Gas Chromatographic–Mass Spectrometric Analysis of Biomarkers Related to Folate and Cobalamin Status in Human Serum after Dimercaptopropanesulfonate Reduction and Heptafluorobutyl…

ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 2008

Impact Factor: 5.64 · DOI: 10.1021/ac8003506 · Source: PubMed

3 AUTHORS:

Petr Simek
Academy of Sciences of the Czech Republic
113 PUBLICATIONS **1,984** CITATIONS
SEE PROFILE

Petr Husek
Academy of Sciences of the Czech Republic
20 PUBLICATIONS **259** CITATIONS
SEE PROFILE

Helena Zahradníčková
Institute of Entomology ASCR
44 PUBLICATIONS **524** CITATIONS
SEE PROFILE

Anal. Chem. **2008,** *80,* 5776–5782

Gas Chromatographic−Mass Spectrometric Analysis of Biomarkers Related to Folate and Cobalamin Status in Human Serum after Dimercaptopropanesulfonate Reduction and Heptafluorobutyl Chloroformate Derivatization

Petr Šimek,*,† Petr Hušek,†,‡ and Helena Zahradníčková†

Biology Centre, Laboratory of Analytical Biochemistry, Academy of Sciences of the Czech Republic, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic, and Institute of Clinical Biochemistry, Faculty Hospital, 17. listopadu 1790, CZ-708 52 Ostrava, Czech Republic

Methylmalonic acid and total homocysteine belong to useful clinical indicators of cobalamin and folate status and are commonly measured separately. A sensitive and rapid method has been developed for simultaneous determination of both biomarkers and related metabolites in serum or plasma by isotope dilution gas chromatography−mass spectrometry (GC/MS). Thiols bound in disulfide bonds were released with 2,3-dimercapto-1-propanesulfonic acid (DMPS), and after deproteinization, they were phase-transfer derivatized with heptafluorobutyl chloroformate (HFBCF) in a single step. The reducing capability of the DMPS agent was comparable to that of the dithiothreitol, but exceeded the latter in much cleaner extracts obtained. The new method enabled GC/MS screening of amino acidic metabolites, including cystathionine and thiol-containing dipeptides, as their N(S,O)-heptafluorobutoxycarbonyl heptafluorobutyl ester derivatives in serum or plasma. Accurate quantitation of seven biomarkers was accomplished by using deuterated internal standards; the detection limits ranged from 7 to 20 nmol/L, the between-day precision from 1.5 to 8.8%, and the recoveries were between 83 and 103%. The results suggest that the new combined procedure of DMPS reduction and HFBCF derivatization make the method efficient for diagnostics of folate and cobalamin status as well as for screening of amino acidic metabolites in body fluids.

Methylmalonic acid (MMA) and total homocysteine (tHcy) measurements in serum or plasma received nowadays considerable attention because of being useful indicators of cobalamin and folate status. Elevated MMA levels in body fluids may reflect impaired cobalamin function, whereas tHcy is elevated in both folate and cobalamin deficiencies. The determination of serum MMA has been a subject of numerous studies, as it presumably has a better diagnostic accuracy than measurement of cobalamin levels.[1,2] Abnormalities of tHcy have also been associated with cardiovascular and thromboembolic diseases.[3,4] It has been further demonstrated that changes in tHcy metabolism affect other thiols, such as cysteine (Cys), cysteinylglycine (CysGly), glutathione, and cystathionine (Cth).[5,6] Thus, Cth, which is formed from Hcy and Ser by cystathionine-β-synthase and further converted to Cys in the transsulfuration pathway, was elevated in patients with cobalamin and folate deficiency[7] and the Cth/Cys ratio increased significantly in renal patients.[8]

The biomarkers of folate and cobalamin status were analyzed almost exclusively by separate methods. MMA, occurring in serum at the 50−400 nmol/L level, was usually quantified in the form of its *tert*-butyldimethylsilyl[7,9] or alkyl ester[10–14] derivatives by isotope dilution GC/MS, and recently also by a method based on determination of the underivatized MMA by liquid chromatography−tandem mass spectrometry (LC−MS/MS).[15] On the other hand, Hcy is predominantly bound to proteins and other thiol metabolites in blood and has to be released from the disulfide bonds prior to analysis. Several reducing agents such as 1,4-dithiothreitol (DTT) or 1,4-dithioerythritol (DTE), borohydrides, trialkylphosphines, and 2-mercaptoethanol have been employed

* To whom correspondence should be addressed. E-mail: simek@bclab.eu.
† Academy of Sciences of the Czech Republic.
‡ Faculty Hospital.

(1) Hoelleland, G.; Schneede, J.; Ueland, P. M.; Lund, P. K.; Refsum, H.; Sandberg, S. *Clin. Chem.* **1999**, *45*, 189–98.
(2) Monsen, A. L. B.; Ueland, P. M. *Am. J. Clin. Nutr.* **2003**, *78*, 7–21.
(3) Nygård, O.; Nordrehaug, J. E.; Refsum, H.; Ueland, P. M.; Farstad, M.; Vollset, S. E. *N. Engl. J. Med.* **1997**, *337*, 230–7.
(4) Herrmann, W.; Herrmann, M.; Obeid, R. *Curr. Drug Metab.* **2007**, *8*, 17–31.
(5) Mansoor, A. M.; Svardal, A. M.; Schneede, J.; Ueland, P. M. *Clin. Chem.* **1992**, *38*, 1316–21.
(6) Mansoor, A. M.; Svardal, A. M.; Ueland, P. M. *Anal. Biochem.* **1992**, *200*, 218–29.
(7) Stabler, S. P.; Lindenbaum, J. L.; Savage, D. G.; Allen, R. H. *Blood* **1993**, *81*, 3403–13.
(8) Herrmann, W.; Obeid, R. *Clin. Chem. Lab. Med.* **2005**, *43*, 1039–47.
(9) Rifai, N.; Hagen, T.; Bradley, L.; Sakamto, M. *Ann. Clin. Bichem.* **1998**, *35*, 633–6.
(10) Rasmusen, K. *Clin. Chem.* **1989**, *35*, 260–4.
(11) Pietzsch, J.; Julius, U.; Hanefeld, M. *Clin. Chem.* **1997**, *43*, 2001–4.
(12) Sass, J. O.; Endres, W. *J. Chromatogr., A* **1997**, *776*, 342–7.
(13) Hušek, P.; Matucha, P.; Vránková, A.; Simek, P. J. *Chromatogr., B* **2003**, *789*, 311–22.
(14) Windelberg, A.; Arseth, O.; Kvalheim, G.; Ueland, P. M. *Clin. Chem.* **2005**, *51*, 2103–9.
(15) Blom, H. J.; van Rooijk, A.; Hovegeen, M. *Clin. Chem. Lab. Med.* **2007**, *45*, 645–50.

