Urinary Excretion of Homocysteine-Thiolactone in Humans

GRAŻYNA CHWATKO1 and HIERONIM JAKUBOWSKI1,2*

Background: A metabolite of homocysteine (Hcy), the thioester Hcy-thiolactone, has been implicated in coronary heart disease in humans. Because inadvertent reactions of Hcy-thiolactone with proteins can lead to cell and tissue damage, the ability to detoxify or eliminate Hcy-thiolactone is essential for biological integrity. We examined the hypothesis that the human body eliminates Hcy-thiolactone by urinary excretion.

Methods: We used a sensitive HPLC method with postcolumn derivatization and fluorescence detection to examine Hcy-thiolactone concentrations in human urine and plasma.

Results: We discovered a previously unknown pool of Hcy-thiolactone in human urine. Urinary concentrations of Hcy-thiolactone (11–485 nmol/L; n = 19) were ~100-fold higher than those in plasma (<0.1–22.6 nmol/L; n = 20). Urinary Hcy-thiolactone accounted for 2.5–28.3% of urinary total Hcy, whereas plasma Hcy-thiolactone accounted for <0.002–0.29% of plasma total Hcy. Urinary concentrations of Hcy-thiolactone, but not of total Hcy, were negatively correlated with urinary pH. Clearance of Hcy-thiolactone, relative to creatinine, was 0.21–6.96. In contrast, relative clearance of Hcy was 0.001–0.003.

Conclusions: The analytical methods described here can be used to quantify Hcy-thiolactone in biological fluids. Using these methods we showed that the human body eliminates Hcy-thiolactone by urinary excretion. Our data also suggest that the protonation status of its amino group affects Hcy-thiolactone excretion.

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Coronary heart disease is the major cause of death in industrialized nations. Despite advances in our understanding of cardiovascular disease, traditional risk factors such as hypertension, smoking, diabetes, and hyperlipidemia do not accurately predict cardiovascular events. Numerous clinical studies have shown that plasma total homocysteine (tHcy) is a risk factor for cardiovascular disease and stroke in humans (1) and predicts mortality independently of traditional risk factors in patients with coronary artery disease (2). Plasma tHcy is also a risk factor for neurodegenerative disorders, including dementia and Alzheimer disease (3). Possible cellular mechanisms by which Hcy may contribute to cardiovascular disease include unfolded protein response, oxidative stress, and the induction of proinflammatory factors (4).

A possible molecular mechanism underlying Hcy toxicity involves metabolic conversion of Hcy to Hcy-thiolactone during the steps leading to protein biosynthesis (5, 6). Because of its similarity to the protein amino acid methionine, Hcy enters the initial steps of protein synthesis and is misactivated by methionyl-tRNA synthetase (MetRS in Scheme 1) to form homocysteinyl-adenylate.

MetRS + Hcy + ATP ⇌ MetRS•Hcy-AMP + PPi

Scheme 1.

Hcy, however, does not complete the protein biosynthetic pathway but is converted to Hcy-thiolactone (Scheme 2) in a quality-control reaction catalyzed by methionyl-tRNA synthetase in all organisms investigated, including humans (7, 8).
Hcy-thiolactone is known to be cytotoxic in experimental animals and in cell cultures. Chronic infusions of Hcy-thiolactone into baboons cause patchy desquamation of vascular endothelium and arterial thrombosis (9), whereas acute infusions into mice and rats cause seizures and death within minutes (10). Exposure of mouse (11), rat (12), or chicken (13) embryos to Hcy-thiolactone causes increased lethality, growth retardation, blisters, and abnormalities of somite development. In one study, Hcy-thiolactone was reported to be nonteratogenic in mouse embryos. The maximum dose used in that study (14), however, was lower than those used in other studies (11–13), so that an embryotoxic dose had not been reached. Hcy-thiolactone induces apoptotic death in cultured human vascular endothelial cells (15) and promyeloid cells (16) and inhibits insulin signaling in rat hepatoma cells (17).

