Measurement of Thiols in Human Plasma Using Liquid Chromatography with Precolumn Derivatization and Fluorescence Detection

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Abstract

A liquid chromatography (LC) method for the simultaneous measurement of the main low molecular mass thiols (i.e., cysteine, cysteinylglycine, homocysteine, and glutathione) in human plasma is described. The sample treatment consists of the reduction of disulfide bounds with tri-*n*-butylphosphine and protein precipitation with trichloroacetic acid followed by precolumn derivatization with a thiol-selective fluorogenic reagent (7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide). The structure of thiol derivatives is assessed using electrospray ionization–mass spectrometry (MS). The stability of resulting adducts in acidic medium (24 h at 10°C) allows the automation of the technique and a high throughput of samples (approximately 50 per day). Separation is complete within 12 min using isocratic reversed-phase mode, and detection is operated by spectrofluorimetry (λ_{ex} = 385 nm and λ_{em} = 515 nm). Quantitation is performed by an internal standardization mode using thioglycolic acid. The LC method is fully validated, and homocysteine concentrations obtained in plasma samples are compared with values measured using either fluorescence polarization immunoassay or capillary gas chromatography–MS; a good correlation is observed between LC and both methods. The method has been applied in daily use to a large-scale study in a human healthy population, and some resulting data are discussed.

Introduction

The measurement of plasma thiols appears to be a relevant parameter in order to investigate the numerous pathological diseases implying oxidative stress processes. As a matter of fact, glutathione (GSH) plays an important role as a scavenger of oxidant species and free radicals (1). The precursors involved into its biosynthesis, cysteine (Cys) and γ-glutamylcysteine (γ-GluCys), and its degradation product resulting from the reaction catalyzed by γ-glutamyltransferase, cysteinylglycine (CysGly), are also of interest to fully evaluate the glutathione homeostasis in a biological system (e.g., cells, tissues, blood, or plasma) (2). Moreover, homocysteine (Hcy) has recently emerged as an important biological parameter, because hyperhomocysteinemia is related to the risk of atherosclerosis and premature cardiovascular diseases (3–5). Concerning this latter thiol, the monitoring in plasma of its total concentration (*t*Hcy) corresponding to its free reduced and oxidized forms and protein-bound forms, has been stated to reflect the potent level of this parameter as a clinical marker (6). Consequently, a reduction step of disulfide-bounds has to be performed before protein precipitation and liquid chromatographic (LC) analysis.

Therefore, the simultaneous measurement of all the cited thiols present a great interest, because most of them are metabolically related and their disturbance can correspond to metabolic disorders. Many chromatographic methods have been reported in the investigation of endogenous thiols, especially LC using thiol-selective fluorogenic reagents (7–20). More recently, capillary electrophoresis has been described as a potential alternative technique for this purpose (21). We presently report a LC method with precolumn derivatization devoted to the simultaneous measurement of the main endogenous low-molecular-mass thiols as their total concentration after reduction in plasma. As an additional point to the validation, results obtained for *t*Hcy using this LC technique are compared with two other methods widely used for its measurement: gas chromatography–mass spectrometry (GC–MS) (22–24) and fluorescence polarization immunoassay (FPIA) (25). Data obtained during a large-scale epidemiological study are also shown.

Experimental

Chemicals, reagents, and standards

All chemicals and solvents were of analytical or LC reagent grade and used without further purification. All the studied

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thiols in their reduced or disulfide forms, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F), and tri-*n*-butylphosphine (TBP) were obtained from Sigma (St. Louis, MO).

The stock solution of each standard thiol was prepared at a concentration of 1.0mM in 0.1M HCl containing 2mM EDTA and kept at –80°C for a maximum of 2 months. Calibration curves including 5 points were generated daily with further dilutions of stock solutions in 9% (w/v) NaCl containing 4mM EDTA at concentrations ranging from 50–300, 10–50, 2.5–15, and 1–10µM for Cys, CysGly, Hcy, and GSH, respectively.

Blood collection and storage conditions

Blood was collected at the antecubital vein of healthy volunteers (informed consent was obtained, and the research protocol was in agreement with the Helsinki Declaration) in a reclined position in tubes containing 1.8 g/L K_3EDTA (Vacutainer Tube, Becton Dickinson, Grenoble, France) previously chilled in crushed ice and put again in ice immediately after collection. Blood samples were centrifuged (1000 × *g* for 15 min at 4°C) within 2 h after collection, and resulting plasma samples were frozen at –80°C until analysis.

The present epidemiologic study was included in the Stanislas Cohort, and the data shown involved 414 healthy subjects (205 men aged 6–59 years, and 209 women aged 7–55 years).

