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ORIGINAL ARTICLE

An on-column derivatization method for the determination of homocysteine-thiolactone and protein *N*-linked homocysteine

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Abstract Homocysteine (Hcy) is incorporated into protein via a reaction of the thioester Hcy-thiolactone with ε-amino group of a protein lysine residue generating *N*-Hcy-protein. This reaction impairs and alters protein's function and has been implicated in atherothrombotic disease. Here, we describe new high-performance liquid chromatography assays for the determination of Hcy-thiolactone, protein *N*-linked Hcy, and Hcy based on an on-column derivatization with *o*-phthaldialdehyde and fluorescence detection. The on-column derivatization generates narrow peaks, which allows fast run times (3–5 min) and facilitates determination of *N*-linked Hcy directly from acid hydrolysates of plasma protein. Utility of these assays was demonstrated with human urine and plasma samples.

Keywords Homocysteine-thiolactone · Protein *N*-linked homocysteine · HPLC · *o*-Phthaldialdehyde · Human plasma · Urine

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Abbreviations

Hcy	Homocysteine
tHcy	Total Hcy, i.e., free Hcy present in a sample after reductive cleavage of disulfide bonds
$(Hcy)_2$	Homocystine
N-Hcy-protein	*N*-homocysteinylated protein
protein *N*-linked Hcy	Hcy bound to protein by an amide linkage to ε-amino group of lysine residue(s)
OPA	*o*-phthaldialdehyde
SPE	Solid phase extraction
CMQT	2-chloro-1-methylquinolinium tetrafluoroborate
TCEP	Tris(2-carboxyethyl)phosphine
CBS	Cystathionine β-synthase
LOQ	Lower limit of quantification

Introduction

Homocysteine (Hcy) is an intermediate in the metabolism of the essential protein amino acid methionine in mammals. Levels of Hcy are regulated by remethylation to Met, catalyzed by Met synthase and betaine-Hcy methyltransferase, and transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β-synthase (CBS). The remethylation requires betaine or vitamin B_{12} and 5-methyltetrahydrofolate, generated by 5,10-methylenetetrahydrofolate reductase (MTHFR) (which requires riboflavin), whereas the transsulfuration requires vitamin B_6. In all organisms examined, including human, Hcy is also metabolized to Hcy-thiolactone by methionyl-tRNA synthetase (MetRS) in an error-editing reaction in protein biosynthesis when Hcy

is mistakenly selected in place of methionine (Jakubowski 1990, 2001a, b; Jakubowski and Goldman 1993).

Hcy-thiolactone is cytotoxic in experimental animals and human cell cultures (Jakubowski 2004, 2006a, 2007, 2008a), mostly because of its ability to modify proteins by forming adducts in which Hcy is *N*-linked to the ε-amino group of protein lysine residues, which impairs or alters protein's function (Jakubowski 1997, 1999, 2000). The bulk of Hcy-thiolactone produced in humans (Chwatko and Jakubowski 2005a) and mice (Chwatko et al. 2007) is cleared by urinary excretion.

Genetic or nutritional deficiencies in Hcy metabolism lead to hyperhomocysteinemia, increased Hcy-thiolactone and *N*-Hcy-protein levels in humans and mice (Chwatko et al. 2007; Jakubowski 2008b; Jakubowski et al. 2009; Perla-Kajan et al. 2007), and are associated with cardiovascular and neurodegenerative disorders, such as dementia and Alzheimer's disease (Lentz 2005). In humans, *N*-Hcy-protein contributes to thrombogenesis and autoimmune activation (Jakubowski 2006a, 2007, 2001b, Jakubowski et al. 2008). In *ApoE*-deficient mice, *N*-Hcy-protein accumulates in atherosclerotic lesions, and the accumulation increase in the animals fed a high Met diet (Perla-Kajan et al. 2008). Recent human clinical studies show that plasma *N*-Hcy-protein is associated with risk of coronary heart disease (Yang et al. 2006), whereas plasma Hcy-thiolactone is associated with the development and progression of diabetic macrovasculopathy (Gu et al. 2008).