10.1021/ac8003506 CCC: $40.75
Published on Web 06/24/2008

to accomplish the reduction.[14–18] The most commonly used DTT reduction step became part of the commercial immunoassay kits used for routine clinical tHcy assays.[16] For the simultaneous determination of tHcy together with the related thiols, HPLC-based methods have been preferred.[16,17,19,20]

The evident complexity of the folate and cobalamin (vitamin B_{12}) metabolism has evoked a need to simultaneously determine levels of MMA, tHcy, and related amino acidic metabolites. Windelberg et al.[14] have elaborated a GC/MS assay based on a successive DTE reduction of the bound thiols, ethanol deproteination, and methyl chloroformate derivatization. The strategy for immediate derivatization of amino acidic compounds in aqueous media by chloroformates was proposed by Hušek.[21,22] Generally, it was utilized in numerous studies and also for the separate determination of tHcy[11–13] and MMA[14] in serum or plasma. The advantage of simultaneous conversion of carboxylic, amino, and thiol groups into N(O,S)-alkoxycarbonyl alkyl ester derivatives, even in complex deproteinized biological fluids, stimulated synthesis and examination of novel fluoroalkyl chloroformates (FCF). FCF reagents are more reactive than the analogues with aliphatic alkyls and readily convert polar compounds into derivatives with favorable features in GC analysis.[23–29]

In our pilot measurements of thiols in body fluids, we observed that alkyl chloroformates generally reacted with the reducing reagents and their oxidation products. As a result, these products coelute particularly with the late-eluting biomarkers and interfere with their GC/MS determination. In this work, we thus focused on developing an approach enabling us to minimize the undesired interferences. For the release of the bound thiols in serum or plasma we examined 2,3-dimercapto-1-propanesulfonic acid (DMPS, sodium salt) and compared the reductant with the commonly used DTT. DMPS, commercially sold as Dimaval, has been known primarily as a chelating agent capable of forming stable complexes with mercury, lead, and arsenic, thus allowing their elimination from the body.[30] DMPS is relatively nontoxic and has been administered per os or intravenously in long-term chelation therapy. Owing to the sulfonic moiety in its structure, it is perfectly water soluble and nearly odorless.[31] To our knowledge, DMPS has been used as a reducing agent only in some studies of disulfide–thiol oxidation–reduction states in proteins and cells.[32–34]

In this study, the DMPS reduction step was combined with the heptafluorobutyl chloroformate (HFBCF) derivatization procedure and examined for the analysis of MMA, tHcy, total Cys (tCys), related thiols, and protein amino acids in serum or plasma by GC/MS. The method was validated by means of the available labeled standards for the determination of MMA, tHcy, tCys, Met, Ser, Gly, and Cth in serum.

EXPERIMENTAL SECTION

Materials. Amino acids, MMA, γ-GluCys, CysGly, pyridine, isooctane (2,2,4-trimethylpentane), DTT, and DMPS were delivered from Sigma-Aldrich (Prague, Czech Republic) at the highest purity available. Perchloric acid (PCA) p.a (>70%) was supplied from Acros Organics (Geel, Belgium). Sodium carbonate, hydroxide, and chloride were obtained from Lachema (Brno, Czech Republic, p.a. grade). d_2-Gly, L-d_3-Met, D,L-d_3-Ser, D,L-d_4-cystine, and D,L-d_8-homocystine were purchased from Cambridge Isotope Laboratories (Andover, MA). d_3-MMA was provided by Per Magne Ueland (University of Bergen, Norway), and D,L-d_4-Cth by Jakub Krijt (Institute of Inherited Metabolic Disorders, Charles University, Prague). The 2,2,3,3,4,4,4-heptafluorobutyl chloroformate (HFBCF) was synthesized in the Biology Centre (Academy of Sciences, České Budějovice, Czech Republic) following a procedure described by Abe.[24] For derivatization, a mixture of HFBCF with isooctane (1:4, v/v) was used.

WARNING! Manipulation of the reagent should be performed in a well-ventilated area (fume hood). After application, autosampler syringes should be rinsed with propan-2-ol to prevent corrosion of their plungers.

Aqueous solutions containing up to 1.5% DMPS or DTT were prepared in 4-mL amber vials every week and kept under argon atmosphere. Perchloric acid (1.2 mol/L) was prepared by diluting the delivered acid (an average concentration of 71%, i.e., 11.76 mol/L) in a ratio of 5:44 (v/v) with water. Carbonate-buffered saline was prepared as a 2% solution of 0.5 mol/L sodium carbonate in a physiological solution. The carbonate–perchlorate buffer was prepared as a 2:5:1 (v/v/v) mixture of 1.2 mol/L PCA, 0.5 mol/L NaOH, and 0.5 mol/L Na_2CO_3 (pH 8–9). The catalytic solution was prepared by mixing pyridine with 50 mmol/L aqueous sodium carbonate in ratio 1:6 (v/v).