Hcy-thiolactone can be detrimental 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 (5–7, 18–22) (Scheme 3).

Modification with Hcy-thiolactone affects protein structure (5, 20, 22), decreases the physiologic activities of proteins (5, 20, 23), and has toxic effects on cells (24). N-Hcy–protein complexes elicit immune responses in rabbits (5, 25, 26) and humans (5, 26). An autoimmune response to N-Hcy–protein complexes is associated with stroke (26).

Human plasma concentrations of Hcy-thiolactone are much lower than those expected from studies with cultured cells. For example, in cultured human diploid fibroblasts (18) or umbilical vascular endothelial cells (19), Hcy-thiolactone represents ~10% of tHcy. However, Hcy-thiolactone represents only <0.8% (7, 8) or 0.2% (27) of plasma tHcy. This apparent discrepancy suggests that the human body possesses mechanisms that prevent of Hcy-thiolactone from accumulating to harmful concentrations.

Because protein N-homocysteinylation can lead to cell and tissue damage, humans most likely evolved mechanisms to detoxify Hcy-thiolactone. The present work shows that the human body eliminates Hcy-thiolactone by selective urinary excretion. In addition, our data suggest that the protonation status of its amino group affects the excretion of Hcy-thiolactone.

**Materials and Methods**

**Preparation of [35S]Hcy-thiolactone and [35S]Hcy**

We converted L-[35S]Met (5 mCi; Amersham Pharmacia Biotech) to L-[35S]Hcy-thiolactone (specific activity, 20 000 Ci/mol) as described previously (22, 28). We prepared [35S]Hcy by hydrolyzing [35S]Hcy-thiolactone with 0.1 mol/L NaOH for 15 min at 37 °C (18).

**Human Plasma and Urine Samples**

We obtained fasting human blood or fresh morning urine from healthy volunteers 30–57 years of age, as approved by the Institutional Review Board. The blood was collected in Vacutainer EDTA tubes, the tubes were chilled on ice, and the plasma was separated by centrifugation at 4 °C and 2000g for 15 min. Plasma samples were stored at −80 °C. This procedure prevents postcollection enzymatic hydrolysis of Hcy-thiolactone by Hcy-thiolactonase/paraoxonase (28, 29) and nonenzymatic reaction of Hcy-thiolactone with protein lysine residues, which strongly depends on temperature (7, 18, 20).

The urine was collected into beakers, and after its volume was recorded, it was stored frozen in 10-mL aliquots at −80 °C. In urine, Hcy-thiolactone is stable for several hours after collection (see Results).

**Stability and Interconversion of [35S]Hcy-thiolactone and [35S]Hcy in Urine**

Urine samples were supplemented with 1 nmol/L [35S]Hcy-thiolactone or [35S]Hcy and incubated at 4, 23, or 37 °C. The remaining [35S]Hcy-thiolactone and [35S]Hcy were measured by thin-layer chromatography on cellulose plates (Analtech, Inc.) with butanol-acetic acid–water (4:1:1 by volume) as the solvent (18).

**Sample Preparation for Hcy-thiolactone Measurements in Human Urine**

Human urine (0.5 mL) was ultrafiltered through a 10-kDa cutoff membrane (Ultrafree 10kDa Biomax; Millipore) at 4 °C. The pH of the ultrafiltrate (0.25 mL) was adjusted to 8.0 by the addition of 1 mol/L K2HPO4 (20 μL) to convert the positively charged form of Hcy-thiolactone to a neutral form [Hcy-thiolactone has a pKₐ=7.1, (30)], which was then adsorbed on 10 mg of phosphate-buffered saline-washed activated charcoal (cat. no. C 7606; Sigma-Aldrich). The charcoal was washed with 1 mol/L NaCl (twice with 0.1 mL each time), 0.1 mL of phosphate-buffered saline, and 0.1 mL of deionized water. The conversion of the adsorbed Hcy-thiolactone to the positively charged form by acidification with 10 mmol/L HCl (three times with 0.1 mL each time) led to the release of Hcy-thiolactone from the charcoal. The acid eluates were lyophilized and dissolved in 100 μL of mobile phase; 10-μL aliquots were then subjected to cation-exchange HPLC. The mean (SD) recovery, assessed with[35S]Hcy-thiolactone (18 000 cpmp) added to each sample as an internal standard, was 32.2 (9.4)%.

