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# Gas chromatographic–mass spectrometric determination of total homocysteine in human plasma by stable isotope dilution: method and clinical applications

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**Abstract**

The detection and quantitation of slight increases of plasma homocysteine levels is of growing interest. This has prompted us to develop a highly sensitive and accurate capillary gas chromatography–mass spectrometry (GC–MS) method. The method proved to be highly sensitive (DL=0.17 μmol/l) with between- and within-run precision less than 6% and 7%, respectively. Reference values of plasma total homocysteine have been determined for men ($n$=39) and women ($n$=36), showing a significant difference ($P$=0.003) between gender. Preliminary results in cerebrovascular accidents and in venous thrombosis are presented. 

*Keywords:* Homocysteine

## 1. Introduction

Mild hyperhomocysteinemia is currently considered as an independent risk factor for atherosclerosis and atherothrombosis [1]. This risk factor is not very rare; mild hyperhomocysteinemia is found in 5–7% of the general population [2]. The determination of total homocysteine (tHcy) in human plasma for detecting moderate increase is of growing interest. tHcy refers to all the forms of homocysteine (free, bound to proteins or oxidized) and requires the reduction of the disulfide bonds prior its quantitation. Most methods use HPLC with precolumn derivatization and only a few gas chromatography–mass spectrometry (GC–MS) [3,4]. The latter are highly reliable, specific and sensitive especially when the isotopic dilution method is used, and in this case, often considered as a reference method. Their most frequently mentioned drawback (costly equipment) is a little bit old-fashioned with the appearance of less expensive benchtop GC–MS instruments. Moreover, the great number of published papers using GC–MS as methodology can be considered as a valuable proof. Actually this equipment is found in many laboratories and among them particularly in university hospital laboratories.

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We report a highly sensitive and accurate GC–MS method for the determination of moderate hyperhomocysteinemia in human plasma and its successful application in clinical use.

## 2. Experimental

### 2.1. Chemicals

DL-Homocysteine and *N*-(*tert.*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) were obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France). DL-Homocystine-3,3,3′,3′,4,4,4′,4′-d8 (98% enriched), used as internal standard, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Macroporous anion-exchange resin (AG MP-1, 100–200 mesh, chloride form) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dithiothreitol, and gradient grade acetonitrile were purchased from Merck (Darmstadt, Germany). Analytical-reagent grade methanol and acetic acid were obtained from Prolabo (Paris, France). Deionized water was prepared using an Elga water purification system (Aquadem, Nice, France).

### 2.2. GC–MS apparatus

An HRGC mega 2 series gas chromatograph with an AS 800 autosampler was coupled to a Trio 1000 quadrupole mass spectrometer (ThermoQuest, Les Ulis, France). Gas chromatographic separation was achieved on a low bleeding BPX5 fused-silica capillary column (12.5 m×0.22 mm I.D., 0.25-μm film thickness) from SGE (Villeneuve St. Georges, France). Electron impact (EI) ionization mode and selected ion recording (SIR) method were performed.

### 2.3. Sample collection

Blood was drawn from overnight fasting subjects and put on crushed ice immediately after venipuncture. In the following 30 min, blood was centrifuged for 10 min at 3500 rpm, plasma was separated without delay, aliquoted and stored at −20°C until analysis. A pilot study involving twelve people among the laboratory staff was conducted for the choice of the anticoagulant. Three kinds of anticoagulants were tested: EDTA, heparin and CTAD (citrate theophylline adenosine dipyridamole) by using Vacutainer blood collection tubes (Becton Dickinson, Meylan, France).