Plasma sample treatment and precolumn derivatization

Plasma samples were quickly thawed at 37°C. A 200-µL aliquot of plasma or standard solution for calibration curves was transferred into a 1.5-mL polypropylene tube put in crushed ice, and 100 µL of the internal standard solution (thioglycolic acid, TGA, at a concentration of 300µM) and 50 µL of a 5% (v/v) TBP solution in dimethylformamide were added. After mixing for 10 s using a vortex, a nitrogen stream was introduced for 10 s into the tube before capping it in order to retard further air oxidation. The resulting mixture was incubated in the dark at 4°C for 30 min. A 10% (w/v) TCA solution (200 µL) was added for protein precipitation. After centrifugation at 1500 × *g* at 4°C for 15 min, 100 µL of the supernatant was transferred into a 1-mL autosampler vial and mixed with 30 µL of 0.5M NaOH, 250 µL of 0.2M borate buffer (pH 9.0), and 50 µL of a 2.3mM ABD-F solution in dimethylformamide. The resulting mixture was incubated at 50°C for 20 min under gentle stirring; then, the derivatization reaction was stopped by adding 50 µL of 1.0M HCl, and resulting samples were cooled in crushed ice.

LC system and operating conditions

The LC system consisted of an isocratic solvent delivery pump (model PU-980, Jasco-Prolabo, Fontenay-sous-Bois, France), an autosampler equipped with a 20-µL injection loop, a cooling sample device and column oven (model AS-300, ThermoQuest, Les Ulis, France), a spectrofluorimetric detector (model FP-920, Jasco), and data processing software (Borwin model, JMBS Developpements, Le Fontanil, France).

The tray compartment containing sample vials was cooled at 10°C, and the LC system was operated overnight. A guard column (4 × 4-mm i.d.) packed with LiChrospher RP_{18} endcapped 5-µm (Merck, Darmstadt, Germany) and an analytical column (70 × 4-mm i.d.) packed with Nucleosil 100 C_{18} 5-µm (Macherey-Nagel, Düren, Germany) were eluted with acetonitrile–0.1M phosphate buffer pH 2.5 (5:95, v/v) at a column temperature of 35°C and a flow rate of 1.0 mL/min. Spectrofluorimetric detection was operated at an excitation and emission wavelength of 385 and 515 nm, respectively.

MS of derivatives

Each thiol (approximately 1 mg) was separately derivatized according to the procedure described previously; the resulting reaction mixture was injected onto a LiChropher 100 RP_{18} endcapped (5 µm) 125- × 4-mm column (Merck) eluted with acetonitrile–water–formic acid (5:95:0.1, v/v/v) at a flow rate of 1.0 mL/min with UV detection at 385 nm. Each derivative peak was collected, and a 20-µL aliquot of the collected fraction was injected into the MS (Platform VG Bio Q model, Micromass, Alltrincham, U.K.). The infusion solvent mixture entering into the MS was acetonitrile–water–formic acid (25:75:0.1, v/v/v) at a flow rate of 10 µL/min. Electrospray in both positive and negative modes was used as an ionization mode (cone energy, 50 V; temperature, 60°C) and data were collected and reprocessed by means of Mass Lynx software (Micromass).

GC–MS method for total homocysteine

The capillary GC–MS was used as previously reported (22–24). Briefly, plasma samples, after the addition of deuterated internal standards (i.e., Hcy, cystathionine, methylmalonic acid) and reduction with 1,4-dithio-D,L-threitol, were purified using anion-exchange chromatography. Then, the *tert*-butyldimethylsilyl derivatives were separated by a capillary GC equiped with an automatic liquid sample injection system (Hewlett-Packard 6890, Waldbronn, Germany) and detected with a mass detector (Hewlett-Packard 5973) in selected ion monitoring (SIM) mode.

Immunoassay method for total homocysteine

The FPIA relied upon the fully automated Abbott IMx assay devoted to the measurement of *t*Hcy in plasma, according to the previously reported technique (25).

Statistical calculation

The comparison between *t*Hcy concentrations in plasma samples measured by LC versus either the GC–MS or FPIA method was made by linear regression analysis and the Bland and Altman (26) plot. Analysis of variance (ANOVA) with repeated measurements was used as a statistical approach to test the significance of differences ($p < 0.05$) between thiol concentrations.