**Sample Preparation for Hcy-thiolactone Measurements in Human Plasma**

Human plasma samples (0.4 mL) were deproteinized by ultrafiltration through Millipore 10-kDa cutoff mem-
branes at 4 °C. The protein-free ultrafiltrate (0.2 mL) was adjusted to pH 8.0 with 0.01 mL of 1 mol/L K₂HPO₄, and Hcy-thiolactone was extracted with 1 mL of chloroform–methanol (2:1 by volume) at room temperature. The Hcy-thiolactone was reextracted from the organic phase with 0.2 mL of 0.1 mol/L HCl. Chloroform–methanol is more selective than charcoal for extracting Hcy-thiolactone from plasma samples. The aqueous phase was lyophilized on a Labconco concentrator, the residue was dissolved in 20 μL of deionized water, and 10-μL aliquots were mixed with 10 μL of mobile phase and analyzed by cation-exchange HPLC. The mean (SD) recovery, assessed with plasma samples supplemented with 5–40 nmol/L Hcy-thiolactone, was 61.8 (7.7)%.

**SAMPLE PREPARATION FOR tHcy MEASUREMENTS IN HUMAN URINE OR PLASMA**

Plasma tHcy was determined as described previously (8) by a procedure that involves conversion to Hcy-thiolactone, which is then quantified by HPLC. Urinary tHcy was determined by a similar procedure. Urine (200 μL) was treated with 10 mmol/L dithiothreitol and ultrafiltered through a Millipore 10-kDa membrane at 4 °C. A 100-μL portion of the ultrafiltrate was treated with 2 μL of 1 mol/L dithiothreitol and 10 μL of 12 mol/L HCl for 30 min at 100 °C. The treated ultrafiltrate was divided into 10-μL aliquots, which were lyophilized and dissolved in 200 μL of mobile phase; 20 μL of this solution was then applied to a cation-exchange HPLC column.

The tHcy assayed here includes not only Hcy obtained by the reductive cleavage of all disulfide bonds in a sample (31) but also Hcy-thiolactone. Standard methods of tHcy determination (31, 32) may also include a contribution from Hcy-thiolactone.

**HPLC SEPARATION, DETECTION, AND QUANTIFICATION**

Hcy-thiolactone was analyzed by a modified version of a cation-exchange HPLC method (8, 21). A Beckman-Coulter System Gold Noveau HPLC instrument, containing a multimode 508 autosampler, advanced gradient solvent delivery module 126, and a Jasco 1520 fluorescence detector, was used. A manual injector (7725i; Rheodyne) with a 0.1-mL loop was used for analysis of single samples. Chromatograms were analyzed by Gold Noveau chromatography workstation software for Windows.

Samples (10–20 μL) were injected into a cation-exchange PolySULFOETHYL aspartamide column [150 × 1 mm (i.d.); 5 μm bead size; 300Å pore size; PolyLC, Inc.]. The column was eluted isocratically with 10 mmol/L sodium phosphate buffer, pH 6.6, containing 5 mmol/L NaCl at a flow rate 0.15 mL/min. Under these conditions, Hcy-thiolactone was well separated from two major interfering compounds: Hcy, which was not bound, and histidine, whose binding to the column was weaker than the binding of Hcy-thiolactone (Fig. 1).