### 2.4. Sample preparation for GC–MS

A 100 μl volume of human plasma, standard or bovine serum (used as quality control) was spiked with 100 μl of deuterated internal standard (10 nmol), 1 ml of deionized water and 100 μl of 1 *M* NaOH containing 10 mg/ml of dithiothreitol. After Vortex mixing, samples were incubated at 40°C for 30 min. After cooling, samples were applied on to small plastic columns containing 100 mg of AG MP1 resin. The resin in bulk had been previously washed with methanol and then deionized water. After samples were applied, the columns were washed three times with 3 ml of water and once with 3 ml of methanol, then amino acids were eluted with 1.1 ml of 0.4 *M* acetic acid in methanol. Eluates were evaporated to dryness under nitrogen and then derivatized by adding 70 μl of MTBSTFA and 30 μl of dry acetonitrile. Derivatization was carried out by heating at 120°C for 60 min.

### 2.5. GC–MS measurements

The *tert.*-butyldimethylsilyl (*t*-BDMS) derivatives of standards and of plasma samples were analyzed in the electron impact ionization mode. The electron energy was set at 70 eV and the source temperature at 200°C. The injector temperature was set to 250°C and the interface temperature at 280°C. The initial temperature of the GC oven was 110°C, which was held for 1 min after sample injection, and then was increased at 25°C/min to 220°C, then at 15°C/min to 250°C, and finally at 25°C/min to 300°C. Helium was used as the carrier gas and the pressure at the column inlet was 35 000 Pa. Derivative volumes of 0.5 μl were injected via the autosampler. Data were collected using the selected ion recording mode. The $[M-57]^+$ ions were monitored using a 80 ms dwell time: 420.30 and 424.30 corresponding, respectively, to homocysteine and 3,3,4,4-d4-homocysteine. Homocysteine in plasma samples was quantitated from the standard curve which was established from different homocysteine standard dilutions (0, 3.7, 7.4, 14.8, and 29.6 μmol/l) containing 10 nmol of 3,3,3′,3′,4,4,4′,4′-d8-homocystine.

### 2.6. HPLC measurements

Instead of dithiothreitol (used for GC–MS measurements), reduction of disulfides (both free and protein-bound) was made with $NaBH_4$. The subsequent free sulfhydryl groups were derivatized with monobromobimane. The thiol–bimane adducts were quantified by reversed-phase liquid chromatography and fluorescence detection [5].

### 2.7. Statistics

All values were calculated as mean±SD. Mean values of Hcy between male and female or between cases and controls were compared by the *t*-test procedure. Paired-sample *t*-tests were used to compare the anticoagulants. The threshold of significance was set at $P \leq 0.05$. Statistical analysis was performed by using SPSS software (Paris, France).

## 3. Results

### 3.1. Analytical performance

#### 3.1.1. Calibration curve

Standard solutions, which contained a variable amount of homocysteine (0–29.6 μmol/l) and a fixed amount of d8-homocystine (10 or 100 μmol/l), were prepared and carried through the entire procedure of extraction, derivatization and analysis outlined above. The height of the $[M\text{-}57]^+$ ions formed from homocysteine (420.3) and d4-homocysteine (424.3) were determined (Fig. 1) and the ratio (420.3/424.3) was plotted as a function of the amount of homocysteine. Only with the 100 μmol/l of d8-homocystine, a linear relationship was observed over the range 0–29.6 μmol/l for tHcy (Fig. 2). Samples with tHcy>30 μmol/l should be diluted

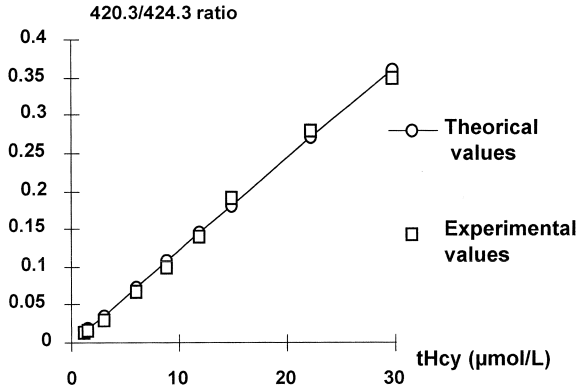


Fig. 2. Linearity of the calibration curve with d8-homocystine concentration of 100 μmol/l. Good agreement between theoretical and experimental values showing that reduction step with DTT is complete.