Results and Discussion

Optimization of thiol derivatization and LC system

The two main problems with the measurement of thiols in biological matrices are their instability during sample treatment and the absence of chromophores for sensitive detection in LC systems. Concerning the first point, conditions for blood collection and plasma storage have been extensively studied and discussed elsewhere (6,7,27,28). In order to overcome the lack of chromophore, the detection of thiols in LC systems can be oper-

ated either directly by electrochemistry (29,30) or more frequently by fluorescence after labelling with various thiol selective reagents. The main fluorogenic reagents used are monobromobimane (mBrB) (8–10), *N*-(1-pyrenyl)maleimide (NPM) (11,12), *ortho*-phthalaldehyde (OPA) (12–15), ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) (16–18,20), and ABD-F (7,16,18,19). NPM and OPA can be used in either a pre- (11,13–15) or postcolumn (12) mode. OPA can only detect a limited number of aminothiols, such as either GSH and γ-GluCys (12,14) or Cys and Hcy (13,15), and the other cited reagents react with all the thiols. mBrB (9) and NPM (11) have the main disadvantage of producing reagent peaks on chromatograms that can overlap thiol peaks or are late-eluted. Both ABD-F and SBD-F do not present these disadvantages: they have no intrinsic fluorescence, but the latter reagent has several strong drawbacks. SBD-F reacts between 6 and 30 times slower than ABD-F (1 h versus 5–20 min) at a higher temperature (60°C versus 50°C) and pH (9.5–11 versus 8–9.3) values, which represents harmful conditions for the stability of thiol derivatives (18,21).

We have presently selected ABD-F (Figure 1) with regard to the different points discussed, and the present work is focused on the optimization of operating conditions for plasma treatment in order to measure total thiols. Therefore, the following points were examined more carefully: (*a*) choice of reducing reagent, (*b*) pH of derivatization medium and reaction time, (*c*) stability of derivatives, and (*d*) LC conditions.

Thiol reducing reagents (*a*) such as 2-mercaptoethanol and 1,4-dithio-D,L-threitol cannot be used in connection with a precolumn derivatization step involving a fluorogenic substance reacting with sulfhydryl groups, because the probe would be fully consumed by the thiol reagent excess. Therefore, non-thiol-reducing compounds such as sodium borohydride (10,30) and TBP (7,16,18,19) have to be selected. TBP has the advantage of being an excellent specific reductant for disulfide bonds able to quantitatively break those links even when used only in a 20% molar excess, as well as being nonreactive toward ABD-F (7) and so not producing side-reaction with the fluorogenic probe. Tris(2-carboxyl-ethyl)phosphine (TCEP) has been recently reported to yield higher reduction rates of Hcy disulfide than TBP (17). TBP was presently used, and we found no difference of thiol recoveries when a plasma pool was fortified with either reduced thiols or their corresponding disulfide (see the Assay validation of thiols section). This demonstrates the nearly 100% yield of the reduction step using TBP (Table I) and verifies its efficiency.

According to Toyo'oka and Imai (18), optimal pH (*b*) for derivatization reactions with ABD-F is between 8 and 9.3; at lower pH values, the reaction does not occur, and in a higher alkaline medium, some byproducts appear (19). The same operating conditions (addition of NaOH and borate buffer) to alkalinize the reaction medium were selected in order to yield the apparent pH value of 9.0 in both aqueous standard solutions and plasma samples. At a temperature of 50°C, the optimum reaction time of 20 min was retained because it corresponds to the maximum formation rate of derivatives (Figure 2).

Thiol derivatives obtained in an alkaline medium and kept at the same pH do not have sufficient stability (*c*) to allow automated injections in a LC system for more than a couple of hours at room temperature. However, they can be stabilized for a longer period by acidification of the reaction mixture; this fact has been demonstrated for derivatives obtained with mBrB (10), NPM (11), and ABD-F (7,16,19). It was found that in an acidic medium (pH between 1 and 2), no significant variation of the peak area corresponding to each thiol derivative was observed for 24 h at 10°C. Therefore, the automation of the LC system was

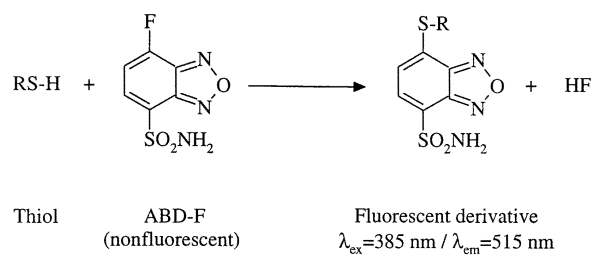

Figure 1. Reaction scheme of the precolumn thiol derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F).

Table I. Recoveries of Thiols from a Plasma Pool Fortified with Their Reduced and Disulfide Forms