Hcy-thiolactone was quantified by postcolumn derivatization with a solution of o-phthalaldehyde (OPA) in NaOH and fluorescence detection (33). Under alkaline conditions, Hcy-thiolactone is almost instantly hydrolyzed to Hcy, which then reacts with OPA to give a highly fluorescent derivative. Only two other naturally occurring compounds, histidine and glutathione, are known to yield fluorescent derivatives after reactions with OPA under alkaline conditions (33). Fluorescence yields of these derivatives depend on the pH. In our experimental setup, derivatization with 2.5 mmol/L OPA in 0.25 mol/L NaOH gave the optimum fluorescence yield for Hcy-thiolactone.

The effluent was mixed in a three-way T-valve with 2.5 mmol/L OPA in 0.25 mol/L NaOH, delivered at a flow rate 0.07 mL/min. The mixture passed through a Teflon tubing reaction coil [0.3 mm (i.d. × 3 m] and then was monitored with a Jasco 1520 fluorescence detector using excitation at 370 nm and fluorescence emission at 480 nm. Examples of HPLC analysis of the Hcy-thiolactone content of human urine are shown in Fig. 1.

The identity of the eluted material as Hcy-thiolactone was confirmed by its comigration with an authentic Hcy-thiolactone standard and by the sensitivity to NaOH. The on-column detection limit was 25 fmol of Hcy-thiolactone. The inter- and intraassay variation was 6.7% and 8.5%, respectively, for plasma samples and 7.1% and 12%, respectively, for urine samples. The assay was linear in the concentration range 0.1–500 nmol/L. Hcy-thiolactone.

**Results**

**STABILITY OF HCY-THIOLACTONE IN BLOOD, PLASMA, AND URINE**

Hcy-thiolactone is inherently unstable in biological fluids at physiologic temperature (18–20, 28, 29). For example,
the half-life of Hcy-thiolactone in human serum at 37 °C is 0.5–1.5 h (29). To examine the stability of Hcy-thiolactone at lower temperatures, samples of EDTA-blood and EDTA-plasma were supplemented with Hcy-thiolactone and kept for up to 24 h at 4 or 23 °C, and the remaining Hcy-thiolactone was assayed. EDTA was used as an anticoagulant to inactivate calcium-dependent serum Hcy-thiolactonase activity (28, 29). We observed no significant losses of Hcy-thiolactone in EDTA-treated samples kept at 4 °C for up to 1 h. After 5 h at 4 °C, 87.8 (3.8)% and 72.4 (1.4)% of the Hcy-thiolactone originally present in plasma and blood, respectively, was detected, and after 5 h at 23 °C, only 43.9% and 34.4%, respectively, of the Hcy-thiolactone originally present in plasma and blood was detected.

Because most urine samples are slightly acidic (pH <7.0) and almost protein-free (compared with plasma), Hcy-thiolactone is expected to be much more stable in urine than in plasma. Indeed, we observed no significant losses of Hcy-thiolactone in urine samples (pH 5.2–6.3) kept for up to 24 h at 4 °C or 9 h at 23 °C, whereas ~20% was lost after 24 h at 23 °C (Table 1). The stability of Hcy-thiolactone in urine was similar to its stability in water, phosphate buffer (pH 6.6), or phosphate-buffered saline (Table 1), suggesting that the components of urine do not appreciably accelerate the hydrolysis of Hcy-thiolactone. In a urine sample with a pH of 7.3, there was essentially no loss of Hcy-thiolactone after 9 h of storage at 4 °C, whereas ~30% was lost after 24 h at 4 °C. However, when the pH 7.3 urine sample was kept at 23 °C for 9 and 24 h, 40% and 70% of the original Hcy-thiolactone, respectively, was lost (not shown).

These data indicate that the stability of Hcy-thiolactone in blood collected into EDTA-containing tubes and processed at 4 °C immediately after collection to obtain plasma is satisfactory for reliable assays. Urinary Hcy-thiolactone is much more stable and can be reliably assayed, even in samples kept at room temperature for a few hours.

