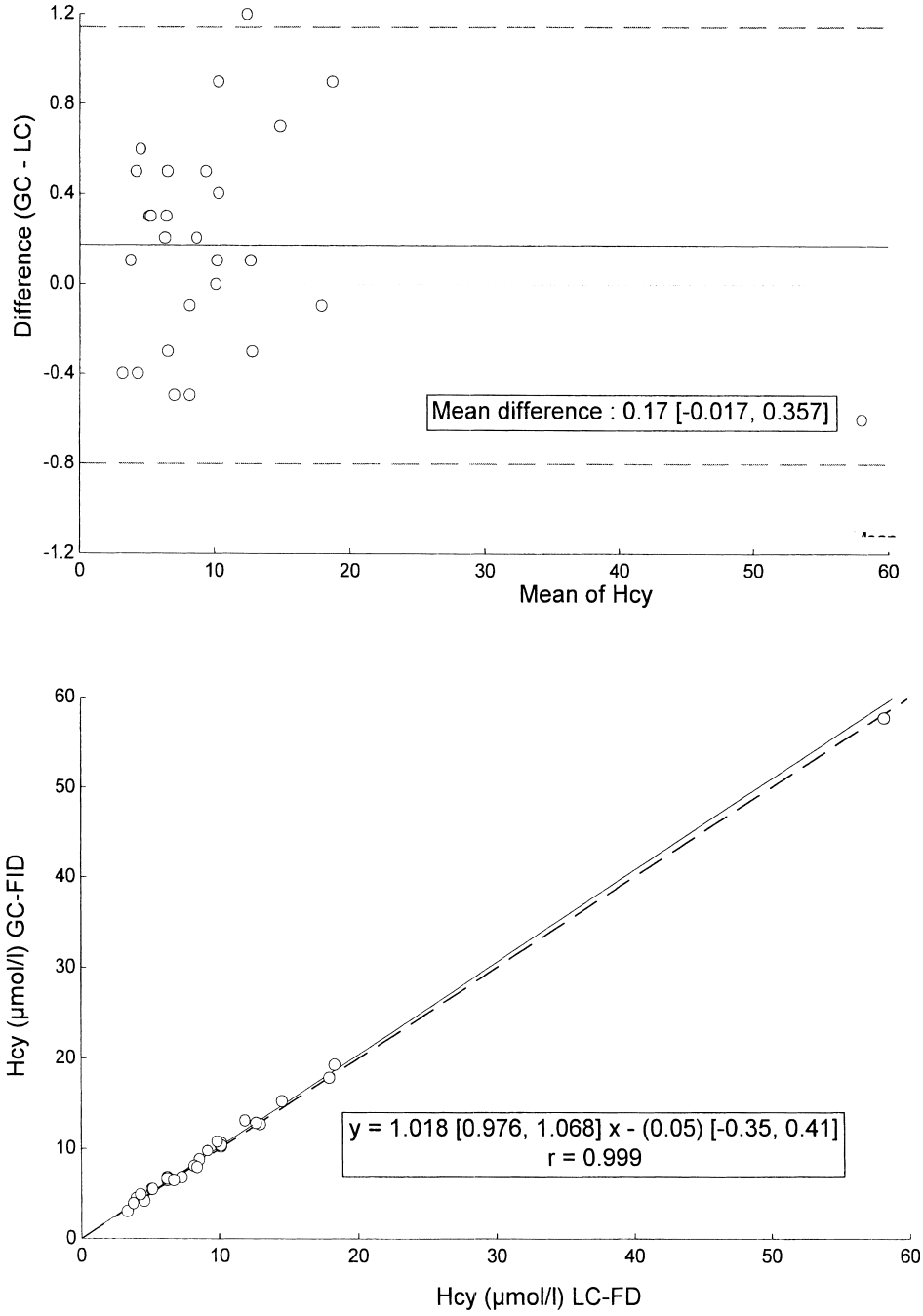

Fig. 1. Comparison of the GC and LC–FD determination of plasma Hcy by means of Passing-Bablok regression and Bland-Altman differences plot (n=27).