Thiol	Concentration (μM) Added	Calculated	Measured	RSD (%) (*n* = 6)	Recovery (%)
Reduced					
Cys	—	—	178*	1.4*	—
	40.0	218	227	0.4	104
	90.0	268	274	3.1	102
	180.0	358	348	3.2	97
CysGly	—	—	27.8*	1.1*	—
	5.0	32.8	30.2	2.2	92
	12.0	39.8	40.6	0.6	102
	27.0	54.8	52.4	2.4	96
Hcy	—	—	7.9*	0.8*	—
	1.7	9.6	9.4	3.5	98
	4.1	12.0	11.4	4.2	95
	8.3	16.2	16.4	2.9	101
GSH	—	—	2.0*	3.1*	—
	0.4	2.4	2.6	2.9	108
	1.1	3.1	3.4	2.9	110
	2.6	4.6	4.5	4.0	98
Disulfide					
Cys-Cys	—	—	183*	2.5*	—
	40.0	223	220	3.5	98
	90.0	273	274	2.8	101
	180.0	363	366	2.2	101
CysGly-CysGly†	—	—	—	—	—
Hcy-Hcy	—	—	8.3*	3.0*	—
	1.7	10.0	9.9	2.9	99
	4.1	12.4	12.9	4.7	104
	8.3	16.6	17.4	3.6	105
GSSG	—	—	2.2*	5.1*	—
	0.4	2.6	2.6	5.5	99
	1.1	3.4	3.4	4.0	106
	2.6	4.5	4.5	5.7	104

* Nonfortified plasma pool.
† Not commercially available.

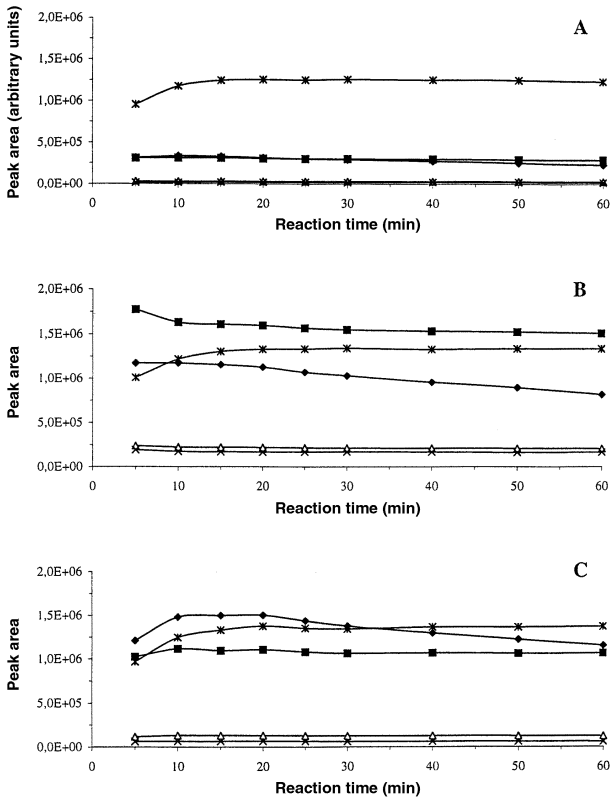

Figure 2. Time dependence for the formation rate and stability of the derivatives resulting from reaction of thiols with ABD-F at pH 9.0. Standard thiol solutions corresponding to the lowest (A) and highest (B) concentrations of the calibration curve; plasma pool (C). u, Cys; n, CysGly; Δ, Hcy; ×, GSH; Q TGA.

Table II. Selectivity of the LC System and Relative Fluorescence Intensity (RFI) Factor of Thiol Derivatives

Thiols	RRT*	RFI
Endogenous thiols		
Cysteine	0.2	1.0
Cysteinylglycine	0.3	5.2
Homocysteine	0.4	2.5
Glutathione	0.6	1.9
γ-Glutamylcysteine	0.7	2.1
Coenzyme A	0.9	2.9
α-lipoic acid	1.2	1.5
Exogenous thiols		
Thioglycolic acid	1.0	1.0
2-Mercaptoethanol	1.4	3.0
N-acetylcysteine	1.5	1.6
2-Mercaptopropionylglycine	2.4	2.9
Cysteamine	3.4	3.1
Captopril	3.5	1.5
D-penicillamine	3.6	1.0

* RRT, relative retention time expressed versus thioglycolic acid selected as internal standard.

† RFI, relative fluorescence intensity defined as (peak area of the considered thiol derivative/thiol molar quantity injected) × (thioglycolic acid molar quantity injected/peak area of thioglycolic acid derivative).

done overnight with a cooling of the autosampler.

LC conditions (*d*) for the analysis of resulting thiol derivatives were selected to provide short retention times with complete separation between peaks. A short length reversed-phase column and isocratic eluting conditions met these requirements. Lowering the pH of the mobile phase from 3.2 to 2.5 increased both retention time and fluorescence signal (approximately 30% and 40%, respectively); thus, the lower value was retained and acetonitrile content (5%) was optimized to give the best compromise between short retention times and full resolution between peaks (Rs, higher than 1.2 for each pair of vicinal peaks).