**Hcy-thiolactone and tHcy in urine and plasma**

Whether Hcy-thiolactone is present in urine was not previously known. We found Hcy-thiolactone in all human urine samples analyzed (Table 2). Urinary Hcy-thiolactone concentrations ranged from 11.0 to 473.7 nmol/L (median, 144.8 nmol/L). Urinary tHcy concentrations were 0.4–5.3 μmol/L (median, 2.3 μmol/L). Urinary Hcy-thiolactone represented 2.5–28.3% (median, 6.0%) of urinary tHcy.

Plasma Hcy-thiolactone concentrations ranged from <0.1 to 22.6 nmol/L (median, 0.56 nmol/L; Table 2). Plasma tHcy concentrations ranged from 3.5 to 18.6 μmol/L (median, 12.2 μmol/L). In plasma, Hcy-thiolactone represented <0.001% to 0.29% (median, 0.01%) of plasma tHcy (Table 2).

**Lack of interconversion of Hcy and Hcy-thiolactone in urine**

Hcy-thiolactone and Hcy can be interconverted by enzymatic and/or nonenzymatic reactions in serum or plasma (5–7, 18–20, 28, 29, 34). To examine whether any of the possible interconversions occur in urine, we supplemented urine samples with [35S]Hcy or [35S]Hcy-thiolactone, incubated the samples at 37 °C for 2–6 h, and analyzed them by thin-layer chromatography. We did not observe any formation of [35S]Hcy-thiolactone in urine samples supplemented with [35S]Hcy. In urine samples supplemented with [35S]Hcy-thiolactone, we observed slow hydrolysis of [35S]Hcy-thiolactone to [35S]Hcy, with 20–40% [35S]Hcy-thiolactone hydrolyzed after 6 h. These observations indicate that interconversions do not contribute significantly to urinary Hcy-thiolactone or tHcy.

<table>
<thead>
<tr>
<th>Sample</th>
<th>9 h at 4 °C</th>
<th>24 h at 4 °C</th>
<th>9 h at 23 °C</th>
<th>24 h at 23 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine (n = 10)</td>
<td>96.8 (9.0)</td>
<td>90.6 (7.7)</td>
<td>88.6 (12.9)</td>
<td>80.8 (12.1)</td>
</tr>
<tr>
<td>Phosphate buffer, pH 6.6 (n = 6)</td>
<td>89.2 (0.4)</td>
<td>77.4 (1.8)</td>
<td>89.4 (4.2)</td>
<td></td>
</tr>
<tr>
<td>Water (n = 6)</td>
<td>86.3 (2.1)</td>
<td>77.4 (1.8)</td>
<td>89.4 (4.2)</td>
<td></td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>103.2 (3.4)²</td>
<td>99.9³</td>
<td>93.2 (2.1)³</td>
<td>89.1²</td>
</tr>
</tbody>
</table>

¹ Samples were supplemented with 10–500 nmol/L Hcy-thiolactone, incubated under the specified conditions, and assayed for the remaining Hcy-thiolactone.
² n = 3.
³ n = 1.

**Table 1. Mean (SD) stability of Hcy-thiolactone in urine.**

**Table 2. Hcy-thiolactone and tHcy concentrations in human urine and plasma.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Urine (n = 19)</th>
<th>Plasma (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hcy-thiolactone, nmol/L</td>
<td>168 (127)</td>
<td>4.1 (6.7)</td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>2.5 (1.6)</td>
<td>10.9 (4.6)</td>
</tr>
<tr>
<td>Hcy-thiolactone/tHcy ratio, %</td>
<td>7.7 (7.0)</td>
<td>0.03 (0.04)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean (SD)</th>
<th>Median</th>
<th>Range</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.0–473.7</td>
<td>144.8</td>
<td>1.7–28.3</td>
<td>0.4–5.3</td>
<td>12.2</td>
<td>3.5–18.6</td>
</tr>
<tr>
<td>0.001 to 0.29%</td>
<td>0.01</td>
<td>&lt;0.001 to 0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Thus, the presence of Hcy-thiolactone and Hcy in urine is attributable to elimination or excretion from the blood.