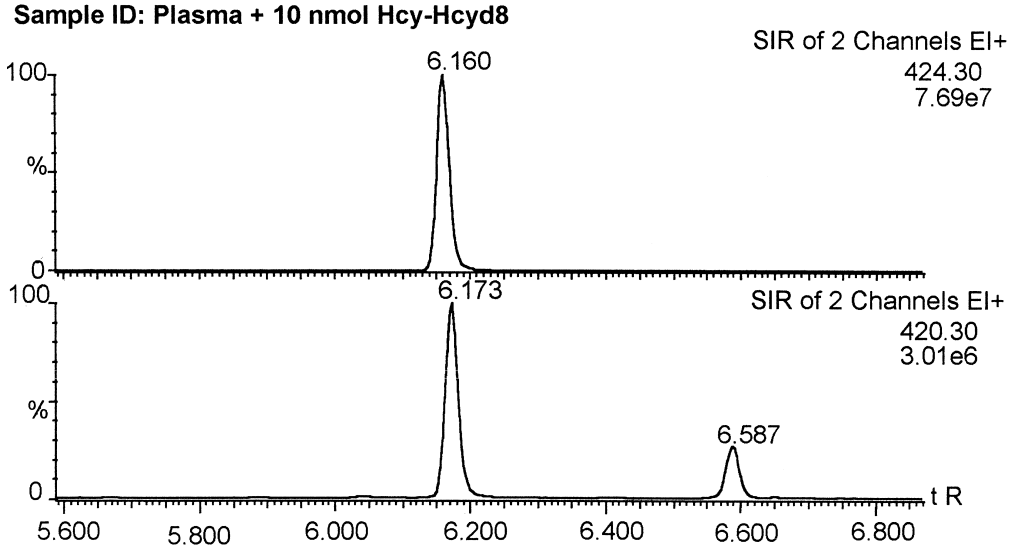


Fig. 1. Selective ion recording at *m/z* 420.3 and 424.3 obtained from a normal human plasma sample. NB: retention time of deuterated molecule is always shorter than that of an unlabelled molecule.

in deionized water. Linear regression of the observed tHcy ($y$) versus the calculated expected tHcy ($x$) gave the following equation:

$$y = 1.007 \times x - 0.003 \; (r = 0.998; S_{y,x} = 0.0078)$$

*3.1.2. Detection limit*

The detection limit (DL) is based on the statistical analysis of the signal difference observed for blanks and samples.The DL was evaluated by the following equation:

$$\mathrm{DL} = (3\,\mathrm{SD_b} \times C_{st})/(R_{st} - R_b)$$

where $SD_b$ is the standard deviation of the blank measurements ($n = 10$), $C_{st}$ is the concentration of a very low standard (0.185 μmol/l), and $R_{st}$ and $R_b$ are 420.3/424.3 ratio for standard and blank, respectively. The DL value was 0.17 μmol/l.

*3.1.3. Precision*

The variations of the between- and within-assay imprecision of the method were estimated after analyzing bovine serum samples in 16 and 10 determinations, respectively. The coefficients of variation for these measurements were 5.7% and 6.8%, respectively.

*3.1.4. Accuracy*

The accuracy of the stable isotope dilution assay was evaluated in two manners. Firstly, by the recovery of a supplement of tHcy (see below) and secondly, by the analysis of a control serum set (Bio-Rad, Munich, Germany). Two levels, control 1 (lot No. 015160) and control 2 (lot No. 015374) were tested in six different series. We found 10.8±0.65 μmol/l for the control 1 (manufacturer-stated target mean value: 9.8 μmol/l; acceptable range: 7.6–12.0 μmol/l) and 36.0±3.1 μmol/l for the control 2 (manufacturer-stated target mean value: 33 μmol/l; acceptable range: 26.0–40.0 μmol/l).