The developed LC method devoted to the main biological plasma thiols was selective against other minor endogenous thiols, exogenous compounds including some pharmacological

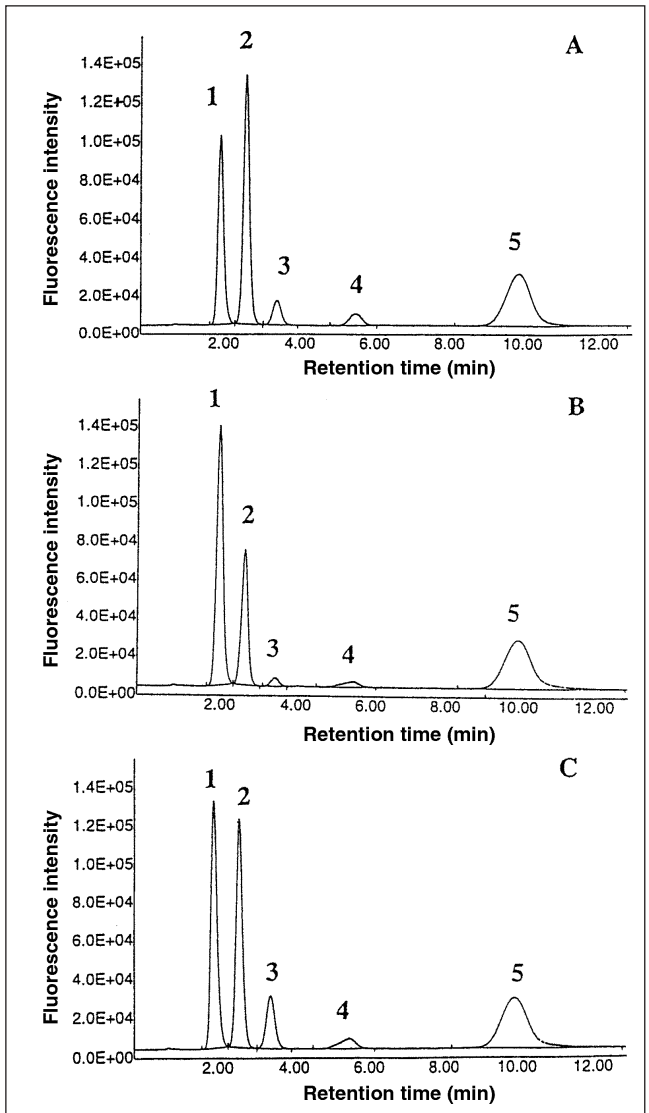

Figure 3. Typical chromatograms corresponding to standard thiol solutions (A) (1, Cys, 300μM; 2, CysGly, 50μM; 3, Hcy, 15μM; 4, GSH, 10μM; 5, internal standard TGA, 300μM) and to plasma samples with low (B) and high (C) *t*Hcy levels. LC operating conditions: Nucleosil 100 C_{18} (5 μm) 70 × 4-mm-i.d. column eluted with acetonitrile–0.1M phosphate buffer pH 2.5 (5:95, v/v) at a flow rate of 1.0 mL/min and at 35°C; fluorescence detection, λ_{ex} = 385 nm and λ_{em} = 515 nm.

active substances (Table II), and also a possible minor reagent peak eluted after the GSH-derivative peak. Its appearance seems to be (in routine practice) a good marker of ABD-F solution degradation. Relative response factors of the different thiol derivatives have been calculated (Table II); data agree with those previously reported (16,28). Typical chromatograms corresponding to standard solutions and plasma samples are shown in Figure 3.

Structural study of thiol derivatives by ESI-MS

No structural assessement of ABD-thiol derivatives has been reported in the literature. The molecular mass of each thiol derivatized with ABD-F was presently determined using MS with ESI as ionization mode. A molecular peak was observed in either positive or negative mode, according to the studied derivative. The MS data indicate the presence of a main protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ion at the expected value (m/z) of each derivative, as illustrated in Figure 4. $[M+H]^+$ values found for ABD-Cys, ABD-CysGly, ABD-Hcy, ABD-GSH, and ABD-γ-GluCys derivatives were 318.95, 375.90, 332.98, 504.93, and 447.97, respectively. $[M-H]^-$ values for ABD-TGA and ABD-*N*-acetylcysteine were 287.97 and 360.85, respectively. For these two latter derivatives, a peak corresponding to the dimeric adduct was also observed.

