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The determination of low-molecular-mass thiols with 4-(hydroxymercuric)benzoic acid as a tag using HPLC coupled online with UV/HCOOH-induced cold vapor generation AFS☆

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ABSTRACT

An alternative analytical method was established for simultaneous determination of main urinary low-molecular-mass (LMM) thiols including cysteine (Cys), cysteinylglycine (Cys–Gly), homocysteine (HCys), γ-glutamyl cysteine (γ-Glu–Cys) and glutathione (GSH) as well as *N*-acetylcysteine (NAC) using RPLC coupled on line with UV/HCOOH-induced cold vapor generation atomic fluorescence spectrometry (UV/HCOOH–CVG–AFS) with 4-(hydroxymercuric)benzoic acid (PHMB) as a tag. The LMM thiols were stabilized and labeled by PHMB allowing the determination of reduced form thiols (R-thiols) and total thiols (T-thiols) without and with Tris-(2-carboxyethyl)-phosphine reduction. UV/HCOOH-induced Hg cold vapor generation was used instead of K_2SO_8–KBH_4/NaOH–HCl and/or $KBrO_3$/KBr–KBH_4/NaOH–HCl systems as an effective interface between RPLC and CVG–AFS. The limits of detection (3σ) of RPLC–(UV/HCOOH)–CVG–AFS with PHMB labeling for Cys, HCys, Cys–Gly, γ-Glu–Cys and GSH as well as NAC were 4.6, 5.9, 5.9, 8.1, 7.3 and 5.9 nM with the RSD of 4.4, 5.1, 3.6, 7.5 4.2 and 3.7% ($n = 6$ at 2 μM), respectively, satisfying the simultaneous determination of the main urinary LMM thiols. This developed method was applied successfully to determine the LMM R-thiols and T-thiols in 10 urine samples contributed by 10 healthy volunteers.

1. Introduction

Low-molecular-mass thiols (LMM thiols) including glutathione (GSH), cysteine (Cys), homocysteine (HCys), γ-glutamyl cysteine (γ-Glu–Cys) and cysteinylglycine (Cys–Gly) as well as *N*-acetylcysteine (NAC) are critical cellular components that play numerous important roles in metabolism and homeostasis. Among them, Cys, HCys and GSH are most important; others are the derivatives of these compounds. GSH is the principal nonprotein thiol involved in the antioxidant cellular defenses [1], which is formed from the reaction of γ-Glu–Cys with Gly while cleaved into Cys–Gly and Glu [2]. Cys is a critical substrate for protein and GSH synthesis [3]. HCys, a product of demethylation of methionine, can be converted to cysteine or remethylated to methionine [4]. NAC is an endogenous product of cysteine metabolism [2,5]. Disorder of these thiols metabolism relates with many diseases. For example, disorder of Cys metabolism can result in high concentration of cystine in the urine; because of the very low solubility of cystine in urine, kidney stone formation is a clinical manifestation of classical cystinuria [4]. In laboratory diagnosis of cystinuria, after a positive sodium nitroprusside test, quantitative analysis of cysteine and homocysteine is required to differentiate between cystinuria and homocystinuria. To elucidate the function of these important LMM thiols in biochemical and clinical practice their identification and determination in urine is essential [4]. The analysis of all these LMM thiols is also necessary and important in the diagnosis of inherited diseases such as homocystinuria and cystinuria, and of acquired metabolic disturbance diseases such as cobalamine and folic acid deficiencies [2], stimulating the interests in determining Cys and related LMM thiols in urine [2,4,6–9].

In order to achieve the determination of the LMM thiols by CE and/or HPLC coupled with UV [4,6,8,10,11], fluorescence [7,9,12,13–17], electrochemical detection [18–21], mass spectrometry [2,22–26] and atomic spectrometry such as atomic fluorescence spectrometry (AFS) [27] and inductively coupled plasma mass spectrometry (ICP–MS) [28], most of them except electrochemical detection need derivatization because of low concentration of thiols in urine, and more importantly, derivatization