High-performance liquid chromatography (HPLC)-based assays for *N*-Hcy-protein rely on the quantification of Hcy-thiolactone liberated from *N*-Hcy-protein subjected to acid hydrolysis under reducing conditions. Hcy-thiolactone is quantified by UV (Jakubowski 2002a) or fluorescence after post-column derivatization with *o*-phthaldialdehyde (OPA) (Jakubowski 2008b). In other HPLC-based methods, protein *N*-linked Hcy is derivatized with 4-fluoro-7-sulfonylbenzofurazan (Uji et al. 2002) or 7-fluorobenzo-2-oxa1,3-diazole-4-sulfonate (Perna et al. 2006), liberated from protein by acid hydrolysis, and quantified by fluorescence.

Hcy-thiolactone assays rely on HPLC with UV detection (Jakubowski 2002b) or fluorescence detection after post-column derivatization with OPA (Chwatko and Jakubowski 2005a, b; Jakubowski et al. 2009; Mukai et al. 2002), or GC/MS after pre-column derivatization with heptafluorobutyric acid anhydride (Daneshvar et al. 2003). Although the post-column OPA derivatization method produces reproducible results, it requires relatively labor-intensive sample work-up, utilizes [^{35}S]Hcy-thiolactone as a radiotracer, and yields wide peaks, a known limitation of post-column derivatization methods. Here, we describe new protein *N*-linked Hcy, Hcy-thiolactone, and tHcy assays that rely on 'on-column' derivatization with OPA, which eliminates limitations of the post-column derivatization method.

Materials and methods

Reagents

D,L-Hcy-thiolactone hydrochloride was purchased from Lancaster (Eastgate, White Lund, Morecambe, England). 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in the laboratory according to the procedure described earlier (Bald and Głowacki 2001). D,L-Hcy and D,L-homocystine $(Hcy)_2$ were from Sigma–Aldrich (St. Louis, MO, USA). Hydrochloric acid, sodium hydroxide, sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), HPLC-grade 2-propanol, methanol, and acetonitrile were from J.T. Baker (Deventer, The Netherlands). OPA was purchased from Fluka (Buchs, Switzerland). Tris(2-carboxyethyl)phosphine (TCEP) was from Merck (Darmstadt, Germany). All reagents were tested and found to be stable for unattended analysis.

Instrumentation

A Hewlett–Packard 1100 Series HPLC instrument (Waldbronn, Germany), containing a quaternary pump, autosampler, thermostated column compartment, vacuum degasser, and 1046A fluorescence detector was used. For instrument control, data acquisition and analysis, Hewlett–Packard ChemStation for LC 3D system, including single instrument ChemStation software and a Vectra computer was used. For SPE extraction vacuum manifold (J.T. Baker, Deventer, The Netherlands) and SPE cartridges (Strata C 18 U, Phenomenex, CA, USA) were used.

Human plasma and urine samples

Human EDTA-plasma was obtained from blood of human volunteers as previously described (Chwatko and Jakubowski 2005a, b). Human urine was collected anonymously from healthy volunteers as previously described (Chwatko and Jakubowski 2005a). Samples were stored at −80°C before analyses.

Sample preparation for urinary Hcy-thiolactone assays

Human urine (2 mL) was supplemented with sodium phosphate buffer (0.2 mL 0.2 M, pH 7.7) and transferred to Strata C18-U (55 µm, 200 mg/3 ml) solid-phase extraction cartridges from Phenomenex. The SPE cartridges were solvated with 2 mL of 2-propanol, followed by 1 mL 0.2 M sodium phosphate buffer, pH 7.7. After loading the sample, the cartridge was washed with 0.25 mL 0.02 M HCl in 50% methanol–water solution to remove unwanted material. Hcy-thiolactone was eluted with 0.25 mL 0.02 M HCl in 70% acetonitrile–water solution and 20 µL of the

resulting solution was applied on a reversed-phase HPLC column.

Sample preparation for urinary tHcy assays

Human urine (50 µL) was diluted with 450 µL sodium phosphate buffer (pH 7.7, 0.2 M) and treated with 10 µL of 0.25 M TCEP for 10 min and 20 µL of the resulting sample was applied on a reversed-phase HPLC column.