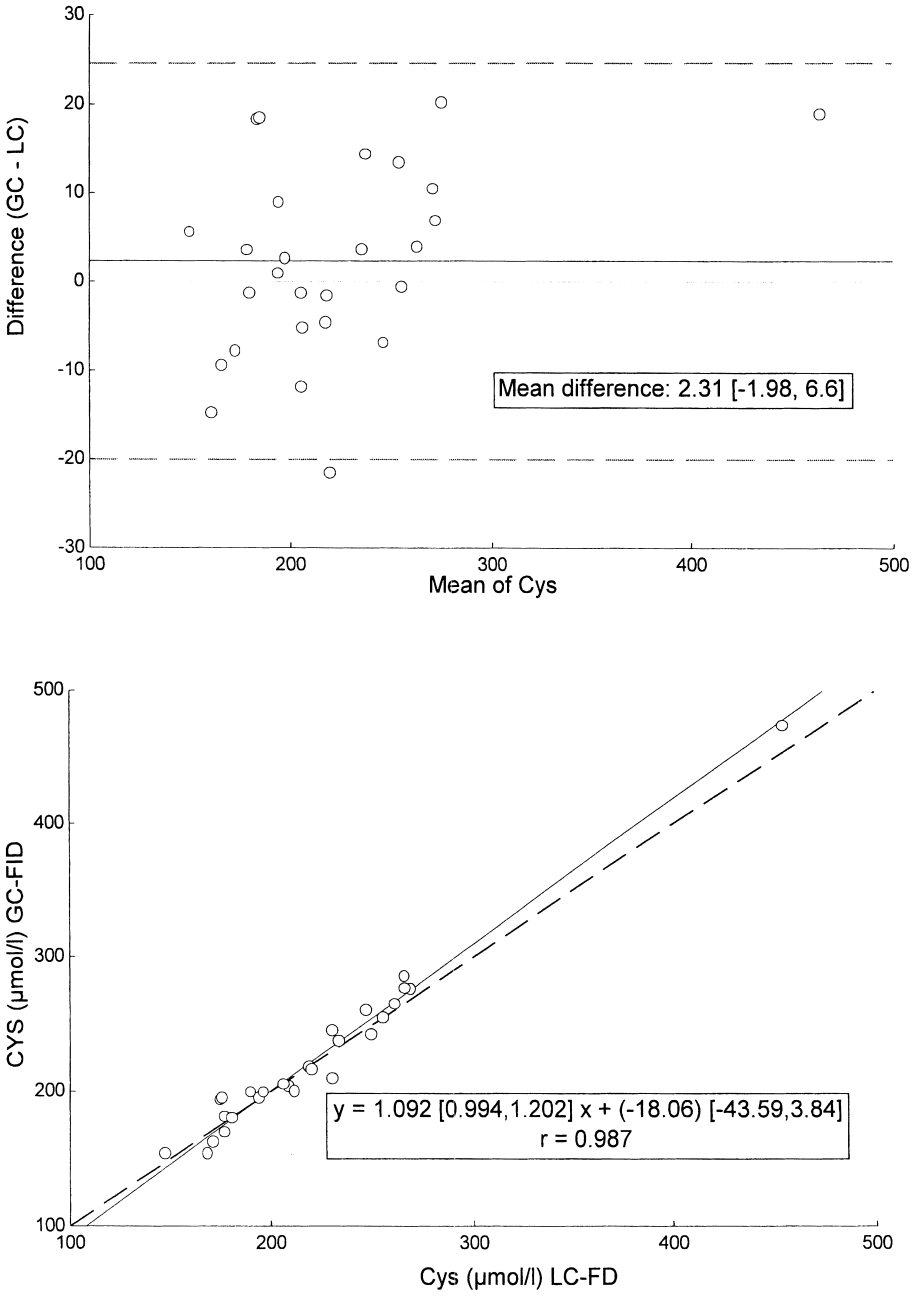

Fig. 2. Comparison of the GC and LC–FD determination of plasma Cys by means of Passing-Bablok regression and Bland-Altman differences plot (n=27).

Moreover, we could demonstrate that Hcy and related amino acids could be determined in a small amount of plasma even with GC–FID, the least expensive chromatographic technique. As a result, nearly the same sensitivity as with selective detectors [24], or with the commonly used GC–MS can be