*3.1.5. Comparison with the HPLC technique*

Plasma samples ($n = 29$) were measured by the described method ($y$) and by an HPLC method developed in our laboratory ($x$). Data resulting from the two assays are analyzed by a linear regression analysis using the Deming procedure:

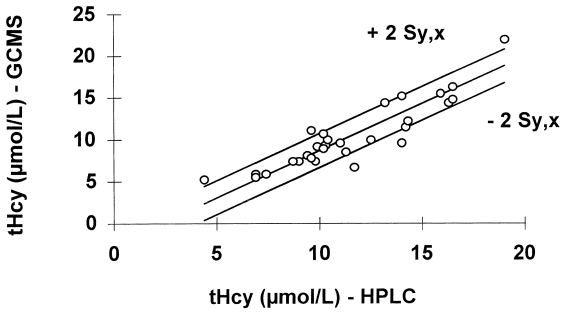


Fig. 3. Comparison of tHcy measured by the GC–MS ($y$) versus HPLC ($x$) using the Deming procedure regression method.

$$y = 1.128 \times x - 2.594 \; (r = 0.942, S_{y,x} = 1.015)$$

as shown in Fig. 3 and indicated satisfactory agreement between the two methods.

*3.2. Choice of the blood-collection medium*

Three anticoagulants (EDTA, CTAD and heparin) were tested on 12 subjects. CTAD was proposed by the hematologists because for thrombophilia diagnosis, several coagulation tests are done using this anticoagulant. Using paired-sample $t$-tests, a significant difference was observed between the three anticoagulants. EDTA plasma samples showed significantly lower values (6.6±2.1 μmol/l, mean±SD) than CTAD (7.4±2.1 μmol/l), which showed significantly lower values than heparin (8.1±2.5 μmol/l).

The recovery of a supplement of tHcy (3.7 μmol/l) was evaluated with the three anticoagulants. Twelve human plasma samples were analyzed with and without this supplement. For each anticoagulant, all the 24 samples were carried through the same trial. We found a recovery of 98.5±8%, 100.2±4.7%, and 99.8±11.9% for heparin, CTAD and EDTA, respectively.

*3.3. Reference values*

Results were obtained from a sample of 75 healthy volunteers (39 men and 36 women) aged from 44 to 64 for the men and from 39 to 64 years old for the women. Mean values of 9.5±1.9 μmol/l for the men and 8.0±2.2 μmol/l for the women were found. A

Table 1
Mean±SD for plasma tHcy concentrations in healthy subjects and patients

| Group | *n* | Mean±SD | *P*[a] |
|---|---|---|---|
| Controls | 75 | 8.8±2.2 | – |
| Venous thrombosis | 74 | 10.6±9.1 | 0.101 |
| Cerebrovascular disease | 63 | 10.3±4.2 | 0.008 |

[a] *P* indicates the *t*-test between cases and controls.

statistical difference of $P=0.003$ was noted between the two groups. A previous study on a smaller group of people ($n=37$), recruited from the preventive medicine center of the hospital, had shown similar results. Mean values of 10.2±1.7 ($n=17$) and 8.0±2.6 μmol/l ($n=20$) were found for the men and women, respectively.

### *3.4. Patients with venous thrombosis or with cerebral infarction*

We evaluated the prevalence of moderate hyperhomocysteinemia in a group of patients with cerebrovascular accident (stroke) and in a group of patients with venous thrombosis. Hyperhomocysteinemia was diagnosed when fasting plasma homocysteine levels exceeded the upper limit of the normal range (mean±2 SD of values obtained in the control group). Table 1 shows that the mean value of tHcy levels in the group of cerebrovascular accident patients was significantly different from those of control subjects. No significant difference was found between the venous thrombosis patients and the control groups. tHcy levels exceeded the upper limit of the normal range in nine patients (14%) with cerebrovascular accident and in seven patients (9%) with venous thrombosis. Among the patients with moderate hyperhomocysteinemia, 100% were men in the group of cerebrovascular accident patients, and in the group of venous thrombosis patients 57% and 43% were men and women, respectively.