Sample preparation for plasma protein *N*-linked Hcy and tHcy assays

Human plasma (50 µL) was diluted with 0.2 M sodium phosphate buffer pH 7.5 (0.2 mL). Disulfide bonds in the sample were reduced by a treatment with 0.25 M TCEP (20 µL). Liberated free sulfhydryl groups were derivatized with 0.1 M CMQT (20 µL) (Bald et al. 2004). Protein was precipitated with 3 M perchloric acid (50 µL). The samples were centrifuged and both supernatants and protein pellets were collected. Supernatants (10 µL), containing CMQT-derivatized low-molecular-weight thiols were applied onto an HPLC column for tHcy determination. The protein pellets were washed two times with 3 mL water, and hydrolyzed with 3 mL 6 M HCl (115°C, 6 h) to liberate protein *N*-linked Hcy. The hydrolysates were dried, dissolved in 0.1 mL 50 mM HCl, and 10 µL of the resulting sample was applied on an HPLC column for protein *N*-linked Hcy determination.

HPLC separation, detection, and quantification

Quantification of Hcy-thiolactone and protein *N*-linked Hcy was carried out by a modification of previous procedures (Mukai et al. 2002; Chwatko et al. 2007; Jakubowski 2008a, b; Głowacki et al. 2010).

Briefly, 10 or 20 µl of final analytical solution (for plasma and urine, respectively) was injected onto a reversed-phase Hamilton PRP-1 column (150 × 4.6 mm, 5 µm; Energy Way, Reno, NV, USA). The column was eluted isocratically at 25°C with a mobile phase containing 0.1 M NaOH, 0.01 M OPA, and 30% acetonitrile at a flow rate of 1 mL/min (urine samples). For plasma samples, separation of Hcy-OPA derivatives was accomplished using mobile phase containing 0.01 M OPA in 0.1 M NaOH, (component A) and acetonitrile (component B). A gradient of acetonitrile was used. The elution profile was as follows: 0–5 min 20–50% B, and 5–6 min 50–20% B. Under both conditions Hcy-thiolactone and Hcy, but not homocystine, yield sharp peaks eluting at 2.1 min (or 3.9 min for plasma under gradient elution) when subjected to HPLC on a Hamilton PRP-1 column (Fig. 1).

The detection and quantification was by fluorescence using excitation at 370 nm and emission at 480 nm. Peaks

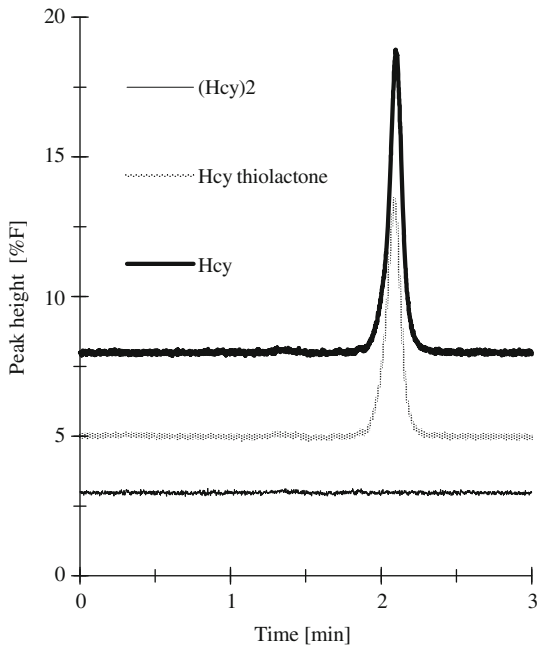

Fig. 1 HPLC chromatograms after on-column derivatization of *Hcy-thiolactone*, *Hcy*, and *(Hcy)$_2$* with OPA and fluorescence detection

of analytes were identified by comparison of their retention times with those of authentic standards. An HPLC run time for each sample was from 3 to 6 min.

Validation procedure

Standard addition method was used for calibration of the methods.