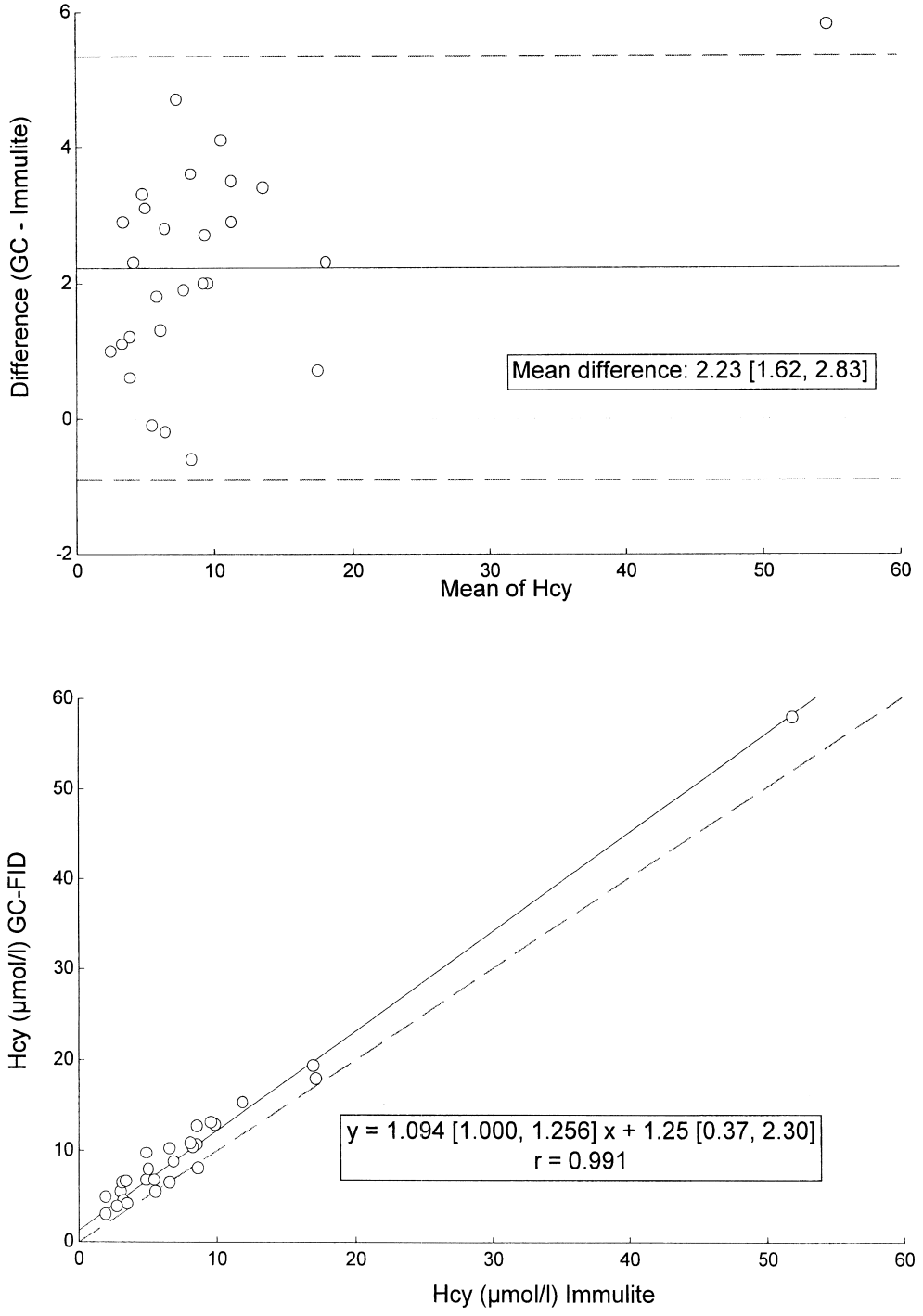

Fig. 3. Comparison of the GC and Immulite-EIA determination of plasma Hcy by means of Passing-Bablok regression and Bland-Altman differences plot (n=27).