## 4. Discussion

The aim of this study was to establish an accurate assay for total homocysteine in plasma in order to determine this parameter in routine investigation in the assessment of vascular risk. We reported reference values for tHcy from healthy subjects and preliminary results of its prevalence in two types of vascular diseases.

This method is an adaptation of a GC–MS method previously described by Stabler et al. [6]. This method is more sensitive (DL=0.1 pmol) than HPLC methods (DL=2–4 pmol), [7] which are the most currently used methods for measuring tHcy in plasma. Moreover, GC–MS assay offers good analytical precision (CV<6–7%) and shows satisfactory correlation with a standard HPLC technique. Its high sensitivity and specificity (deuterated Hcy as internal standard) show this method is well suited to the reliable determination of Hcy at low levels in clinical investigations.

However, our GC–MS method measures slightly lower concentrations than the HPLC method, showing a lack of standardization in tHcy measurements as it has been previously reported for chromatographic procedures [8]. The lower values obtained by GC–MS can be attributed to the use of an internal standard (deuterated Hcy) that equilibrates with endogenous homocysteine during the reduction step and remains equilibrated all through the sample preparation procedure.

The pre-analytical step – choice of anticoagulant and handling of blood samples after venipuncture – is the most important step, but also the most difficult to codify. Several publications discussed the best procedure of blood sample handling [8–12]. The big constraint to overcome is the immediate storage of the whole blood on crushed ice after venipuncture and the rapid separation of blood cells from plasma after centrifugation. The use of some anticoagulants as preservatives in whole blood, such as sodium fluoride [10] or acidic citrate [11], has been proposed but seems actually to be not adequate enough, at least for sodium fluoride [12]. The most commonly used anticoagulant is EDTA but in this study the lowest plasma tHcy values were observed on EDTA-containing tubes ($P=0.00$ between EDTA and heparin or EDTA and CTAD). The difference between using CTAD or heparin is less important ($P=0.022$). A higher osmolarity of EDTA than CTAD and heparin, leading to a higher plasma volume, could be proposed as an explanation of the highest significant difference. A difference between the concentrations of tHcy measured in EDTA and in

acidic citrate has already been reported [11]. However, the effect of anticoagulant on plasma tHcy concentration needs to be investigated on a great number of samples. If a difference between anticoagulants on tHcy concentrations was confirmed, this would imply the need to establish reference values for each anticoagulant. Independently of blood collection medium, immediate storage at 0°C after venipuncture and the rapid plasma separation were considered as the two main elements of the pre-analytical step for accurate tHcy determinations [12].

The following conditions (CTAD as the anticoagulant, the use of crushed ice immediately after venipuncture and prompt centrifugation) were selected for tHcy plasma level determinations. Our values for healthy volunteers are in good agreement with those reported in previous studies [8]. Mean tHcy concentrations were significantly lower in women than in men. The gender difference for tHcy was also found in the smallest sample of people taken from the preventive medicine center. The age of the two groups was of the same order of magnitude, although, the smallest group was slightly younger (from 31 to 59 and from 26 to 59 years old for men and women, respectively). No correlation was found between age and tHcy levels in this small group. This could be related to the few cases of lower age and/or to the fact that this correlation is true only above 60 years old [13,14]. No significant difference was observed between the pre ($n=26$) and postmenopausal ($n=10$) healthy women volunteers. One of the reasons could be the small difference in the age of the two subgroups: $44\pm5$ (mean$\pm$SD) and $55\pm5$ years old, respectively for pre and postmenopausal women. Another one could be the use of a hormone replacement therapy by postmenopausal women.