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will stabilize the unstable reduced form thiols and/or make them spectroscopic detectable. Among numerous derivative reagents such as imidazole [6], bromobimane [7], 5,5′-dithio-bis-nitrobenzoic acid [10], maleimide [13], 3-diazole-4-sulfonate [14], 3-iodoacetylaminobenzanthrone[17] and mercurials [27,28], the used of both Hg(II) and 4-(hydroxymercuric)benzoic acid (PHMB) derivatization of sulfhydryl followed by HPLC coupled on-line with atomic spectrometry receive a significant impulse after the fundamental studies conducted by Bramanti et al. Organic mercurial compounds are very specific and sensitive reagents for reaction with sulfhydryl(s) because of the strong mercury–sulfur affinity [27–36] which has been applied for protein determination by AFS [29–34] and ICP–MS [35] as well as counting sulfhydryls and disulfide bonds in peptides and proteins by ESI–MS [36]. Mercury (Hg) in Hg-labeling thiols could be reduced into Hg^0 using K_2SO_8–KBH_4/NaOH–HCl and/or $KBrO_3$/KBr–KBH_4/NaOH–HCl and determined by AFS [27]. However, compared with the above mentioned reduction systems for Hg-labeled thiols determination with AFS, UV-induced cold vapor generation (UV–CVG) is a better choice for determination of Hg [37–41] which has comparable CVG efficiency and much simple reduction system. It can be easily used as an interface to hyphenate RPLC and AFS for more accurate determination of Hg-labeled thiols.

In this study, we aimed to develop a simple and accurate UV/HCOOH–CVG–AFS for the determination of the LMM thiols labeled with 4-(hydroxymercuric)benzoic acid (PHMB). Combined with RPLC and Tris-(2-carboxyethyl)-phosphine (TCEP) reduction as well as using β-mercaptoethanol (β-ME) as an internal standard, the reduced form LMM thiols (R-thiols) and total thiols (T-thiols) in urine can be quantified.

2. Experimental

2.1. Chemicals and stock solutions

PHMB ($NaOCOC_6H_4OHgOH$) was obtained from Fluka (Buchs, Switzerland) and used as received. 1 mM stock solution of PHMB was prepared by dissolving the corresponding sodium salt in 0.01 M NaOH solution to improve its solubility. The solution was stored at 4 °C in dark and necessary dilution was performed just before use. GSH, Cys, HCys, γ-Glu–Cys, Cys–Gly, NAC and β-ME, TCEP and creatinine were purchased from Sigma–Aldrich (St. Louis, MO, USA). The corresponding compound was respectively dissolved in 0.01 M Tris (hydroxymethyl) aminomethane–HCl (Tris–HCl, pH 7.0) containing 1 mM EDTA to prepare 1 mM stock solution each. Trifluoroacetic acid (TFA) used as a component of RPLC mobile phase was also purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (UPW; 18 MΩ) was prepared with a Milli-Q system (Millipore Filter Co., Bedford, MA, USA) and used throughout this study. Formic acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) used for UV-induced Hg CVG was diluted freshly by UPW (v/v). Other chemicals were at least of analytical grade unless stated.

2.2. Instrumentation

RPLC for separating the PMHB-labeled thiols was performed with a Shimadzu LC-2010A system (Kyoto, Japan) equipped with a quaternary pump, an autosampler (10–100 μL), a thermostated column compartment, a vacuum degasser which were controlled by Shimadzu Class-VP software. Separations were carried out with an Agilent C8 reversed phase column (150 mm × 4.6 mm I.D.; particle size, 5 μm; porosity, 300 Å). All solutions were filtered through a 0.45 μm cellulose acetate filter (Millipore) before use. Chromatographic grade CH_3OH (mobile phase A) and 0.1% TFA solution (B) (pH 3.5 adjusted with ammonia solution (25% w/v)) were used to achieve a gradient elution programmed as 0–10 min 5% A, 10–20 min from 5% A to 15% A, 20–35 min 15% A to 60% A and 35–40 min 60% A to 5% A under a linear gradient at 25 °C for one run. The flow rate of the mobile phase was 1.0 mL/min and the sample injection volume was 20 μL.

Table 1
Optimized instrumental parameters for the AFS determination of mercury.

Parameters	Settings
Mercury hollow cathode lamp	253.7 nm
Lamp current	30 mA
Voltage for photomultiplier tube	−310 V
Observation height	7 mm
Carrier argon flow rate	300 mL/min
Quantification mode	Peak area

Hg determination was performed on a non-dispersive atomic fluorescence spectrometer (Beijing Rayleigh Analytical Instrument Corporation, China) equipped with a high performance Hg hollow cathode lamp (253.7 nm, Beijing Institute of Vacuum Electronics Research, China). The instrumentation for UV-induced cold vapor generation was detailed in our pervious work [40–42]. Briefly, because β-ME was used as an internal standard in this study, formic acid, another effective radical precursor under UV illumination, was used instead of β-ME in this study, in which a homemade 40-W low-pressure Hg-lamp (254 nm, 25 mm O.D. × 130 mm in length) was used as an illumination source, and six quartz tubes (0.8 mm I.D. × 15 mm in length) were arranged in parallel around the UV-lamp. The optimized instrumental parameters for the AFS determination of Hg are listed in Table 1. Data acquisition and post disposal was done by HWH software version 1.0 [43].