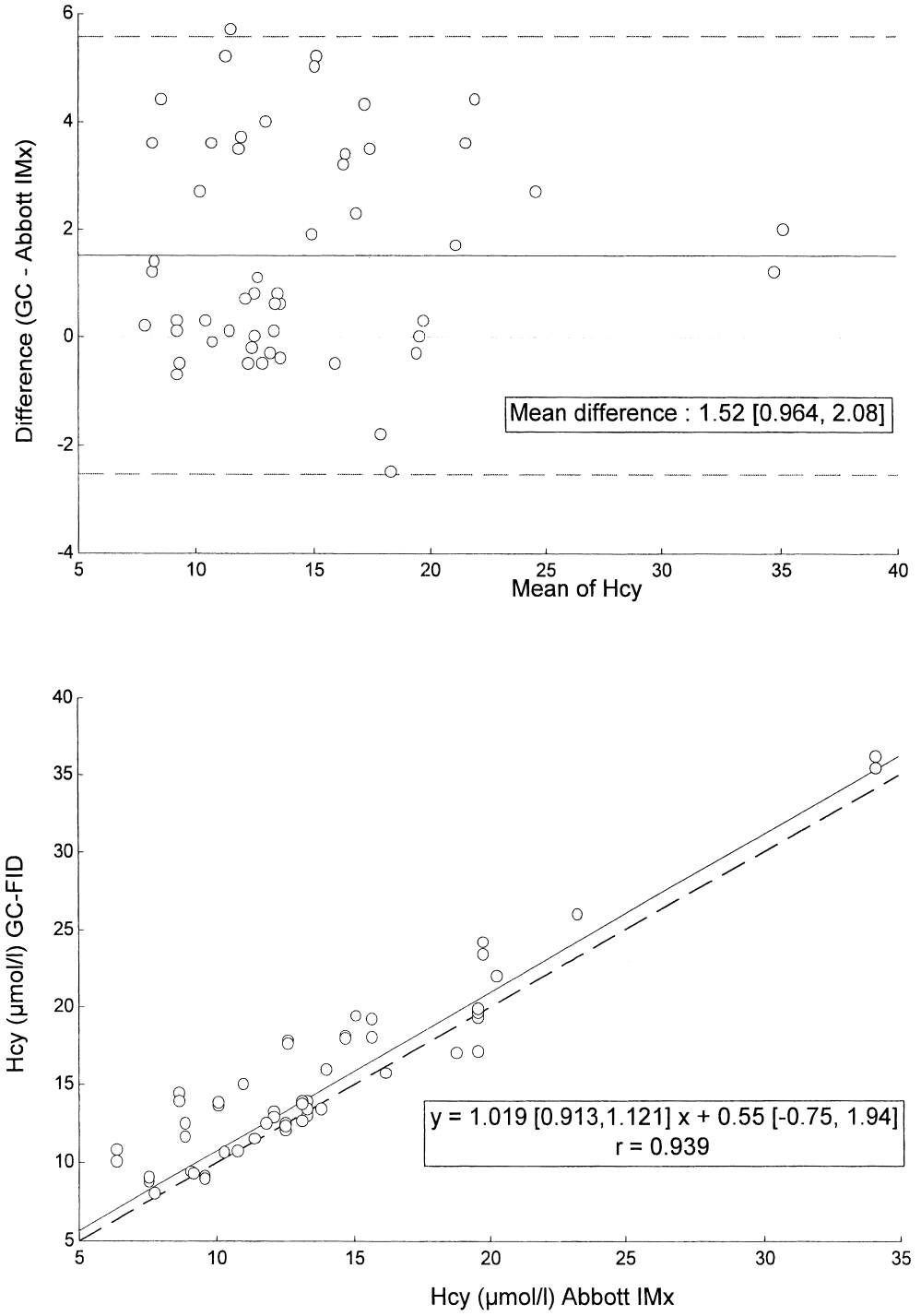

Fig. 4. Comparison of the GC and Abbott IMx determination of plasma Hcy by means of Passing-Bablok regression and Bland-Altman differences plot (n=52).

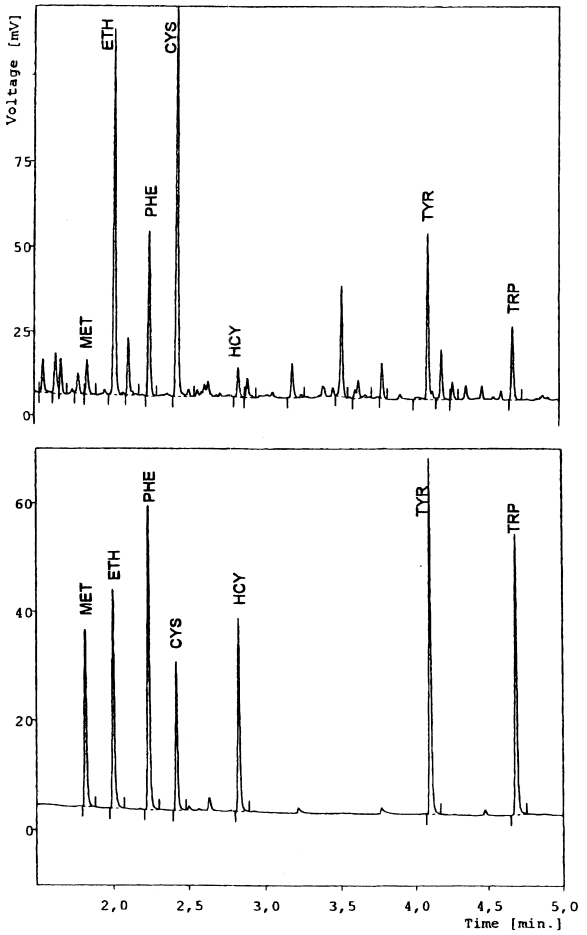

Fig. 5. GC–FID analysis of equimolar mixture of chromatographic standards (bottom, initial amount 10 nmol each) and plasma amino acids with a slightly elevated Hcy concentration (15, 2 μmol/l). Ethionine added as I.S. in amounts of 20 nmol per 0.1 ml plasma.

obtained. Nothing prevents the latter technique to be applied in connection with the presented sample preparation. As mentioned previously [4], the derivatives maintained sufficient stability for at least 3 days and more than 100 samples could be processed completely within a day. Cysteine-glycine dipeptide and reduced glutathionine were not assayed successfully by GC under the present conditions. To make the procedure more precise for LC–MS determination the use of deuterated standards (D_8-homocystine) is currently under testing.

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