Moderate hyperhomocysteinemia is well recognized as a possible risk factor for thrombotic vascular events and its determination in clinical investigation of vascular thrombosis is now requested. Our preliminary results of moderate plasma tHcy levels in cerebrovascular accidents (14%) and in venous thrombosis (9%) showed a non-negligible prevalence of this risk factor. Case-control studies have previously found a high prevalence of mild hyperhomocysteinemia (from 14% to 42%) in cerebrovascular disease [15–17]. The strongest association ($>30\%$) could be explained by including a methionine loading test to detect heterozygosity for cystathionine β synthase [15,17]. In contrast to the great number of papers on the association between moderate hyperhomocysteinemia and arterial disease, the association with venous disease has been less studied until recently. Case-control studies reported a prevalence of hyperhomocysteinemia from 10% to 25% among patients with venous thrombosis [18,19]. Our preliminary results showed a quite similar frequency of this abnormality and its stronger association among men than women. However, a great number of data on the relationship between mild hyperhomocysteinemia and deep venous thrombosis are still unclear (interactions with thrombophilic factors) or lacking (interactions with non-heriditary factors). Thus a larger ($n=200$) case-control study with age and sex pairing is in progress to examine the prevalence of mild hyperhomocysteinemia as well as its association with other risk factors among patients suffering from deep venous thrombosis.

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## References

[1] G.N. Welch, J. Loscalzo, New Engl. J. Med. 338 (1998) 1042.
[2] P.M. Ueland, H. Refsum, L. Brattström, in: R.B. Francis Jr. (Ed.), Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function, Marcel Dekker, New York, 1992, p. 183.
[3] S.P. Stabler, P.D. Marcell, E.R. Podell, R.H. Allen, Anal. Biochem. 162 (1987) 185.
[4] J.O. Sass, W. Endres, J. Chromatogr. A 776 (1997) 342.
[5] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[6] S.P. Stabler, J. Lindenbaum, D.G. Savage, R.H. Allen, Blood 81 (1993) 3404.
[7] D.W. Jacobsen, V.J. Gatautis, R. Green, K. Robinson, S.R. Savon, M. Secic, J. Ji, J.M. Otto, L.M. Taylord Jr., Clin. Chem. 40 (1994) 873.
[8] P.M. Ueland, H. Refsum, S.P. Stabler, M.R. Malinow, A. Andersson, R.H. Allen, Clin. Chem. 39 (1993) 1764.

[9] R. Probst, R. Brandl, M. Blümke, D. Neumeier, Clin. Chem. 44 (1998) 1567.
[10] J. Moller, K. Rasmussen, Clin. Chem. 41 (1995) 758.
[11] 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.
[12] M.P. Hughes, T.H. Carlson, M.K. McLaughlin, D.D. Bankson, Clin. Chem. 44 (1998) 2204.
[13] L. Brattström, A. Lindgren, B. Israelsson, A. Andersson, B. Hultberg, J. Int. Med. 236 (1994) 633.
[14] K. Rasmussen, J. Moller, M. Lyngbak, A.M. Holm Pedersen, L. Dybkjaer, Clin. Chem. 42 (1996) 630.
[15] R. Clarke, L. Daly, K. Robinson, E. Naughten, S. Cahalane, B. Fowler, B.I. Graham, New Engl. J. Med. 324 (1991) 1149.
[16] C.J. Boushey, S.A.A. Beresford, G.S. Omenn, A.G. Motulsky, Jama 274 (1995) 1049.
[17] M. Candito, P. Bedoucha, M.H. Mahagne, G. Scavini, M. Chatel, J. Chromatogr. B 692 (1997) 213.
[18] P. Simioni, P. Prandoni, A. Burlina, D. Tormene, C. Sardella, V. Ferrari, L. Benedetti, A. Girolami, Thromb. Haemostas. 76 (1996) 883.
[19] M. den Heijer, T. Koster, H.J. Blom, G.M.J. Bos, E. Briet, P.H. Reitsma, J.P. Vandenbroucke, F.R. Rosendaal, New Engl. J. Med. 334 (1996) 759.