Tapered polypropylene 1.1-mL reaction vials were supplied by Continental Laboratories (San Diego, CA). Adjustable 50- and 100-μL transferpettor pipet with glass capillary were delivered by Merck (Darmstadt, Germany).

Sample Collection. Fasting serum or plasma samples were aliquots of clinical material remaining after routine tHcy determination. Venous blood samples were collected by a standardized procedure into blood collecting tubes without additives for the

(16) Ducros, V.; Demuth, K.; Sauvant, M. P.; Quillard, M.; Causse, E.; Candito, M.; Read, M.-H.; Dray, J.; Garcia, I.; Gerhardt, M. F. *J. Chromatogr., B* **2002**, *781*, 207–26.
(17) Krijt, J.; Vacková, M.; Kožich, V. *Clin. Chem.* **2001**, *47*, 1821–8.
(18) Valerio, A.; Baldo, G.; Tessari, P. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 561–7.
(19) Andersson, A.; Isaksson, A.; Brattstrom, L.; Hultberg, B. *Clin. Chem.* **1993**, *39*, 1590–7.
(20) Nolin, T. D.; McMenamin, M. E.; Himmelfarb, J. *J. Chromatogr., B* **2007**, *852*, 554–61.
(21) Hušek, P. J. *Chromatogr., B* **1995**, *669*, 352–7.
(22) Hušek, P.; Simek, P. *Curr Pharm. Anal.* **2006**, *2*, 23–43.
(23) Dalene, M.; Skarping, G.; Tinnerberg, H. *J. Chromatogr., B* **1994**, 319–28.
(24) Abe, I.; Fujimoto, N.; Nishiyama, T.; Terada, K.; Nakahara, T. *J. Chromatogr., A* **1996**, 221–7.
(25) Maurino, V.; Minero, C.; Pelizzetti, E.; Angelino, S.; Vincenti, M. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 1328–36.
(26) Vincenti, M.; Ghiglione, N.; Valsania, M. C.; Davit, P.; Richarson, S. D. *Helv. Chim. Acta* **2004**, *87*, 370–5.
(27) Vincenti, M.; Biazzi, S.; Ghiglione, N.; Valsania, M. C.; Richardson, S. D. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 803–13.
(28) Hušek, P.; Simek, P.; Hartvich, P.; Zahradníčková, H. J. *Chromatogr., A* **2008**, *1186*, 391–400.
(29) Zahradníčková, H.; Hušek, P.; Simek, P.; Hartvich, P.; Maršálek, B.; Holoubek, I. *Anal. Bioanal. Chem.* **2007**, *388*, 1815–22.
(30) Campbell, J.; Clarkson, T. W.; Omar, M. D. *J. Am. Med. Assoc.* **1986**, *256*, 3127–30.
(31) Aposhian, H. V.; Maiorino, R.; Gonzalez-Ramirez, D.; Zuniga-Charles, M.; Xu, Z.; Hurlbut, K. M.; Junco-Munoz, P.; Dart, R. C.; Aposhian, M. M. *Toxicology* **1995**, *97*, 23–38.
(32) Bennett, T. A.; Edwards, B. S.; Sklar, L. A.; Rogelj, S. *J. Immunol.* **2000**, *164*, 4120–9.
(33) Chigaev, A.; Zwartz, G. J.; Buranda, T.; Edwards, B. S.; Prossnitz, E. R.; Sklar, L. A. *J. Biol. Chem.* **2004**, *279*, 32435–43.
(34) Hsu, M-F.; Chen, Y-S.; Huang, L.-J.; Tsao, L.-T.; Kuo, S-C.; Wang, J-P. *Eur. J. Pharmacol.* **2006**, *535*, 43–52.

Table 1. Concentration of isotope-labeled internal standards (IS) and analytes used in the study

Deuterated IS for Quantitation of the Analytes							
	d3-Cth	d3-MMA	d8-$(Hcy)_2$	d3-Met	d3-Ser	d4-$(Cys)_2$	d5-Gly
stock solution (mmol/L)	0.1	0.1	1	5	40	20	40
takeoff per 15 μL (μL)	3	3	3	3	1.5	1.5	1.5
final concentration (μmol/L)	1	1	20(40)	50	400	200(400)	400

Analytes and Levels Used in the Calibration Studies							
	Cth	MMA	$(Hcy)_2$	Met	Ser	$(Cys)_2$	Gly
stock solution (nmol/μL)	1	1	10	50	50	25	100
takeoff per 10 μL (level 1, μL)	1	1	1	1	2	2	2
level 1[a] (μmol/L)	10	10	200(400)[b]	500	1000	500(1000)	2000
level 2	2	2	40(80)	100	200	100(200)	400
level 3	0.4	0.4	8(16)	20	40	20(40)	80
level 4	0.1	0.1	2(4)	5	10	5(10)	20
level 5	0.02	0.02	0.4(0.8)	1	2	1(2)	4

[a] Levels 2−5 were prepared by dilution of the first level with water to concentrations of 1/5, 1/20, 1/100, and 1/500. [b] Concentrations of the liberated thiols, i.e., Hcy and Cys, are given in parentheses. $(Hcy)_2$, homocystine; $(Cys)_2$, cystine.