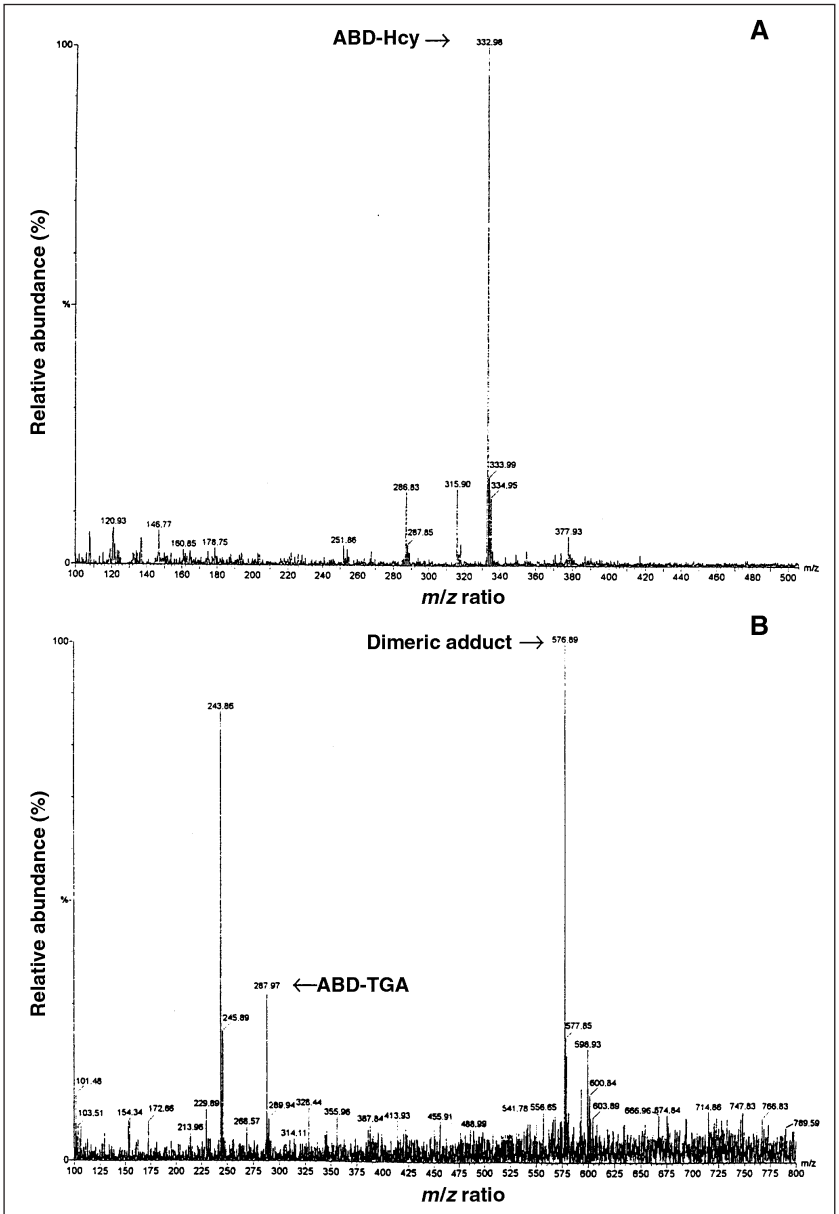

Figure 4. Mass spectra of ABD-thiol derivatives obtained by ESI-MS. Arrows indicate the molecular ion $[M+H]^+$ of ABD-Hcy derivative in positive mode (A) and $[M-H]^-$ of ABD-TGA derivative in negative mode (B).

Assay validation of thiols

The choice of an internal standard was made after testing different exogenous thiols for selectivity (Table II): TGA showed the shortest retention time (approximately 12 min) among them and was thus selected.

The assay validation was achieved using the internal standardization mode for quantitation; linearity, limit of detection (Table III), repeatability, and recoveries (Table I) were studied. The linearity range for each thiol was selected according to their physiological values and included the "cut-off" value between physiological and pathological concentrations of *t*HCy (i.e., 15µM) (3,10).

Limits of detection found for a signal-to-noise ratio of 3 agreed with previous data in the literature (19).

Recoveries were calculated by using a plasma pool fortified with thiols in their reduced and disulfide forms at 20, 50, and 100% of each thiol concentration measured before spiking. A previous report stated some improvements when using the disulfide form of Hcy for calibration (25,29). The high recoveries found in our experiments for both forms of each thiol demonstrate that optimal operating conditions for reduction and extraction steps have been selected and also validate the choice of aqueous standard solutions of reduced thiols for routine calibration. Repeatability was evaluated simultaneously with the recovery study, and resulting relative standard deviation (RSD) values were lower than 3% (6 replicates). Interday variation was evaluated using the same plasma pool, and RSDs for the total forms of Cys, CysGly, Hcy, and GSH were 5.9, 6.3, 7.7, and 9.3%, respectively (n = 6 days).

Comparison of homocysteine measurement by LC, GC–MS, and immunoassay

*t*Hcy concentrations obtained in plasma samples with the LC method presently described were compared with values measured using either GC–MS (22–24) or FPIA (25), both techniques being widely used in the literature.