ESI ion trap mass spectrometer (ESI–MS) (Bruker Daltonics, Bremen, Germany) used for analyzing PHMB labeled GSH was performed in the positive-ion mode. The operational parameters were as follows: nebulizer, 22 psi; dry gas, 12 L/min; dry temperature, 300 °C; capillary, −3500 V; endplate offset, −500 V; skim 1, 35.0 V; skim 2, 6.0 V; capillary exit offset, 60.0 V; octopole, 2.80 V; lens 1, −5.0 V; lens 2, −60.0 V; trap drive, 55.0; and max accumulation time, 50.00 ms.

2.3. Sample pretreatment and derivatization procedures

Urine samples from spontaneous micturition were contributed by 10 healthy volunteers, and either analyzed without delay or stored at −20 °C until analysis. 750 μL samples were acidified with 240 μL sulfosalicylic acid (SSA) (1 M) containing 1 mM EDTA, followed by the addition of 10 μL β-ME (1 mM) as an internal standard. And then centrifuged (15,000 × *g*, 15 min, 4 °C) to deproteinize, finally, the supernatant was divided into aliquots and stored at −20 °C until use.

One aliquot (200 μL) was mixed with 795 μL of 0.01 M Tris–HCl buffer (pH 7.0) containing 100 μM PHMB for the measurement of R-thiols after 6 μL ammonia (25% w/v) was added for neutralizing the excess sulfosalicylic acid to about pH 7; while the other aliquot was reduced using 400 μL 2.5 mM TCEP (final concentration: 1 mM) in 0.01 M Tris–HCl buffer (pH 7.0), and then the mixture was incubated at 37 °C for 30 min (optimized condition) for the measurement of T-thiols. After 30 min incubation, 395 μL 0.01 M Tris–HCl buffer (pH 7.0) containing 1 mM PHMB was added to label the thiols and to exhaust the excess TCEP. After 10 min labeling time at room temperature (25 °C), 20 μL of the solution obtained was injected into the RPLC–(UV/HCOOH)–CVG–AFS system for analyzing the labeled thiols. Through out the above procedures, the analyzing sample was diluted 6.7-folds as compared with the original urinary sample.

2.4. Quantification of the LMM thiols

For calibration experiments, appropriate amount of the LMM R-thiols with 2 μM β-ME, which was used as an internal standard, were derivatized by PHMB (5 times excess to the R-thiols) in Tris–HCl buffer (0.01 M, pH 7.0). The peak area ratios of PHMB labeled R-thiols to that of internal standard were respectively plotted versus the R-thiol concentrations.

2.5. Determination of creatinine

Creatinine was determined using an HPLC method reported elsewhere [4]. In brief, 20 μL of an UPW-diluted urine sample (1:50) was injected on to the RPLC system (the same as used for the thiol determination) using a mobile phase composed of 10 mM phosphate buffer and methanol (98:2, v/v, pH 6.8) at the flow rate of 1 mL/min. It was determined at 236 nm according to the peak area obtained. Identification of the creatinine peak was based on comparison of the retention time and diode array spectra with the corresponding data obtained from the authentic standard compound.

3. Results and discussion

3.1. Thiols labeling with PHMB

In order to optimize the reaction conditions of R-thiols labeled with PHMB and confirm the formation of PHMB labeled thiols, GSH was chosen as a model of the target LMM thiols owing to its more complicated stereo-hindrance effect of tripeptide compared with other target dipeptides (Cys–Gly and γ-Glu–Cys and NAC) and amino acids (Cys and HCys). Labeling reaction time and pH dependence were investigated on the labeling efficiency of PHMB to GSH. The results obtained indicated that the labeling reaction completed within 10 min and GSH could be labeled quantitatively with PHMB from pH 2 to 7. It was confirmed by ESI–MS (Fig. 1), the peak at *m*/*z* 629.8 belongs to PHMB–GSH, 483.9 the fragment of PHMB–GSH losing a Glu and 651.8 the adduct of (PHMB–GSH) Na while the peak at *m*/*z* 307.9 of GSH completely disappears, indicating that a quantitative labeling was achieved. Moreover, the formation of PHMB–GSH was also indicated by the distribution of the multiple peaks around *m*/*z* 629.8 which is contributed from Hg natural isotopic distribution [36,44]. PHMB-labeled GSH was stable at least for the length of a working day. Increasing pH to 7.5 significantly decreased the labeling reaction, and above pH 8 nearly no GSH was labeled with PHMB. This phenomenon is in accordance with the results reported in ref. [45] for the binding of PHMB to egg albumin. PHMB exists