Imprecision and accuracy were determined by the addition of the analyte standards to the urine or plasma samples. Three concentrations representing the entire range of the calibration curves were studied: one near the lower end, one near the center, and one near the upper boundary of the standard curve. The samples were fivefold analyzed according to the elaborated procedures for Hcy thiolactone, tHcy, and *N*-Hcy determination. Measured concentrations were assessed by application of calibration curves obtained on that occasion. Accuracy was calculated using the following formula:

$$\text{Accuracy}\,(\%) = [(\text{measured amount} - \text{endogenous content})/\text{added amount}] \times 100\%.$$

The LOQ values were determined by spiking a proxy matrix (0.9% NaCl in pH 7.4 10 mM phosphate buffer) with decreasing concentrations of analyte until signal to noise ratio of 6:1.

Specificity, the ability to assess unequivocally the analyte in the presence of metabolically related compounds was established by analyzing plasma or urine samples

spiked with cysteine, cysteinylglycine, glutathione and γ-glutamylcysteine, as well as other protein amino acids lysine, leucine, tryptophan, threonine, glutamic acid, serine, alanine, and methionine.

Results and discussion

Sample preparation for Hcy-thiolactone assay

Studies were carried out to establish optimal conditions for Hcy-thiolactone extraction. Different parameters were optimized. The effect of the volume of the flushing solvent and concentration of eluent on the extraction of Hcy thiolactone efficiency has been studied.

The effect of eluent volume on back-extraction efficiency was studied by passing the same sample volumes through the cartridges and eluting retained analyte with different volumes of elution mixture (0.02 M HCl, 70% acetonitrile, 30% H_2O). The amount of eluted Hcy thiolactone was measured chromatographically. The signal height of each eluted solution was compared with standard Hcy-thiolactone sample. As shown in Fig. 2, eluent volume significantly affects recovery. Similar experiments were carried out to optimize acetonitrile content in the eluent and flushing solution volume.

Because it is mostly neutral at a pH 7.7 (the pK_a of its amino group is 6.7) (Jakubowski 2006b), Hcy-thiolactone is expected to be hydrophobic and retained on a C18 SPE cartridge. Thus, to assure Hcy-thiolactone binding, SPE cartridge was flushed with 2-propanol and then equilibrated with pH 7.7 phosphate buffer. Urine sample was also brought to pH 7.7 by the addition of phosphate buffer. Under these conditions all Hcy-thiolactone present in the sample was retained on the SPE cartridge. In contrast, Hcy was not retained under these conditions. At acidic pH Hcy-thiolactone is positively charged and can be back-extracted into the liquid phase. Thus, elution of Hcy-thiolactone from the SPE cartridge was achieved with 20 mM HCl. Typical HPLC analysis of the extract is shown in Fig. 3. A peak eluting at 2.1 min (OPA labeled Hcy) is present in human urine (*middle trace*) and is increased in urine spiked with Hcy-thiolactone (*upper trace*). The identity of the 2.1 min peak was further confirmed by its disappearance after a treatment of urine with NaOH and H_2O_2, which converts Hcy-thiolactone to $(Hcy)_2$ (*bottom trace*).