**RELATIONSHIPS BETWEEN HCY-THIOLACTONE AND tHCY IN URINE AND PLASMA**

The individual values for urinary Hcy-thiolactone and the corresponding urinary tHcy values are plotted in Fig. 2A. A similar plot showing the relationship between plasma Hcy-thiolactone and tHcy is shown in Fig. 2B. There was no correlation between Hcy-thiolactone and tHcy concentrations in urine, whereas there was a weak but significant correlation between Hcy-thiolactone and tHcy in plasma ($r = 0.51; P = 0.02$; Fig. 2B). For the five individuals for whom both plasma and urine samples were available (Table 4), there was a marginally significant correlation between urinary and plasma Hcy-thiolactone concentrations ($r = 0.82$) and no correlation between urinary Hcy-thiolactone and plasma tHcy ($r = 0.16$).

**DAILY URINARY EXCRETION OF HCY-THIOLACTONE**

To determine its daily flux, we monitored Hcy-thiolactone in urine samples from two volunteers for a period of 3 days. The amount of urinary Hcy-thiolactone varied from 9% to 27% (volunteer 1) and 3% to 13.8% (volunteer 2) of urinary tHcy, depending on the time of the day (not shown).

The mean (SD) daily Hcy-thiolactone excretion was 286 (16) nmol for volunteer 1 and 415 (15) nmol for volunteer 2. These amounts of excreted Hcy-thiolactone represented 17.8% and 7.5% of daily tHcy excretion for volunteers 1 and 2, respectively (Table 3).

**CORRELATION OF URINARY HCY-THIOLACTONE WITH pH**

The pH values of fresh morning urine samples varied from 5.2 to 7.3. Such variations in pH affect the ionization status of the amino group of Hcy-thiolactone, which has a $pK_a$ of 7.1 (30). To determine whether ionization may affect urinary excretion of Hcy-thiolactone, we examined the relationship between urinary Hcy-thiolactone concentrations and pH. As shown in Fig. 3A, there was a significant negative correlation ($r = -0.63; P < 0.01$) between urinary Hcy-thiolactone and pH, suggesting a pH-dependent mechanism of excretion. In contrast, there was no correlation between tHcy and pH in urine (Fig. 3B), suggesting a different, pH-independent mechanism of excretion.

**RELATIVE CLEARANCES OF HCY-THIOLACTONE AND tHCY**

To determine how efficiently Hcy-thiolactone is excreted, we calculated relative clearances of Hcy-thiolactone for five individuals. To allow for age-, gender-, and health-dependent differences in glomerular filtration rates, we calculated Hcy-thiolactone clearances relative to creatinine clearances. As shown in Table 4, the relative clearances of Hcy-thiolactone were high but exhibited interindividual variability from 0.21 to 6.96, nevertheless indicating very efficient elimination by the kidney. In contrast, the relative clearances of tHcy were very low and exhibited lower variability from 0.001 to 0.003 (Table 4), indicating insignificant elimination by the kidney, consistent with published data (35, 36).

Fig. 2. Relationships between Hcy-thiolactone and tHcy in human urine (A) and plasma (B). Spearman correlation coefficients are shown.