Table 2. Analytical Recovery of the Assay Examined on Pooled Serum Samples

	concentration, μmol/L						
		added		detected (SD)		recovery, % (CV%)	
analyte	endogenous	low	high	low	high	low	high
Cth	0.09	0.10	2.00	0.17 (0.02)	1.86 (0.05)	91 (3.3)	89 (2.3)
MMA	0.16	0.10	2.00	0.25 (0.02)	2.14 (0.01)	96 (5.4)	99 (4.5)
tHcy	10.5	4.0	80	14.7 (0.5)	89 (0.3)	101 (2.2)	98 (3.5)
Met	24	5.0	100	29.6 (1.0)	119 (0.5)	102 (2.5)	95 (3.6)
Ser	149	10	200	156 (1.7)	360 (1.5)	98 (1.2)	103 (3.6)
tCys	321	10	200	338 (4.0)	517 (5.6)	103 (2.6)	99 (5.4)
Gly	363	20	400	371 (1.2)	779 (1.8)	97 (1.5)	102 (3.2)

Table 3. Precision of the Assay (n = 10)

	supplemented to serum (μmol/L)			within-day (CV %)			between-day (CV %)		
analyte	low	medium	high	low	medium	high	low	medium	high
Cth	0.10	0.50	2	7.3	2.6	3.3	8.8	5.4	7.2
MMA	0.10	0.50	2	2.5	2.9	2.8	3.2	3.2	2.4
tHcy	4.0	20	80	2.0	1.3	2.1	1.8	1.2	4.1
Met	5.0	25	100	1.6	1.1	2.0	2.5	2.6	2.9
Ser	10	50	200	1.0	0.6	2.7	2.8	2.8	3.8
tCys	10	50	200	1.1	0.6	2.3	2.1	1.7	2.3
Gly	20	100	400	0.4	0.7	2.2	2.6	1.5	2.0

preparation of serum. The plasma samples were obtained by collecting the blood into EDTA tubes and cooled immediately in ice water. Serum and plasma were stored at −20 °C until analysis. Informed consent was obtained from every subject and the study was performed in conformance with the Declaration of Helsinki ethical guidelines.

Procedure. Calibration solutions and blends of the deuterated and nondeuterated standards of MMA and amino acids, but without Cys and Hcy, were prepared in 50−100 mmol/L HCl at concentrations similar to those in serum. The composition of the deuterated and native standards and the concentration levels used, are summarized in Table 1.

Serum or plasma samples (150 μL) were spiked with 50 μL of the standard solution, containing 15 μL of the deuterated standards, plus 10 μL of the saline blank or native standard solution and 25 μL of carbonate-buffered saline, in 1.5-mL polyethylene vials. The disulfide bonds were reduced by incubation with 50 μL of 1.5% DMPS (or DTT) for ∼3 min. The samples were then deproteinized with 50 μL of 1.2 mol/L perchloric acid, vortexed, and after standing for 2−3 min, the vials were centrifuged at 2000g for 5 min. A 150-μL aliquot of the supernatant was transferred into the 1.1-mL polypropylene vial and alkalized by adding 50 μL of NaOH−Na_2CO_3 (9:1, both bases 0.5 mol/L) to pH 8−9 (we recommend checking this value by means of pH paper strips). After addition of 75 μL of HFBCF−isooctane 4:1 (v/v) and brief vortexing to create an emulsion, 50 μL of catalytic pyridine solution was added after ∼1 min and the milky organic phase cleared by brief vortexing. Finally, 75 μL of isooctane was added, the content was gently mixed, and an aliquot of the upper organic phase was transferred into an autosampler vial for GC/MS analysis.

Instrumental Analysis. All analyses were performed with a DSQ quadrupole mass spectrometer (Thermo Electron, San Jose, CA) equipped with an electron ionization (EI, 70 eV) ion source and directly coupled to a Trace Ultra gas chromatograph with a

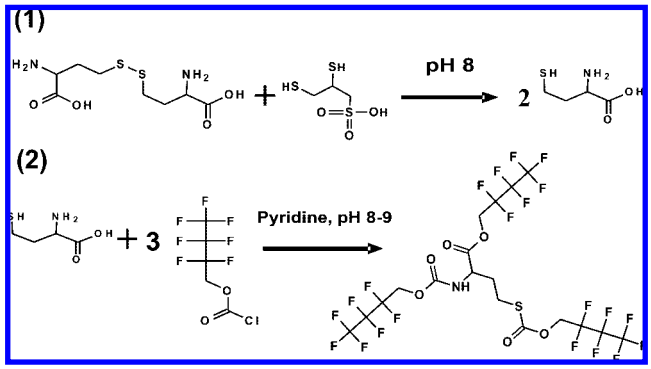

Figure 1. Release of Hcy with DMPS from the homocystine disulfide bonds (1), and derivatization with HFBCF (2).

programmed temperature vaporizing injector. The analytes were separated on a 15 m × 0.25 mm/0.25 μm Varian VF-5 ms fused-silica capillary column. The carrier gas was helium at a flow rate of 1.2 mL/min and a constant velocity mode; the injector temperature was 240 °C; the injection mode was splitless for 1 min; and the injection volume was 0.5 μL. The ion source temperature was 200 °C; the transfer line temperature was 250 °C. The GC oven temperature program started at 50 °C, held for 1.5 min, programmed at 20 °C/min to 170 °C first, and at 30 °C/min to 275 °C. All analytes in the samples were detected in the full-scan mode, mass range of 55−1050 mass units, and scan speed, 7 scans/s. Quantitative analysis of the target analytes was performed in the selected ion monitoring (SIM) mode with dwell time of 20−50 ms for a particular analyte ion.

The SIM ion pairs (native/labeled) were m/z 283 + 438/286 + 441 for MMA/d3-MMA, 256/258 for Gly/d2-Gly, 268/271 for Ser/d3-Ser, 557/560 for Met/d3-Met, 528/530 for Cys/d2-Cys, 282/285 for Hcy/d4-Hcy, and 328/332 for Cth/d4-Cth. Different retention windows were used for each analyte/internal standard pair.

For the purpose of method comparison, tHcy was measured in serum or plasma samples by a chemiluminescence immunoassay (ICL) on an Immulite 2000 Analyzer (DPC, Los Angeles, CA). Reagents, supplies, and instructions for analysis were obtained from the manufacturer.

Data were acquired and processed using Xcalibur software (version 1.3, Thermo Electron). A Passing-Bablok regression[35] was used for comparison of tHcy concentrations obtained from the GC/MS and ICL analyses.