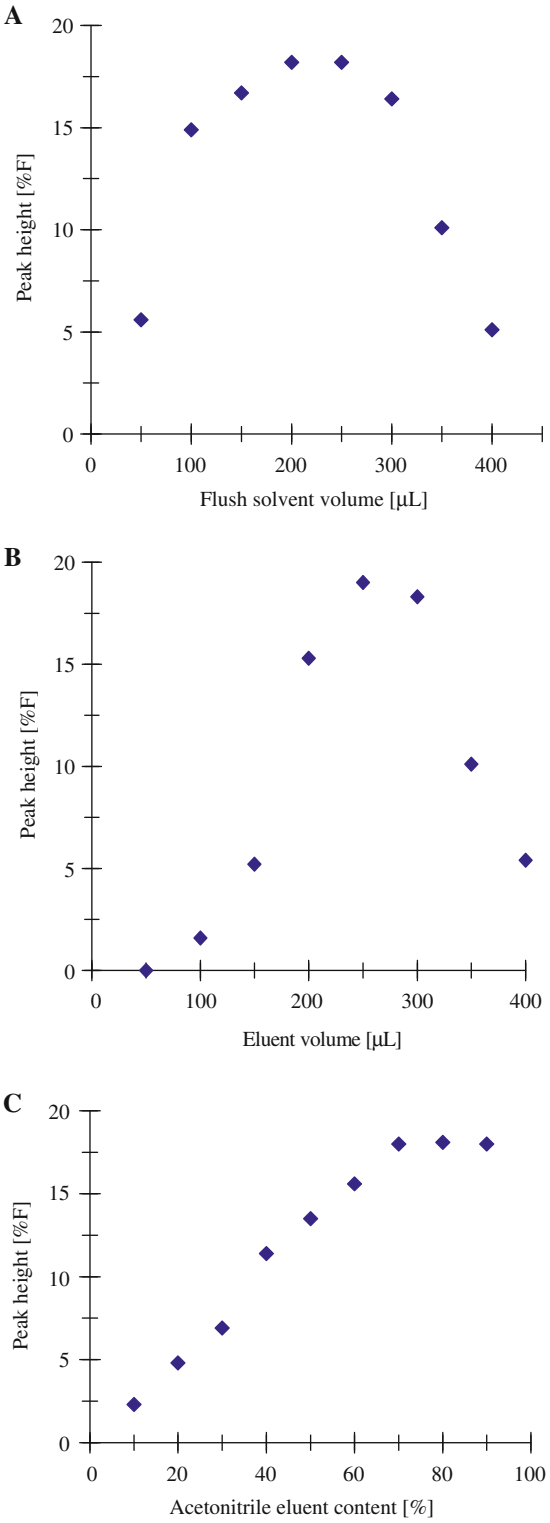

Fig. 2 Extraction efficiency depends on **a** flush solvent volume; **b** eluent volume; and **c** acetonitrile content

Calibration lines for urinary Hcy-thiolactone and tHcy

Human urine was spiked with Hcy-thiolactone or $(Hcy)_2$, to give final concentrations of 0.02–3 or 0.25–32 µM, respectively. Seven-point calibration plot was constructed using four determinations per concentration. The peak heights of Hcy-OPA derivative were plotted versus analyte concentration and the curves were fitted by least-square

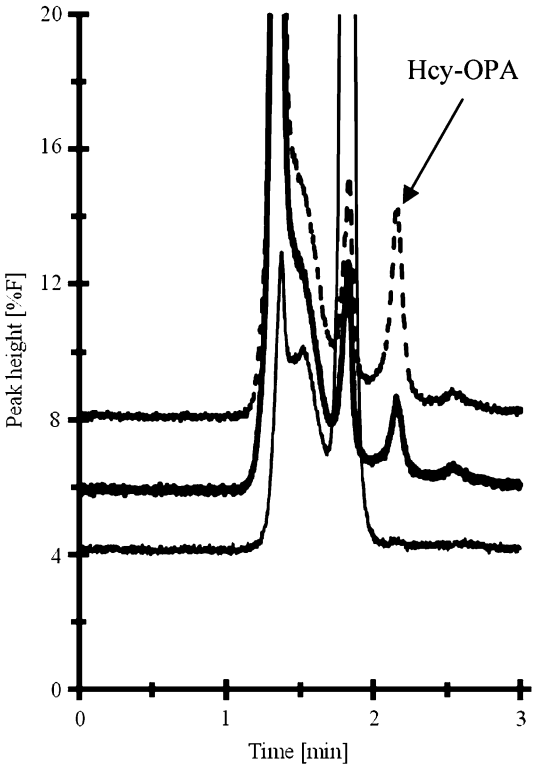

Fig. 3 HPLC analyses of human urine for Hcy-thiolactone using on-column derivatization with OPA and fluorescence detection. *Top trace* human urine spiked with 0.4 μM Hcy thiolactone; *middle trace* normal human urine; *bottom trace* normal human urine treated with NaOH and H_2O_2

linear regression analysis. The equations for the linear regression for Hcy thiolactone and tHcy, were $y = 0.0195x + 1.3$ ($R^2 = 0.9986$) and $y = 1.002x + 2.2$ ($R^2 = 0.9994$), respectively. For urine samples spiked with 0.02–3 μM Hcy-thiolactone, interassay and intraassay variation was 2.5–7%. For urine spiked with 0.25–32 μM Hcy the variation was 1.2–6.3%.