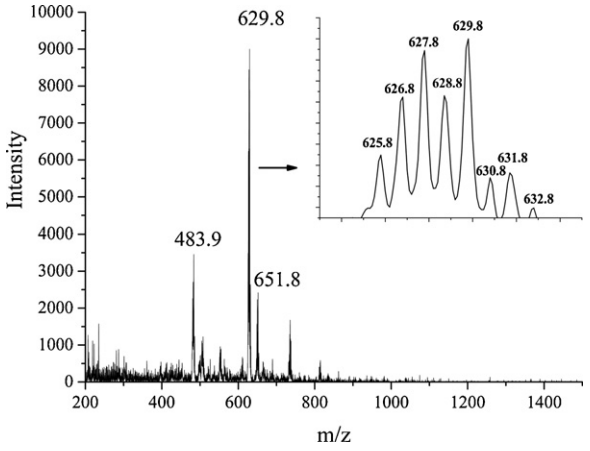

Fig. 1. ESI–MS spectra of PHMB-labeled GSH. Peaks at *m*/*z* 626, 627, 628, 629, 630, 631, 632, and 633 were contributed from the natural isotopic distribution of Hg.

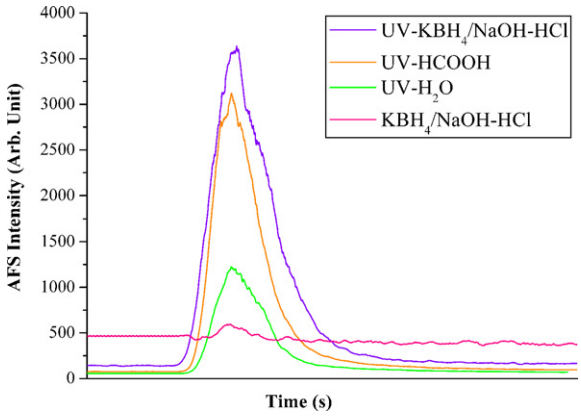

Fig. 2. Comparison of different methods for Hg cold vapor generation of PHMB-labeled GSH (1 μM). The flow rate of 0.05% KBH_4/0.5% NaOH (w/v), 5% HCl (v/v) and 15% HCOOH (v/v) were 1.5, 1.5 and 2.0 mL/min, respectively. And the reaction coil (I.D. 0.8 mm) length was 90 cm.

mainly in the state of undissociated hydroxide at pH above 8, it can not bind easily with thiols. Considering the solubility of PHMB and the labeling efficiency, pH 7 was chosen for all the target thiols labeling within 10 min.

3.2. Determination of PHMB-labeled thiols by UV/HCOOH–CVG–AFS

Among many radical precursors used for UV-induced CVG, HCOOH [37–40] and β-ME [41] were considered as the simplest and most effective ones. Considering that β-ME was used as an internal standard in this study, HCOOH was chosen as the precursor for UV-induced Hg cold vapor generation. The concentration of HCOOH for PHMB-labeled thiols reduction was optimized from 1% to 50% ($HCOOH/H_2O$, v/v), the results obtained indicated that 15% HCOOH was most effective for the CVG of Hg in the PHMB-labeled thiols with a flow rate of 2 mL/min. Comparison with UV–KBH_4/NaOH–HCl, KBH_4/NaOH–HCl and UV–H_2O systems for Hg CVG of the PHMB-labeled GSH is depicted in Fig. 2. Obviously, UV–HCOOH system could get a comparable sensitivity as UV–KBH_4/NaOH–HCl but much higher than the other two system used. In the case of UV–H_2O, somewhat Hg cold vapor were also generated but with low efficiency. Considering about the bond dissociation energy in the cases of Hg–S with 213 kJ/mol and Hg–C in phenyl–Hg–phenyl with 285 kJ/mol [46], UV of 254 nm (471 kJ/mol) is enough for cleaving the bond between Hg and C as well as that between Hg and S. Moreover, the generation of HCOOH radical under UV illuminating [47] accelerated the reduction of Hg species, behaving a similar efficiency as the most common used CVG system of UV–KBH_4/NaOH–HCl, and much simpler than those described in refs. [27,29–34].

3.3. Separation of PHMB-labeled thiols by RPLC

Various combinations of buffer solution and organic solvent were examined to discover the most suitable mobile phase for the separation of the six PHMB-labeled LMM thiols on the C8 column. TFA–methanol appeared promising. The retention factor (*k*) of each PHMB-labeled thiol as a function of the mobile phase pH of 0.1% TFA–methanol (adjusted to a final pH with ammonia, eluted conditions were described in experimental section) was investigated. At acidic pH, the *k* values of Cys (9.2), Cys–gly (12.5), γ-Glu–Cys (17.38), and GSH (19.2) increased with the number of amino acids. The *k* values decreased along with the increase in pH from 3.0 to 4.5, depending on the number of carboxylic groups in the corresponding labeled thiols, which was attributed to the dissociation

Table 2
Analytical features of merit.[a,b].