First, concerning the comparison between LC and GC–MS methods (Figure 5A), it was observed that (*a*) the equation of the regression line shows a slope and an intercept that are not significantly

different from 1 and 0, (*b*) the mean bias was low ($S_{y/x}$ = –0.1; $p < 0.05$), and (*c*) the distribution of concentration differences between methods was inside the limits of agreement (mean ± 2 SD). It can be concluded that these two methods are well correlated, as already demonstrated between the same GC–MS method and different LC techniques (7,22–24).

In a similar way, Figure 5B shows the linear regression calculated between *t*Hcy plasma concentrations measured by LC (*x*) and FPIA (*y*). The resulting slope value (i.e., 0.910) was lower than values previously reported in the literature, ranging from 1.1 to 1.3 (25,32) except for Frantzen et al. (33) who found a slope of 0.993. Such differences could result from analytical bias: different reference substances and treatment sample modes were used in each method, and the range of *t*Hcy concentrations measured in the present study (5 to 20µM) was narrower than in other reports (0 to 300µM). However, the mean bias observed was low ($S_{y/x}$ = –0.4; $p < 0.05$) and the distribution of concentration differences between methods was within the limits of agreement (mean ± 2 SD). It can therefore be concluded that the two methods gave equivalent concentrations for *t*Hcy, as in a previous study (34).

According to these data, a good correlation was observed between the presently described LC technique and both GC–MS and FPIA methods devoted to the measurement of *t*Hcy.

Clinical study

The method is currently applied to a large-scale study involving 1000 families (4000 healthy subjects), the Stanislas Cohort (35). Presently, 414 samples have been analyzed. Figure 6 shows the age- and sex-related distribution for each thiol investigated. Significant differences in relation to age and sex are observed for *t*CysGly, *t*Hcy, and *t*GSH concentrations, but not for *t*Cys. *t*Hcy and *t*GSH levels in plasma increase and decrease with age in both sexes, respectively. These data confirm previous results in which homocysteinemia (3) and reduced glutathione in plasma (36) are correlated with age and sex. *t*CysGly is also modified as a function of age but in a reverse way in men and women; this fact has never been previously pointed out. The present data are of great importance to understand the increasing risk of pathological diseases linked with oxidative stress and need an extended evaluation to the overall population of the epidemiological study in order to obtain more precision in the statistical analysis. Other defense signals against oxidative stress will also be also investigated and correlated to thiols.

Table III. Linearity and LOD of the LC Method

Reduced thiol	Concentration range (µM)	Slope * (× 10^3)	Intercept (× 10^3)	Correlation coefficient (*r*)	LOD (nM)
Cys	50–300	2.9	93.0	0.9867	625.0
CysGly	10–50	22.3	9.3	0.9984	125.0
Hcy	2.5–15	10.9	–1.7	0.9969	312.5
GSH	1–10	10.9	0.1	0.9952	250.0

* Calculated by internal standardization: (thiol peak area/TGA peak area)/thiol concentration.
† Expressed in arbitrary units.
‡ Measured for a signal-to-noise ratio of 3.