Hcy-thiolactone and tHcy levels in normal human urine

The elaborated procedures were applied to authentic human urine samples. Concentrations of Hcy-thiolactone in human urine from 15 subjects varied from 25 to 297 nM with a mean of 103 ± 89 nM. Concentrations of tHcy varied from 1.5 to 6 μM with a mean of 2.6 ± 1.1 μM. These Hcy-thiolactone and tHcy values are similar to the values obtained previously by a post-column OPA derivatization method (Chwatko and Jakubowski 2005a). Similar to previous study (Chwatko and Jakubowski 2005a), there was no correlation between urinary Hcy-thiolactone and tHcy levels. The maximum imprecision values (RSD %, $n = 4$) for determination of Hcy thiolactone and tHcy, in urine were 6.1 and 7.5%, respectively. Notably, such good precision was obtained with no outliners excluded and without including an internal standard.

Sample preparation for plasma protein *N*-linked Hcy and tHcy assays

In addition to protein *N*-linked Hcy, protein *S*-linked Hcy, and low-molecular-weight forms of Hcy are present in plasma (Jakubowski 2007, 2006a, 2001b, 2002a). Sample preparation involves treatment with TCEP to reduce disulfide bonds, derivatization of thiols with CMQT (Bald et al. 2004; Bald and Głowacki 2005), protein precipitation with perchloric acid, and centrifugation to separate CMQT-derivatized Hcy (supernatant) from *N*-Hcy-protein (pellet). The supernatant is applied on HPLC for tHcy determination (Fig. 4), whereas the pellet is processed for *N*-Hcy-protein assay. CMQT-Hcy derivative is more hydrophilic than Hcy itself (Bald et al. 2004), which facilitates its quantitative removal from the protein pellet. In contrast to assays of other Hcy forms, for which sample work-up involves relatively mild conditions, *N*-linked Hcy is liberated from *N*-Hcy-protein by hydrolysis with 6 M HCl (Jakubowski 2002a). Under these drastic conditions CMQT-Hcy-protein derivative decomposes with Hcy-thiolactone formation, which is then determined by an on-column OPA derivatization method.

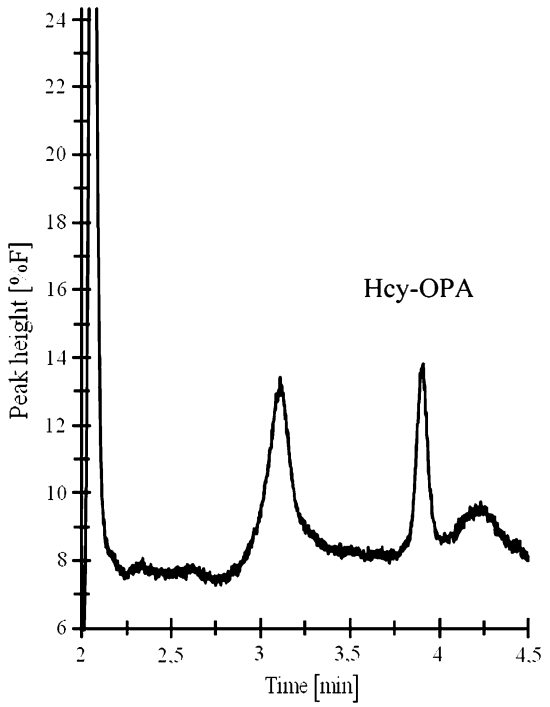

Fig. 4 HPLC analysis of plasma sample for tHcy after on-column derivatization with OPA