Sample	Retention time (min)	Calibration range (μM)	Calibration plot correlation		Regression coefficient	LOD (nM)	LOQ (nM)[c]
			Correlation Slope	Intercept			
PHMB–Cys	11.89	2–50	0.8158	0.0240	0.9997	4.6	15.3
PHMB–Cys–Gly	15.77	0.1–10	0.6592	−0.0135	0.9994	5.9	19.7
PHMB–HCys	18.54	0.1–10	0.6409	−0.0638	0.9996	5.9	19.7
PHMB–Cys–Glu-γ	21.50	0.1–10	0.4682	0.0438	0.9984	8.1	27.0
PHMB–GSH	23.63	0.1–5	0.5115	−0.0527	0.9990	7.3	24.3
PHMB–NAC	27.72	0.1–5	0.6763	−0.0437	0.9986	5.9	19.7

[a] Results from six duplicate runs.
[b] Reactions were performed in Tris–HCl buffer (pH 7.0).
[c] Limit of quantification (LOQ) was calculated based on 10 times signal/noise ratio.

of the carboxyl moiety [14]. The higher *k* value of HCys (14.8) than Cys–Gly (12.5) might be related to the increase of a methylene group with the decrease of polarity compared with Cys–Gly. Combination of acetyl with amino group in NAC decrease the polarity thus increases the *k* value of NAC (22.8). The internal standard β-ME as the most hydrophobic one results in the *k* value of 27.3. In addition, the effects of ion-pair formation with TFA anions should play a substantial role in the peptide retention. From these findings, 0.1% TFA-methanol (pH 3.5) was chosen as the suitable mobile phase for the separation of PHMB-labeled LMM thiols, their retention time is in the order of PHMB–Cys, PHMB–Cys–Gly, PHMB–HCys, γ-Glu–Cys–PHMB, PHMB–GSH, NAC–PHMB and β-ME–PHMB as shown in Fig. 3a. Each PHMB-labeled thiol exhibited a sharp peak, and baseline separation was achieved. Isocratic elution during initial 10 min was adopted for PHMB–TCEP elution in order not to disturb the determination of PHMB-labeled Cys when a urine sample was analyzed. A typical chromatogram of one urine sample with the addition of β-ME used as an internal standard after TCEP reduction and derivatization with PHMB is shown in Fig. 3b. In order to verify that the peaks in Fig. 3b are really due to thiols, thiols in urine sample were blocked with *N*-ethylmaleimide [44] before SSA acidification and PHMB labeling. The result obtained is depicted in Fig. 3c demonstrating that the peaks in Fig. 3b almost disappeared, proving that the peaks in Fig. 3b are ascribed to the labeled thiols in the urine sample. It is also a strong evidence for the high selectivity of mercury towards sulfhydryl.