Method Validation. For the calibration studies, the composition of the medium was analogous to the neutralized serum supernatant after the deproteinization step. To 25 μL of mixed standards (containing 15 μL of the deuterated standards and 10 μL of the native standards at each particular level; see Table 1), 75 μL of the carbonate-buffered saline and 25 μL of 1% DMPS (or DTT) were added. The content was gently mixed and allowed to react for 2−3 min. Then, 75 μL of both the carbonate−perchlorate buffer and the organic reagent was added, and the content was further treated as described in the Procedure section.

Calibration curves were constructed by plotting the peak-area ratios of the analytes to their deuterated counterparts against their concentration. The limit of detection (LOD) was determined as a signal-to-noise ratio of 5:1.

For the recovery studies, endogenous concentrations of the analytes were determined. Thereafter, the serum was divided into three portions and two portions were supplemented with two levels of all analytes (low, high; refer to Table 2). The recovery was calculated according to the following formula: (measured concentration − endogenous concentration)/added concentration.

Precision studies were performed with the same serum divided into three portions and spiked with all analytes at three levels (low, medium, high; Table 3). Within-day precision was assessed by replicate measurements (n = 10) of the analytes on 1 day.

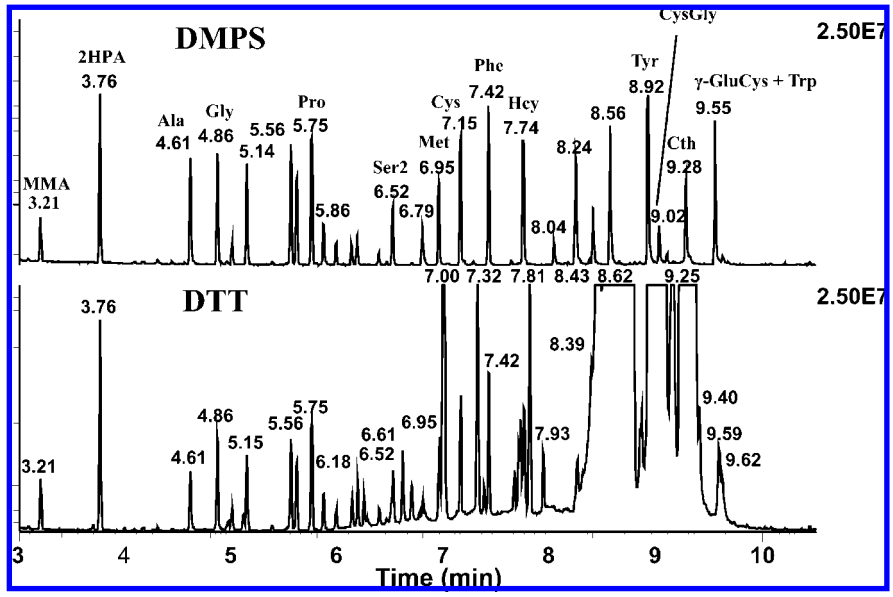

Figure 2. GC/MS TIC chromatograms of the HFBCF-treated amino acid standards (30 μmol/L each, MMA, cystine, and homocystine, 15 μmol/L each), and lactic acid (2HPA,120 μmol/L) after treatment with DMPS (top) or DTT (bottom) and separation on a 15-m VF-5-ms capillary column, as described in the Experimental Section. Retention data (min) and abbreviations, refer also to Table 4. 3.21 MMA, 3.76 2HPA, 4.61 Ala, 4.86 Gly, 5.14 Val, 5.56 Leu, 5.61 Ile, 5.75 Pro, 5.86 Thr1, 6.18 Asp, 6.52 Ser2, 6.79 Glu, 6.95 Met, 7.15 Cys, 7.42 Phe, 7.74 Hcy, 8.04 Gln, 8.24 Orn, 8.40 - His, 8.56 Lys, 8.92 Tyr, 9.02 CysGly, 9.28 Cth, 9.55 γ-GluCys, and 9.57 Trp.