Calibration lines for plasma tHcy and *N*-Hcy

For tHcy calibration, human plasma was spiked with homocystine $(Hcy)_2$ to give final concentrations of Hcy from 0.5 to 150 μM. For *N*-Hcy calibration, the protein pellets, after supernatant removing and water washing and after reconstitution with 3 mL of 6 M HCl, were spiked with Hcy to give final concentrations of Hcy from 0.25 to 10 μM. Next, the pellets were hydrolyzed and processed according to the procedure described in "Sample preparation for plasma protein *N*-linked Hcy and tHcy assays". Nine-point calibration plots, both for tHcy and *N*-Hcy, were constructed using triplicate of the final analytical solution. The peak heights of Hcy-OPA derivative were plotted versus analyte concentration and the curves were fitted by least-square linear regression analysis. The equations for the linear regression for tHcy and *N*-Hcy were $y = 0.638x + 17.5$ ($R^2 = 0.9998$) and $y = 6.4646x + 2.79$ ($R^2 = 0.9997$), respectively. For spiked plasma samples assayed for tHcy and *N*-Hcy, interassay and intraassay variations (RSD) were 0.9–5.9 and 1.4–3.5%, respectively.

HPLC separation for plasma protein *N*-linked Hcy and tHcy assays

After injection of supernatant into the column, equilibrated with 0.1 M NaOH and 10 mM OPA, the CMQT-Hcy derivative undergoes instant hydrolysis to Hcy (Głowacki and Bald 2009) which then reacts with OPA, and is eluted as OPA-Hcy derivative from the HPLC column (Fig. 4). To determine the validity of our new method for protein *N*-linked Hcy, native human albumin (known to contain small amounts of protein *N*-linked Hcy) (Jakubowski 2002a; Głowacki and Jakubowski 2004) and *N*-Hcy-albumin (prepared from human albumin by treatment with Hcy-thiolactone) (Jakubowski 1999; Głowacki and Jakubowski 2004) have been analyzed. A peak corresponding to the OPA-Hcy derivative, eluting at 3.9 min (Fig. 5a), was present in the