<table>
<thead>
<tr>
<th>Table 3. Mean (SD) daily urinary excretion (n = 6) of Hcy-thiolactone and tHcy in two volunteers. a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hcy-thiolactone, nmol/day</td>
</tr>
<tr>
<td>tHcy, nmol/day</td>
</tr>
<tr>
<td>Hcy-thiolactone/tHcy ratio, %</td>
</tr>
</tbody>
</table>

a Mean (SD) urine flows were 1.417 (0.053) and 1.133 (0.202) L/day for volunteers 1 and 2, respectively.
**Discussion**

Hcy-thiolactone is a highly reactive metabolite of Hcy that has been implicated in cardiovascular disease (5–8, 18–22, 26, 28, 29). Because generally applicable analytical methods for Hcy-thiolactone have been developed only recently [Refs. (8, 21, 27, 33); this work], metabolism of Hcy-thiolactone in the human body has remained virtually unexplored. Findings that the ratio of Hcy-thiolactone to tHcy in human plasma is very low [Refs. (7, 8); this work], much lower than the ratios predicted from human tissue culture studies (18, 19), suggested the existence of an unaccounted for pool of Hcy-thiolactone in the human body. The present study accounts for the “missing” Hcy-thiolactone by demonstrating that a previously unknown pool of Hcy-thiolactone exists in human urine. The urinary Hcy-thiolactone pool is ~100-fold larger than the plasma Hcy-thiolactone pool.

Hcy-thiolactone has been detected in human plasma previously. Using HPLC with a reversed-phase C18 column combined with ultraviolet detection, which had an on-column detection limit of 5 pmol, Jakubowski (8) found a median Hcy-thiolactone of 35 nmol/L (n = 6), which represented 0.8% of plasma tHcy in the six individuals studied. Using a gas chromatography–mass spectrometry method with a detection limit of 1.7 nmol/L, Daveshvar et al. (27) found a median Hcy-thiolactone 21.5 nmol/L (n = 2), which represented 0.2% of plasma tHcy in the two individuals studied. The most likely reason for an apparent discrepancy between the Hcy-thiolactone concentrations measured in previous studies and those found in the present study, in which the mean plasma Hcy-thiolactone was 0.56 nmol/L and represented 0.04% of tHcy in 20 individuals (Table 2), is the very limited number of samples analyzed in previous studies.

We found that Hcy-thiolactone represents a much larger fraction of tHcy in urine (2.5–28%) than in plasma (<0.002% to 0.29%; Table 2). The weak correlation between Hcy-thiolactone and tHcy in plasma and a lack of correlation in urine (Fig. 2) suggest that Hcy, a metabolic precursor of Hcy-thiolactone, is not a major determinant of Hcy-thiolactone concentrations in humans. Apparently, other determinants of Hcy-thiolactone concentrations, such as folic acid, methionine (19), and the Hcy-thiolactonase activity of HDL (19, 28, 29), may also be important. In addition, because Hcy-thiolactone can be synthesized by leucyl- and isoleucyl-tRNA synthetases, in addition to methionyl-tRNA synthetase, leucine and isoleucine concentrations would also affect Hcy-thiolactone concentrations (5, 7).

In the present study, we found that the amounts of

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**Table 4. Mean (SD) concentrations and relative clearances of Hcy-thiolactone and tHcy in five volunteers.**

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Urine</th>
<th>Plasm</th>
<th>Relative clearance*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hcy-thiolactone, nmol/L</td>
<td>tHcy, μmol/L</td>
<td>Creatinine, nmol/L</td>
</tr>
<tr>
<td>1</td>
<td>82 (2)</td>
<td>1.4 (0.03)</td>
<td>7.1 (0.1)</td>
</tr>
<tr>
<td>2</td>
<td>389 (34)</td>
<td>4.4 (1.2)</td>
<td>27.4 (0.5)</td>
</tr>
<tr>
<td>3</td>
<td>280 (44)</td>
<td>1.3 (0.2)</td>
<td>9.3 (0.3)</td>
</tr>
<tr>
<td>4</td>
<td>95 (27)</td>
<td>2.3 (0.3)</td>
<td>12.6 (0.6)</td>
</tr>
<tr>
<td>5</td>
<td>183 (15)</td>
<td>1.4 (0.2)</td>
<td>9.0 (0.5)</td>
</tr>
</tbody>
</table>

* Calculated relative to creatinine (Cr) using the formulas: (Hcy-thiolactoneurine/Hcy-thiolactoneplasma)/(Crurine/Crplasma) and (tHcyurine/tHcyplasma)/(Crurine/Crplasma). Creatinine was assayed by the Jaffe reaction method as described by Heinegaard and Tiderstrom (39).