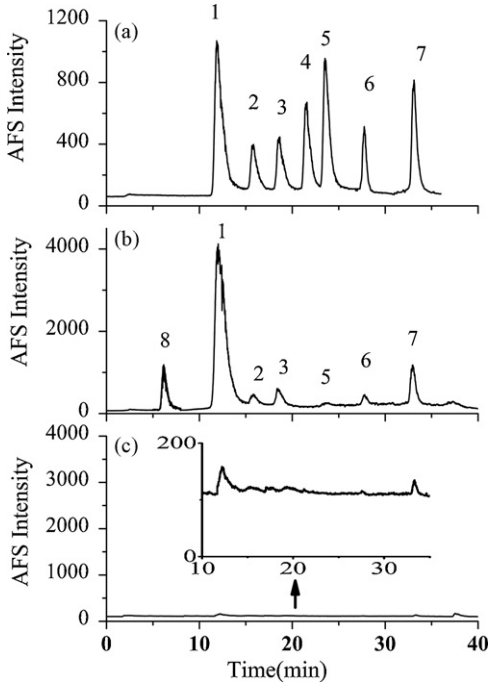

Fig. 3. (a) RPLC–(UV/HCOOH)–CVG–AFS chromatogram of PHMB-labeled LMM thiols; (b) RPLC–(UV/HCOOH)–CVG–AFS chromatogram of one urine sample after TCEP reduction and derivatization with PHMB (β-ME used as an internal standard); (c) RPLC–(UV/HCOOH)–CVG–AFS chromatogram of one urine sample after blocked R-thiols with *N*-ethylmaleimide (1 mM), followed by the SSA acidification and PHMB (0.5 mM) labeling. 1, PHMB-labeled Cys (2.0 μM); 2, PHMB-labeled Cys–Gly (1.2 μM); 3, PHMB-labeled HCys (1.3 μM); 4, PHMB-labeled γ-Glu–Cys (2.0 μM); 5, PHMB-labeled GSH (2.0 μM); 6, PHMB-labeled NAC (1.5 μM); 7, PHMB-labeled β-ME (2.0 μM); 8, PHMB–TCEP. Gradient elution program: 0–10 min 5% mobile phase A (CH_3OH) and 95% mobile phase B (0.1% TFA, pH 3.5); 10–20 min 5% A to 15% A; 20–35 min 15% A to 60% A and 35–40 min 60% A to 5% A at the flow rate of 1.0 mL/min. Sample volume injected: 20 μL. UV/HCOOH–CVG–AFS was used for Hg determination, in which 15% HCOOH at the flow rate of 2 mL/min was used.

3.4. Quantification of thiols by RPLC–(UV/HCOOH)–CVG–AFS

Calibration results obtained according to the peak area ratio of PHMB labeled thiol to that of β-ME (internal standard) plotted versus the LMM thiol's concentration under the optimized conditions are shown in Table 2, indicating that good correlations between the peak area ratios and the concentrations of the corresponding PHMB-labeled thiols (R is from 0.9984 to 0.9997). Limits of detection (LOD) (3σ) under the optimum RPLC–(UV/HCOOH)–CVG–AFS conditions for Cys, HCys, Cys–Gly, γ-Glu–Cys, GSH and NAC were 4.6, 5.9, 5.9, 8.1, 7.3 and 5.9 nM with RSD of 4.4, 5.1, 3.6, 7.5, 4.2 and 3.7% ($n = 6$ at 2 μM), respectively (Table 2). Considering the quantitative labeling of the LMM thiols with PHMB, the different sensitivity might be attributed to the different CVG efficiency of the labeled thiols, which is ascribed to the different bond intensities between Hg in PHMB and the –SH in the thiols [27,29]. Limits of quantification (LOQ) (10σ) for the LMM thiols were calculated to be 15.3 (Cys), 19.7 (Cys–Gly), 19.7 (HCys), 27.0 (γ-Glu–Cys), 24.3 (GSH), 19.7 nM (NAC), respectively.

The LODs for the LMM thiols with the RPLC–(UV/HCOOH)–CVG–AFS method are somewhat higher than those obtained by RPLC–$KBrO_3$/KBr–KBH_4–HCl–AFS [27] (approximately 1 nM), HPLC–electrochemical detection (2–6 nM) [21], HPLC–fluorescence detection (3 nM) [13,17], but are approximately one or two orders of magnitude lower than those of CE with fluorescence detection (approximately 0.1 μM) [11] and HPLC–UV (0.12 μM) [4]. It should be noted that although HPLC–electrochemical detection has as the advantage of simultaneous detecting both disulfide and R-thiols, but it suffers interferences from many compounds (e.g. oxygen) due to the high oxidation potential and long-term instability [27,48,49], limiting its application to real samples. The comparison of the LODs of different methods for the LMM thiols determination was summarized in Table 3.

3.5. Urine sample analysis

In the ten urine samples, Cys, Cys–Gly, HCys, GSH and NAC were determined in the range from 0.4 to 457.5 μM. γ-Glu–Cys was not

Table 3
Comparison of different methods for determination of LMM thiols.

Method	Analyst	Derivatization	LOD (nM)	Ref.
HPLC–UV	Cys, HCys, Cys–Gly	2-*S*-quinolinium	12–25	[4]
HPLC–electrochemical detection	Cys, HCys, GSH, disulfide, PCs	None	2–6	[21]
HPLC–fluorescence detection	Cys, HCys, GSH, NAC	3-Iodoacetylaminobenzanthrone	1–2.3	[17]
HPLC–MS/MS	Cys, HCys, Cys–Gly, GSH, NAC	Ferrocene-based maleimide	30–110	[2]
CE–fluorescence detection	Cys, HCys, Cys–Gly, GSH, γ-Glu–Cys, thiol drug	5-(Bromomethyl)fluorescein	61–183	[11]
HPLC–$KBrO_3$/KBr–KBH_4/NaOH–HCl–AFS	Cys, HCys, Cys–Gly, GSH	4-(Hydroxymercuric)benzoic acid	0.6–1	[27]
Our method	Cys, HCys, Cys–Gly, GSH, NAC, γ-Glu–Cys	4-(Hydroxymercuric)benzoic acid	5–8	

Table 4
$C_{R\text{-thiols}}$ $C_{T\text{-thiols}}$, creatinine normalized $C_{T\text{-thiols}}$ and the fraction of R-thiol to T-thiol determined in ten urine samples.[a].