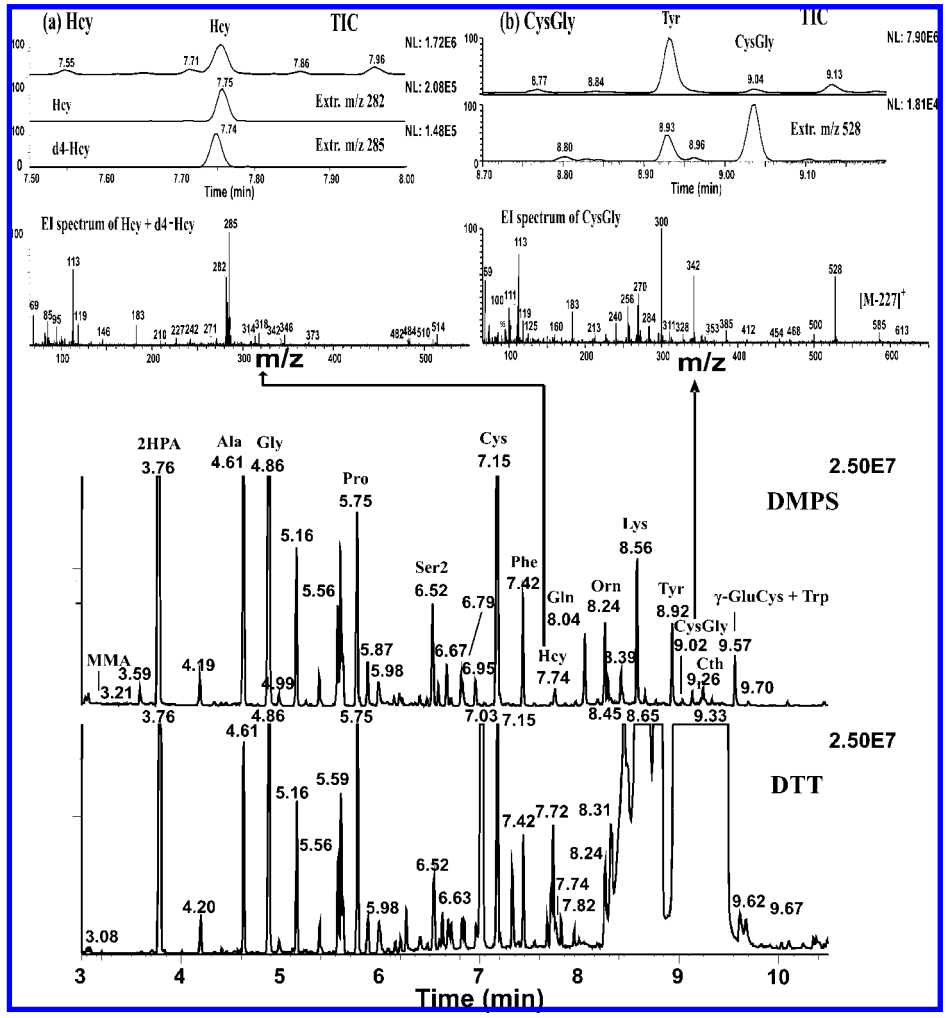

Figure 3. GC/MS TIC chromatograms of the HFBCF-treated serum analyzed on the same VF-5-ms GC column as in Figure 2. The reduction was made with DMPS (top) or DTT (bottom) as described in the Experimental Section. The TIC peaks of Hcy and CysGly and their EI spectra are depicted in detail above the chromatograms. For the detected components, refer also to Table 4.

Between-day precision was determined by analysis of the samples on 20 different days during a month.

RESULTS AND DISCUSSION

Studies dedicated to chelating effects and metabolism of DMPS have shown that DMPS is rapidly oxidized to disulfides in both saline and human blood,[36,37] and already 5 h after DMPS administration, more than 60% of the total plasma DMPS was bound to proteins.[38] Nevertheless, initial trials revealed that DPMS is capable to reduce cystine and homocystine efficiently to their corresponding thiols in both aqueous bicarbonate and serum. Concentration of the reductant, pH of the reaction medium, and incubation time were therefore studied to optimize the reduction process. DMPS concentrations of 0.15−0.5% were examined and 0.3% DMPS in serum or plasma (16 mM, calculated to the free acid) was found to be optimal. For the reduction of the standards in aqueous buffered solutions, however, the DMPS concentration could be lowered to 0.2%, because that of 0.3% and higher led to diminished reaction yields of all analytes. We found that disulfide bonds were reduced with DMPS at pH 7−8 within 1−2 min. DTT showed similarly rapid reduction under the otherwise identical reduction conditions in body fluids as we reported earlier.[13] The reduction process is evidently influenced by the pH of the medium, which might explain the different reduction rate reported for DTT in acidic medium. In this case, 1 mol/L DTT concentration, 70 °C reduction temperature, and 1 h reaction time were required to produce Cys and Hcy with high yields.[39]

The DMPS reduction followed by the HBCF-mediated derivatization provided the expected conversion of the protic functional groups, i.e., carboxyls into the corresponding esters and amino/thiol groups into the respective carbamates and thiocarbonates (Figure 1). Unlike other alkyl chloroformates, facile esterification of the carboxylic groups proceeds without the corresponding alcohol, i.e., heptafluorobutanol. Additionally, the HFBCF reagent is capable of alkylating aliphatic hydroxyls present in Ser and Thr, providing thus the O-carbonate derivatives (Ser2, Thr2) with

(35) Passing, H.; Bablok, W. *J. Clin. Chem. Clin. Biochem.* **1983**, *21*, 709–20.
(36) Islinger, F.; Gekle, M.; Wright, S. H. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 741–7.
(37) Lungkaphin, A.; Chatsudthipong, V.; Evans, K. K.; Groves, C. E.; Wright, S. H.; Dantzler, W. H. *Am. J. Physiol. Renal. Physiol.* **2004**, *286*, F68–F76.
(38) Maiorino, R. M.; Xu, Z. F.; Aposhian, H. V. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 375–84.
(39) Huang, J. M.; Whitman, S. *J. Liq. Chromatogr.* **2006**, *29*, 2513–20.

Table 4. List of N(O,S)-Heptafluorobutoxycarbonyl Heptafluorobutyl Ester Derivatives of Serum Metabolites and Internal Standards (Figure 3) Detected by GC-EI/MS Method