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Fig. 3. Urinary concentrations of Hcy-thiolactone (A), but not tHcy (B), are negatively correlated with urinary pH. Spearman correlation coefficients are shown.
Hcy-thiolactone and tHcy eliminated by urinary excretion were 286–415 nmol/day and 1.6–5.5 μmol/day, respectively (Table 3). This urinary tHcy excretion rate is consistent with previous findings of other investigators (35, 36). Calculations based on a normal glomerular filtration rate of 180 L/day and a free plasma Hcy concentration of 3 μmol/L indicated that 99% of filtered Hcy is reabsorbed (35). A similar calculation for Hcy-thiolactone [0.12–2.4 nmol/mL in plasma (Table 4) and 286–415 nmol/day eliminated with urine (Table 3)] indicated that only 0.4–3.8% is reabsorbed and that >95% of filtered Hcy-thiolactone was excreted in the five individuals examined in the present study. Taken together, these observations suggest that Hcy-thiolactone is efficiently eliminated by urinary excretion, which is typical for the waste products of normal human metabolism. These observations are consistent with a view that Hcy-thiolactone is most likely a toxic metabolite in humans. Although it contributes very little to the daily flux of tHcy in a healthy individual [estimated at 1.3 mmol (36)], renal excretion does remove a large fraction of Hcy-thiolactone, which would otherwise modify proteins.

Because modification by Hcy-thiolactone is detrimental to protein function (5, 6, 20, 22) and could cause cell and tissue damage (5, 23–26), protective mechanisms that detoxify Hcy-thiolactone or prevent its formation have evolved. For example, removal of Hcy by transmethylation to methionine and transulfuration to cysteine inside cells and by the formation of S-Hcy-albumin (20, 28, 31, 32, 34, 37) in the plasma effectively prevents its conversion into Hcy-thiolactone (18, 19).

In addition to elimination by excretion by the kidney, as described in the present work, Hcy-thiolactone is also detoxified by two other mechanisms in the blood: (a) enzymatic hydrolysis by Hcy-thiolactonase activity of the PON1 protein carried on HDL (28, 29); and (b) nonenzymatic reactions with protein lysine residues, which have a half-life of ~1 h (20, 28). Clearance of Hcy-thiolactone can also take place in other organs. However, because there are no data on in vivo metabolic clearance of Hcy-thiolactone, the contribution of urinary elimination to total Hcy-thiolactone clearance cannot be estimated.

A possible mechanism facilitating the accumulation of Hcy-thiolactone in the urine may involve a gain of a positive charge, which could prevent reabsorption by the renal tubules. Hcy-thiolactone is chiefly neutral in the blood (pH 7.4), whereas in the urine (pH ~6), it is positively charged (30). Our finding that urinary Hcy-thiolactone is negatively correlated with pH (Fig. 3A) suggests that a neutral form of Hcy-thiolactone is excreted in the urine, whereas a positively charged form of Hcy-thiolactone is not reabsorbed in the tubules. A similar mechanism operates during renal elimination of other compounds that have pK_a values in the range of pH values observed in human urine, such as the drug pseudoephedrine (38).

In conclusion, we show that a sizeable urinary pool of Hcy-thiolactone exists in the human body and suggest that urinary excretion may be an important mechanism by which Hcy-thiolactone is eliminated. We also show that Hcy-thiolactone can contribute significantly to tHcy pools, particularly in urine. Our findings and the observations of other investigators suggesting that Hcy-thiolactone has adverse effects on physiologic function underscore the importance of examining urinary and plasma Hcy-thiolactone, in addition to tHcy, in the context of human disease.

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References
15. Mercie P, Garnier O, Lacostee L, Renard M, Closse C, Durrieu F,