Sample	1	2	3	4	5	6	7	8	9	10
C_{Cys} (μM)	69.5 ± 4.1	87.6 ± 2.5	59.8 ± 2.5	27.8 ± 2.0	34.3 ± 1.3	73.1 ± 5.6	168.6 ± 19.1	96.7 ± 10.1	76.6 ± 5.9	40.7 ± 2.9
$C_{T\text{-}Cys}$ (μM)	263.4 ± 11.5	342.2 ± 17.4	183.4 ± 17.7	112.6 ± 6.0	177.8 ± 8.4	245.1 ± 14.7	457.5 ± 6.0	412.8 ± 15.7	328.5 ± 9.6	158.4 ± 10.3
Fraction of Cys to T-Cys (%)	26.4	25.6	32.6	24.7	19.3	30.0	36.8	23.4	23.3	25.7
$C_{T\text{-}Cys}$ (mmol/mol of creatinine)	31.5	17.9	23.4	34.8	20.6	34.6	15.0	12.4	24.3	22.8
$C_{Cys\text{–}Gly}$ (μM)	2.5 ± 0.3	7.3 ± 0.8	6.7 ± 0.7	1.7 ± 0.2	3.0 ± 0.1	5.3 ± 0.1	4.4 ± 0.1	20.2 ± 0.5	4.4 ± 0.1	4.8 ± 0.2
$C_{T\text{-}Cys\text{–}Gly}$ (μM)	4.9 ± 0.3	27.2 ± 0.4	21.2 ± 0.3	3.9 ± 0.2	11.9 ± 0.8	11.3 ± 0.2	16.3 ± 1.0	27.4 ± 3.4	22.0 ± 1.4	10.9 ± 0.4
Fraction of Cys–Gly to T-Cys–Gly (%)	51.0	26.8	31.6	43.6	25.2	46.9	27.0	35.2	20.0	44.0
$C_{T\text{-}Cys\text{–}Gly}$ (mmol/mol of creatinine)	0.6	1.4	2.7	1.2	1.4	1.6	0.5	1.7	1.6	1.6
C_{HCys} (μM)	0.8 ± 0.1	5.6 ± 0.3	3.6 ± 0.3	0.9 ± 0.1	2.1 ± 0.1	4.0 ± 0.3	7.7 ± 0.7	7.6 ± 0.4	2.8 ± 0.1	2.0 ± 0.1
$C_{T\text{-}HCys}$ (μM)	2.1 ± 0.2	16.7 ± 0.4	18.0 ± 1.0	4.6 ± 0.2	11.5 ± 0.7	10.0 ± 0.2	16.5 ± 0.8	22.6 ± 1.6	14.0 ± 0.1	12.3 ± 1.1
Fraction of HCys to T-HCys (%)	38.1	33.5	20.0	19.6	18.3	40.0	46.7	33.6	20.0	16.3
$C_{T\text{-}HCys}$ (mmol/mol of creatinine)	0.3	0.9	2.3	1.4	1.3	1.4	0.5	0.7	1.0	1.8
C_{GSH} (μM)	nd	2.1 ± 0.1	nd	nd	0.7 ± 0.1	0.6 ± 0.1	1.5 ± 0.1	2.3 ± 0.1	nd	0.4 ± 0.1
$C_{T\text{-}GSH}$ (μM)	0.9 ± 0.1	6.9 ± 0.2	1.9 ± 0.2	0.9 ± 0.1	2.3 ± 0.1	1.8 ± 0.1	4.3 ± 0.2	6.5 ± 0.3	1.9 ± 0.1	1.8 ± 0.1
Fraction of GSH to T-GSH (%)		30.4			30.4	33.3	34.9	34.6		22.2
$C_{T\text{-}GSH}$ (mmol/mol of creatinine)	0.1	0.4	0.2	0.3	0.3	0.3	0.1	0.2	0.1	0.3
C_{NAC} (μM)	nd	2.8 ± 0.1	2.1 ± 0.2	nd	1.4 ± 0.1	4.0 ± 0.3	4.1 ± 0.4	6.9 ± 0.7	nd	2.0 ± 0.2
$C_{T\text{-}NAC}$ (μM)	nd	19.3 ± 1.0	9.8 ± 0.7	6.5 ± 0.3	12.8 ± 0.6	15.2 ± 0.4	27.3 ± 1.5	33.5 ± 1.0	8.1 ± 0.3	11.6 ± 0.7
Fraction of NAC to T-NAC (%)		14.5	20.4		10.9	26.3	15.0	20.6		17.2
$C_{T\text{-}NAC}$ (mmol/mol of creatinine)		1.0	1.3	2.0	1.5	2.1	0.9	1.0	0.6	1.7