				diagnostic m/z ions (% of rel. abundance)[b]				
RT (min)	analyte	abbreviations	MW[a]	m/z (100)	m/z	m/z	$[M-227]^+$	$[M-199]^+$
3.19	d3-methylmalonic acid (IS)	d3-MMA	485	286	258(17)	441(5)	258(17)	286(100)
3.21	methylmalonic acid	MMA	482	283	255(19)	438(5)	255(19)	283(100)
3.76	lactic acid	2HPA	498	227	271(43)	299(3)	271(43)	299(3)
4.51	sarcosine	Sar	497	270	113(36)	226(19)	270(100)	
4.61	alanine	Ala	497	270	113(10)	227(7)	270(100)	
4.85	d2-glycine (IS)	d2-Gly	485	258	113(18)	214(10)	258(100)	
4.86	glycine	Gly	483	256	113(16)	212(10)	256(100)	
4.99	aminobutyric acid	ABU	511	284	113(25)	84(22)	284(100)	
5.15	valine	Val	525	298	98(48)	283(39)	298(100)	
5.25	β-alanine	3Ala	497	98	269(79)	256(65)		270(6)
5.39	norvaline (IS)	Nva	525	298	256(91)	113(28)	298(100)	
5.56	leucine	Leu	539	312	256(93)	270(29)	312(100)	
5.61	isoleucine	Ile	539	312	283(99)	256(73)	312(100)	
5.75	proline	Pro	523	296	297(8)	113(5)	29(100)6	
5.87	threonine1	Thr1	527	283	100(28)	113(20)	300(18)	
5.95	pipecolic acid	Pip	537	310	82(8)	113(8)	310(100)	
6.01	serine1	Ser1	513	283	286(56)	86(53)	286(56)	
6.14	asparagine	Asn	522	295	113(29)	95(17)	295(100)	
6.18	aspartic acid	Asp	723	254	296(37)	496(12)	496(12)	
6.39	threonine2	Thr2	753	282	227(70)	113(67)	526(8)	
6.51	d3-serine2 (IS)	d3-Ser2	742	271	183(17)	315(4)	515(7)	
6.52	serine2	Ser2	739	268	183(16)	312((8)	512(2)	
6.79	glutamic acid	Glu	737	282	310(40)	82(35)	510(24)	
6.94	d3-methionine (IS)	d3-Met	560	64	283(26)	560(5)	333(6)	
6.95	methionine	Met	557	61	283(43)	557(26)	330(12)	
7.14	d2-cysteine (IS)	d2-Cys	757	113	302(16)	530(16)	530(16)	
7.16	cysteine	Cys	755	113	300(51)	528(33)	528(33)	
7.42	phenylalanine	Phe	573	91	330(79)	131(19)	346(7)	
7.73	d4-homocysteine (IS)	d4-Hcy	773	285	514(8)	85(69)	545(2)	
7.74	homocysteine	Hcy	769	282	82(34)	283(32)	542(4)	
8.04	glutamine	Gln	554	84	282(95)	327(66)	327(66)	
8.24	ornithine	Orn	766	296	256(21)	566(4)		
8.39	histidine	His	789	307	562(47)	546(45)	562(47)	
8.56	lysine	Lys	780	310	256(44)	380(8)		
8.92	tyrosine	Tyr	815	333	289(62)	588(5)	588(5)	
9.02	cysteinylglycine	CysGly	812	113	300(78)	528(36)	585(4)	
9.26	d4-cystathinone (IS)	d4-Cth	1042	332	284(52)	514(8)		
9.28	cystathionine	Cth	1038	328	282(69)	510(8)		
9.46	d4-cystine (IS)	d4-(Cys)2	1060	498	270(38)	530(10)		
9.47	cystine	(Cys)2	1056	496	268(31)	528(31)	829(1)	857(1)
9.55	γ-glutamylcysteine	γ-GluCys	1066	113	285(56)	270(46)		
9.55	tryptophan	Trp	612	130	385(4)	612(3)	385(4)	
10.27	d8-homocystine (IS)	d-8(Hcy)2	1078	514	318(56)	692(11)		
10.29	homocystine	(Hcy)2	1070	510	282(50)	314(35)		

[a] MW, nominal molecular weight of the derivatives. [b] Diagnostic m/z ions suitable for identification involve the most intensive m/z (100%) and other 2−4 fragment ions, including $[M - 227]^+$, $[M - 199]^+$ ions arising from the loss of the heptafluorobutoxy radical $[M - 199]^+$, followed by the CO loss $[M - 227]^+$.

advantagous chromatographic and detection properties. The bulky perfluoroalkyl groups make the original polar analytes highly volatile and "inert" to adsorption sites of a stationary phase. Consequently, larger amino acids and dipeptide thiols, including Cth, CysGly, and γ-GluCys, are easily eluted and thus amenable to GC/MS analysis.

Full-scan GC/MS analysis of the equimolar standard mixture of protein amino acids together with MMA, lactic acid, and the target sulfur amino acids subjected to incubation with either DTT or DMPS are demonstrated after the HFBCF derivatization in Figure 2. Typical full-scan TIC GC/MS records of serum samples (the identical scale), treated with either DTT or DMPS and then HFBCF, are depicted in Figure 3. The substantial difference in the sample records is apparent. Remarkably large peaks were observed on the chromatograms, when the standards and serum or plasma samples were reduced by DTT. The impurity peaks evidently originate from DTT, and its reaction products arise in the course of the reduction and derivatization process. Unfortunately, the impurity peaks contribute to excessive background, which hampers the detection of the later eluting analytes, particularly those eluted after Ser. On the other hand, the interference components were nearly absent when the DMPS was applied as the reducing agent, presumably because the ionic sulfonate group of DMPS is not esterified and the reaction byproducts remain preferably in the aqueous phase. Furthermore, higher yields of most amino acid derivatives (of the dibasic particularly) were also obtained (the data not presented here). The DMPS structure, possessing a liphophilic thiol and a strongly hydrophilic sulfonate parts, may function as a surfactant improving miscibility of the aqueous and organic phase during the reaction course. The derivatization conditions, however, should be maintained at optimum pH range of 8−9, as Hcy tends to form a

thiolacton at pH higher than 9. Outside that pH range, the yields of all analytes decline rapidly.

Using full-scan GC/MS analysis, more than 40 analytes useful in clinical diagnostics could be identified in serum, including Cth and thiol dipeptides, CysGly, and γ-GluCy. Retention data, molecular weight of the (N,O,S)-heptafluorobutoxycarbonyl heptafluorobutyl ester derivatives, and diagnostic ions in their EI spectra are summarized in Table 4. For the EI spectra of the analytes, for which the method was validated, refer to the Supporting Information available.