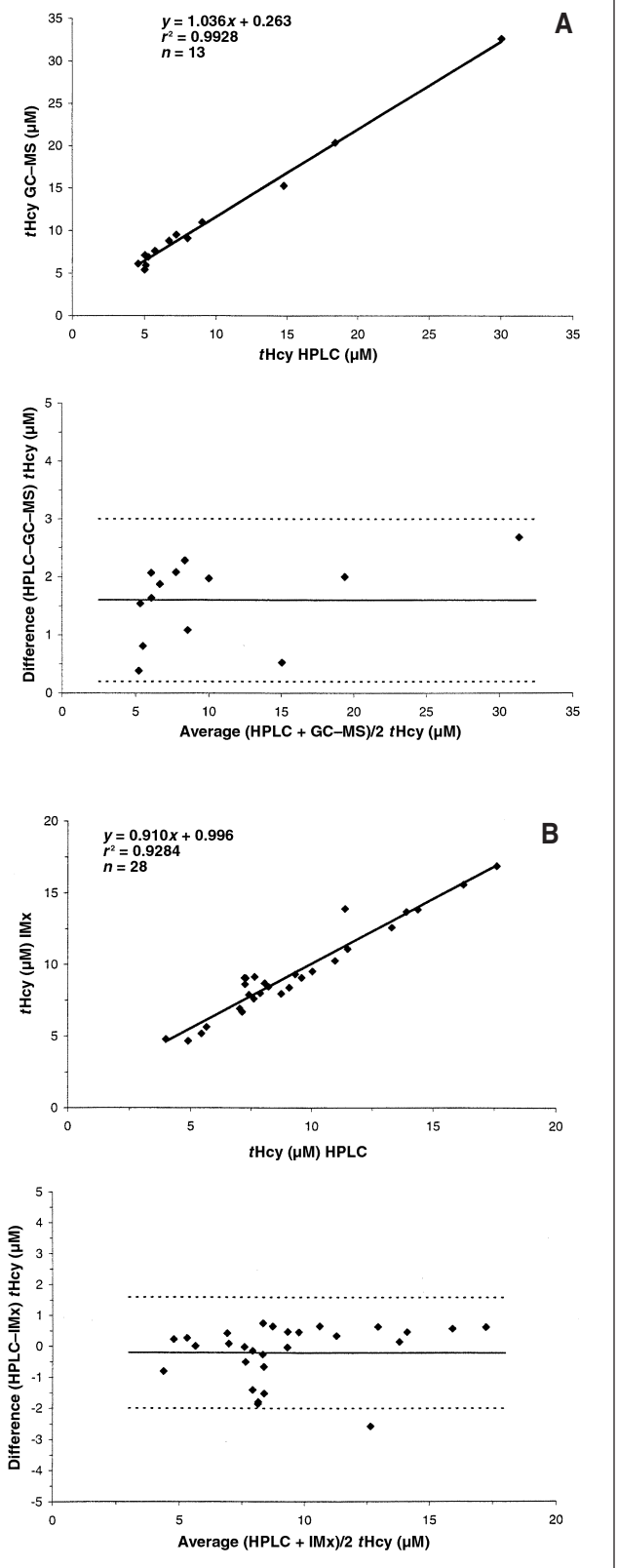

Figure 5. Comparison of *t*Hcy concentrations measured by LC (*x*) and GC–MS (*y*) (A) and FPIA (B) with linear regression analysis and Bland–Altman plot (24), which shows the difference in *t*Hcy results between the two methods as a function of their value with mean difference (solid line) and limits of agreement corresponding to mean ± 2 SD (dashed lines).

Conclusion

The present method has the main advantages over previously reported methods of (*a*) measuring not only one single thiol (e.g., GSH or Hcy) but allows the assay of all the endogenous thiols and thus presents versatility when considering applications, (*b*) offering a high throughput of samples, and (*c*) automation because of the long term stability of the derivatives.

Validation data obtained in the present study demonstrate the feasability of the technique for a daily use in a large-scale study of a human population. The technique has also been applied in our laboratory to other biological matrices, especially cultured cells.

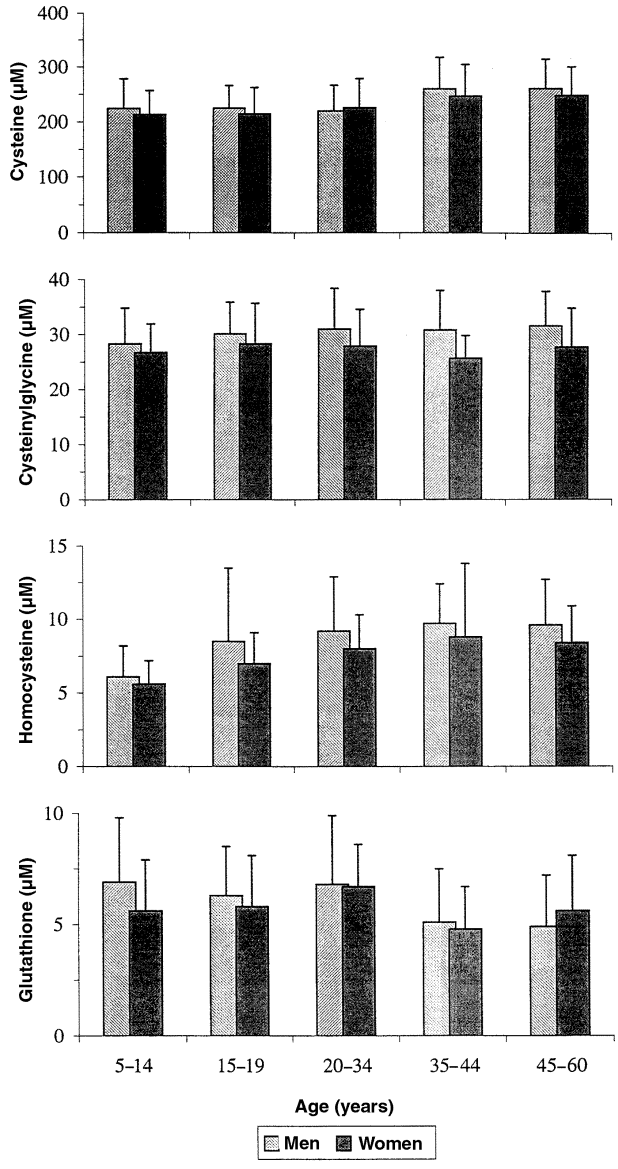

Figure 6. Age- and sex-related distribution of total plasma thiol concentrations (mean + SD) in healthy subjects of the Stanislas Cohort (205 men, ages 6–59, and 209 women, ages 7–55).

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