[a] Results from triplicate runs; nd denotes that the thiol was not determined.

found in the urine samples, which is in line with those reported in refs. [2] and [4,6–9]. A typical RPLC–(UV/HCOOH)–CVG–AFS chromatogram for a urine sample after TCEP reduction and labeled with PHMB is depicted in Fig. 3b. The concentration of the thiols in urine depends largely on the diet, lifestyle, and ambient temperature of the different individuals [4]. To facilitate the comparison between the data, the analytical results for urinary thiols were normalized to creatinine. For the ten urine samples, the concentration of creatinine was measured to be in the range of 3.3–33.4 mM, higher than those reported in ref. [4] (1.92–17.71 mM), which might be ascribed to the individual difference of different clime. The concentrations of R-thiols and T-thiols in the ten urinary samples were determined, respectively. The results are summarized in Table 4. In the urine samples contributed from ten healthy people living in Xiamen China, the creatinine-normalized T-Cys concentration ($C_{T\text{-}Cys}$), for example, was found to be present in the range from 12.4 to 34.8 mmol/mol with an average value of 23.8 ± 7.8 mmol/mol, and the fraction of Cys to T-Cys measured to be from 19.3% to

Table 5
Results from study of precision and recovery of the LMM thiols from urine, $n = 3$.

Thiols	Sample concentration (μM)	Amount added (μM)	Amount measured (μM)	Recovery (%)
Cys	112.6	50.0	160.9 ± 5.5	96.6
	112.6	100.0	215.2 ± 6.8	102.6
Cys–Gly	3.9	2.0	6.0 ± 0.4	104.8
	3.9	5.0	8.7 ± 0.4	95.3
HCys	4.6	2.0	6.5 ± 0.3	94.5
	4.6	5.0	9.4 ± 0.6	96.2
GSH	0.9	1.0	1.8 ± 0.2	88.0
	0.9	2.0	2.8 ± 0.2	92.1
NAC	6.5	5.0	11.3 ± 0.5	95.4
	6.5	10.0	16.4 ± 0.7	98.6

32.6%, which is in well accordance with the values (between 11% and 33%) reported in ref. [50]. Although it is not so reasonable to compare the urinary LMM thiols data from different people living different area [2,4,50], the creatinine-normalized LMM thiol's concentrations in the urinary samples of the volunteers in Xiamen China determined by RPLC–(UV/HCOOH)–CVG–AFS, indicating that $C_{T\text{-}Cys\text{–}Gly}$ were in the range from 0.5 to 2.7 mmol/mol with an average value of 1.5 ± 0.6 mmol/mol, and the fraction of Cys–Gly to T-Cys–Gly was from 20.0% to 51.0%; Those of T-HCys were in the range from 0.3 to 2.3 mmol/mol with the average value of 1.2 ± 0.6 mmol/mol and the fraction of HCys to T-HCys was measured to be from 16.3% to 46.7%. $C_{T\text{-}NAC}$ was determined to be in the range from 0.6 to 2.1 mmol/mol, and the fraction of NAC to T-NAC from 10.9% to 26.3% in seven of the ten urine samples, which is similar to the results obtained in ref. [2] from 10.3 to 19.3. GSH was also determined to be containing in the samples, with $C_{T\text{-}GSH}$ ranging from 0.1 to 0.4 mmol/mol, and the fraction of GSH to T-GSH from 22.2% to 34.9% in six of the ten urine samples. As an additional proof of the principle, standard additional experiments were performed for validating the method developed because of lacking certified reference materials, and the recovery for the 5 main urinary LMM thiols including Cys, Cys–Gly, HCys, GSH and NAC were from 88.0 to 104.8. Details are depicted in Table 5 indicating that this method is useful for the diagnosis of people healthy status.

4. Conclusion

An alternative analytical method was established based on PHMB labeling using RPLC–(UV/HCOOH)–CVG–AFS for quantification of six biological important LMM thiols including Cys, Cys–gly, HCys, γ-Glu–Cys and GSH as well as NAC in urine. It allows the determination of LMM R-thiols and T-thiols without and with TCEP reduction, providing comparative results with the conventional thiols determination methods. Although we were not in possession of urine samples from patients suffering from disorders of thiol metabolism, for example, cystinosis, cystinuria or homocystinuria, we believe this method would be a simple and accurate approach in routine diagnosis of these diseases. Further research will be focused on the novel and compact design of the photochemical CVG interface and new Hg tag exploring to further improve the LODs of the LMM thiols.

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