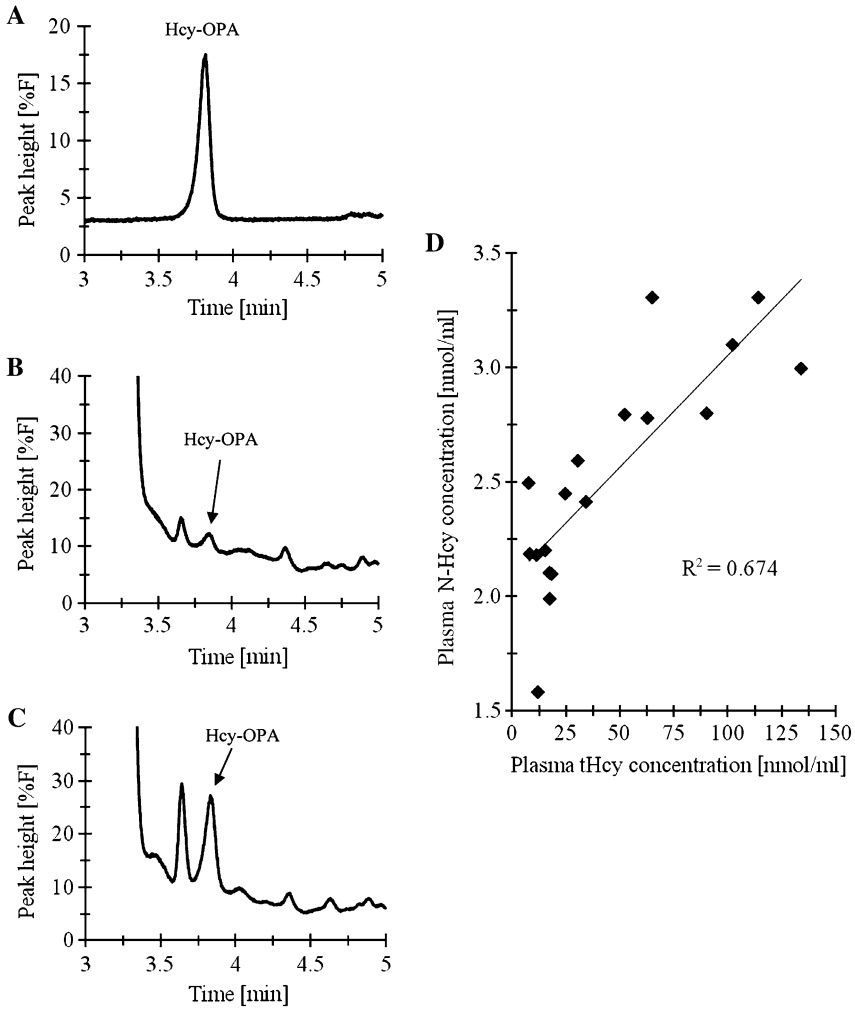

Fig. 5 HPLC determination of protein *N*-linked Hcy content in human serum albumin and plasma using on-column derivatization with OPA and fluorescence detection. **a** Hcy standard; **b** untreated human serum albumin; **c** albumin modified with Hcy-thiolactone. **d** Shows a plot of individual values of plasma protein *N*-linked Hcy versus corresponding values of plasma tHcy for human subjects

Table 1 Imprecision, accuracy, and LOQ

Analyte	Nominal concentration (μM)	Imprecision (RSD, %)	Accuracy (%)	LOQ (μM)
Urine				
Hcy thiolactone	0.025	6.5	96.3	0.02
	1.5	3.7	106.1	
	2.8	4.7	97.1	
tHcy	0.3	4.3	96.4	0.25
	16	1.6	105.2	
	30	6.5	101.1	
Plasma				
tHcy	0.6	1.4	104.1	0.25
	100	1.2	100.1	
	130	3.7	102.1	
N-Hcy	0.3	3.6	96.6	0.25
	5	2.9	100.2	
	9	1.2	103.1	

hydrolysate of human albumin (Fig. 5b) and was increased in Hcy-thiolactone modified albumin (Fig. 5c).

Protein *N*-linked Hcy and tHcy levels in human plasma

Concentrations of protein *N*-linked Hcy in human plasma from 18 subjects varied from 1.6 to 3.3 μM with a mean of 2.52 μM. Concentrations of tHcy varied from 7.7 to 134 μM. These *N*-Hcy-protein and tHcy values are similar to the values obtained previously by a post-column OPA derivatization method (Jakubowski 2008b). Similar to previous studies (Jakubowski 2002a; Jakubowski et al. 2008, 2009), there was a positive correlation between plasma *N*-Hcy-protein and tHcy levels (Fig. 5d). The maximum imprecision values (RSD %, $n = 4$) for determination of tHcy and *N*-Hcy in plasma were 6.3 and 8.2%.

Specificity, accuracy, imprecision, and limit of quantification

Method validation protocol encompassed specificity, linearity, imprecision, recovery, and limit of quantification (LOQ).

Specificity of the method was determined by spiking the samples with metabolically related thiols cysteine, cysteinylglycine, glutathione, and γ-glutamylcysteine as well as other protein amino acids lysine, leucine, tryptophan, treonine, glutamic acid, serine, alanine, and methionine, and the chromatograms were visually inspected for interfering peaks. Under on-column derivatization conditions applied in our experiments only Hcy gives the chromatographic signal. These results concerning cysteine, cysteinylglycine, and γ-glutamylcysteine are in a good agreement with earlier findings (Mukai et al. 2002). Even if some of them react with OPA under the derivatization conditions, the derivatives either elute earlier or do not give fluorescence signals when 370 and 480 nm as excitation and emission wavelengths, respectively, were used.

Details on imprecision, recovery, and limit of quantification estimated according to the procedures described in "Materials and methods" are shown in Table 1.

Conclusions

The methodology described in the present communication allows facile and accurate determination of urinary Hcy-thiolactone and tHcy, as well as plasma protein *N*-linked Hcy and tHcy. The new methodology has several advantages over our previous assays. First, in contrast to our previous methods which utilize [^{35}S]Hcy-thiolactone as a radiotracer, the new *N*-linked protein Hcy assay does not require radioactive materials. Second, the new assay uses fewer steps in sample work-up. Third, the application of the on-column derivatization methodology results in sharper peaks and shortens the time of HPLC analyses for Hcy-thiolactone and tHcy to just 3 min. Furthermore, the on-column derivatization greatly simplifies the procedure by allowing direct application of plasma protein hydrolysates on the column, which significantly speeds up the protein *N*-linked Hcy assay. These features are compatible with routine analyses of clinical specimen. The new assay should help in elucidating the role of protein *N*-homocysteinylation in health and disease.

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