The bulkier heptafluorobutyl moieties in the derivative structures increase the molecular weight of the polyfunctional sulfur biomarkers (Cth, CysGly, γ-GluCy, and also cystine and homocystine) above 1000. Nevertheless, the perfluoroalkylated analytes, in comparison with those prepared by the treatment with classical chloroformates (methyl, ethyl), exhibit excellent volatility and are easily eluted from a standard capillary column at much lower temperature (below 275 °C) in a few minutes. The analytes are eluted from the GC columns according to their boiling points, approximately, and their retention properties can assist in the interpretation of the EI spectra of unknowns, particularly isomers. Molecular ions ($M^{+\cdot}$) of the HFBCF-derived analytes are low abundant in their EI spectra, because cleavage of the heptafluorobutoxy radical (M-$CF_3CF_2CF_2CH_2O\cdot$, $[M - 199]^+$) is strictly preferred in the case of MMA and other carboxylic acids, followed by the carbonyl loss ($M - 199 - CO$, i.e., $[M - 227]^+$), a dominant cleavage process at the 2-amino acid derivatives. The EI spectra of the dibasic amino acids (Orn, Lys) and the sulfur amino acids are more complex, but still contain highly specific diagnostic ions allowing direct identification of the metabolites from their full-scan EI spectra in body fluid samples, if present, at low micromole per liter levels. This feature is exemplified by the direct detection of two biomarkers, Hcy and CysGly, occurring in body fluids of healthy subjects at 7−15 and 25−35 μmol/L levels.[5,16] Peaks of both biomarkers (Hcy, 7.74 min, I.S. = d4-Hcy, 7.73 min) and CysGly (9.02 min) were directly observable in the TIC chromatogram of a normal serum sample, and the corresponding EI spectra, after the background subtraction, were very well searchable with the EI spectra obtained from the standards. The data support clearly the efficacy of the new extraction procedure and confirm suitability of the method for GC/MS screening of the amino acidic metabolites in serum or plasma.

The developed GC/MS method was validated for seven biomarkers with the available deuterated standards, i.e., MMA, tHcy, tCys, Met, Ser, Gly, and Cth. The LODs (signal-to-noise ratio, 5:1) were 0.01 μmol/L for Cth, MMA, Met, and Ser, 0.008 μmol/L for tHcy, 0.02 μmol/L for tCys, and 0.007 μmol/L for Gly. The equations for the regression lines were as follows: $y = 0.001 + 0.282x$ ($r^2 = 0.9977$) for Cth, $y = 0.024 + 12.334x$ ($r^2 = 0.9987$) for MMA, $\log y = -0.124 + 0.935 \log x$ ($r^2 = 0.9992$) for tHcy, $\log y = -0.925 + 0.876 \log x$ ($r^2 = 0.9984$) for Met, $y = 0.060 + 0.032x$ ($r^2 = 0.9978$) for Ser, $y = 0.026 + 0.041x$ ($r^2 = 0.9979$) for tCys, and $\log y = -1.4575 + 0.858 \log x$ ($r^2 = 0.9992$) for Gly.

The LOD values were substantially lower than those reported by Windelberg et al.,[14] particularly for Ser, where the LOD was improved owing to the properties of the OH-protected HFBOC−HFB derivative in 3 orders of magnitude.

Recoveries for all analytes were between 89 (Cth) and 103% (tCys, Ser) and are shown in Table 2. The results of the precision studies are summarized in Table 3. Within-day CVs ranged from 0.4 (Gly) to 7.3% (Cth), and between-day CVs from 1.2 (tHcy) to 8.8% (Cth).

For further evaluation, the GC/MS method was used to analyze 30 fasting serum control subjects (mean age 37.3 years). The median concentrations (in μmol/L) (interquartile range) were the following: tHcy 10.7 (8.1−12.0), Cth 0.09 (0.04−0.13), MMA 0.14 (0.07−0.20), Met 20 (18−24), Ser 99 (82−126), Gly 216 (190−269), tCys 292 (249−328), and total CysGly 31 (23−42). The measured values for all analytes met well the reported clinical values in healthy individuals.[7,40,41] The tHcy concentrations were also compared with the ICL method validated for a routine clinical Hcy assay. A Passing−Bablok regression showed a good agreement ($r = 0.980$) between both the determinations. The intercept ($y = 0.943x + 0.322$) was not significant, the slope was significant at $p > 0.05$. Bland−Altman plot of differences[42] revealed a negative bias of 0.769 to the Immulite ICL assay.

CONCLUSIONS

DMPS is reported as an efficient reduction agent for the release of bound thiols from serum or plasma. In comparison to other reductants, the DMPS reaction products remain preferably in aqueous phase, whereas the N(S,O)-heptafluorobutyryl heptafluorobutyl ester derivatives of the polar analytes are transferred after derivatization into the organic phase, which is directly amenable to GC/MS analysis. The serum or plasma workup involves simple operations of pipetting, mixing, and centrifugation and results in very clean extracts. The more inert, volatile derivatives provide diagnostic EI spectra and enable the developed method to be extended to the screening of the amino acidic metabolites in body fluids and to the diagnostics of biomarkers related to folate and cobalamin status including the sulfur amino acids Cth, CysGly, and γ-GluCys. After the method development, more than 1000 sample analyses have been performed without evident deterioration of the injection liner, column life or ion source of the GC/MS system. The reducing properties of DMPS and automation of the presented method deserve further attention and it will be subject of further studies.

ACKNOWLEDGMENT

This work was supported by the Grant Agency of the Czech Republic, project 303-06-1674. We thank Marie Texlová for her skilled technical assistance, Zdeněk Švagera (Faculty Hospital Ostrava, Czech Republic), and Marie Kašparová (Hospital České Budějovice, Czech Republic) for supplying the serum and plasma samples and for the tHcy clinical assays. Per Magne Ueland (University of Bergen, Norway) is acknowledged for stimulation of this study and cooperation in the Homocysteine Locus research.

SUPPORTING INFORMATION AVAILABLE

Additional information. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 19, 2008. Accepted May 20, 2008.

AC8003506

(40) Allen, R. H.; Stabler, S. P.; Savage, D. G.; Lindenbaum, J. *FASEB J.* **1993**, *7*, 1344–53.
(41) Ueland, P. M.; Refsum, H.; Stabler, S. P.; Malinow, M. R.; Andersson, A.; Allen, R. H. *Clin. Chem.* **1993**, *39*, 764–79.
(42) Bland, J. M.; Altman, D. G. *Lancet* **1986**, *1*, 307–10.