Measurement of Homocysteine and Other Aminothiols in Plasma: Advantages of Using Tris(2-carboxyethyl)phosphine as Reductant Compared with Tri-n-butylphosphine

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Background: Aminothiols have been implicated in the pathogenesis of arteriosclerosis, and reliable methods are needed to determine their concentrations in body fluids. We present a comparison of two analytical methods and focus on the reduction of low-molecular weight and protein-mixed disulfides of homocysteine, cysteine, cysteinyl-glycine, and glutathione.

Methods: The plasma total aminothiol profile was determined by HPLC with fluorescence detection after derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. Disulfides and protein-bound aminothiols were reduced by either tri-n-butylphosphine (the TBP method) or tris(2-carboxyethyl)phosphine (the TCEP method); the effects of temperature, time of reduction, and concentration of reductants were evaluated.

Results: The intraassay imprecision (CV) was <3% for all aminothiols using both methods. The interassay CVs for total cysteine (tCys), total cysteinyl-glycine (tCys-Gly), and total homocysteine (tHcy) were <4% and <8% for the TCEP and TBP methods, respectively, whereas for total glutathione (tGSH) the interassay CV was >12% for both methods. Deming regression and Bland–Altman difference plots showed positive biases for total aminothiol concentrations determined by the TCEP method relative to the TBP method. The mean proportional biases were 65%, 27%, 6%, and 60% for tCys, tCys-Gly, tHcy, and tGSH, respectively. The calculated concentrations of total aminothiols by the TCEP method were less influenced by changes in temperature and concentration of reducing agent or by calibrator matrix.

Conclusions: The agreement between the TCEP and TBP methods was considerably lower for the determination of tCys, tCys-Gly, and tGSH than for tHcy. For total-aminothiol determination, the TCEP method yields better reproducibility and is more robust than the TBP method.

Altered metabolism of aminothiols has been implicated in human pathology (1). In numerous studies, total Hcy (tHcy) concentrations were found to be consistently increased in patients with arteriosclerosis (2), renal failure (3), complicated pregnancies (4), and other diseases. In contrast, analysis of cysteine and glutathione metabolism in these conditions was reported only rarely (5–7). Because homocysteine exhibits prooxidative properties and glutathione antioxidative, and because there is extensive interconversion between these metabolites, their simultaneous analysis in biological samples is necessary to examine their role in human disease.

A frequently used method for total plasma aminothiol measurement is reversed-phase HPLC with fluorescent detection after derivatization of plasma aminothiols with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) (8–11). Plasma aminothiols exist as free reduced and oxidized compounds or as protein-bound ones. The mechanisms of the formation of albumin-bound aminothiols have been published recently (12, 13).

One of the most critical steps in the sample-processing procedure is the reduction of disulfide bonds before derivatization (14). Trialkylphosphines represent power-
ful reductants, which in aqueous solutions stoichiometrically and irreversibly reduce disulfides and are nonreactive toward many other functional groups (15):

\[ R_3P + RSSR + H_2O \rightarrow R_3P=O + 2 RSH \]

The rate-determining step of the reaction is the attack of the phosphine nucleophile on the disulfide bond (15). Among the trialkylphosphines widely used as reducing agents is tri-n-butylphosphine (TBP), despite its irritating odor and the use of toxic dimethylformamide as a solvent. In 1997, Gilfix et al. (16) introduced a novel water-soluble reductant for determination of tHcy: tris(2-carboxethyl)phosphate (TCEP). Comparisons of the method that uses TCEP as a reductant (the TCEP method) with the method that uses TBP (the TBP method) were published (17, 18), but the comparisons were focused solely on tHcy determinations. In the present study, we examine the use of the two reductants under different conditions and assess the suitability of the two methods for use in the study of aminothiol metabolism.

**Materials and Methods**

**REAGENTS**

All chemicals were of analytical or HPLC grade. l-Homocysteine, d,l-homocysteine, cysteinyl-glycine, glutathione (reduced form), N-(2-mercaptopropionyl)-glycine, TCEP, TBP, N,N-dimethylformamide, sodium tetraborate decahydrate, and EDTA were purchased from Sigma. l-Cysteine, SBD-F, acetonitrile (gradient grade), phosphoric acid, trichloroacetic acid, and phosphate-buffered saline (PBS; pH 7.4) were purchased from Fluka.

**PLASMA SAMPLES**

All plasma samples used in this study were aliquots of clinical material remaining after routine tHcy determination. For preparation of calibration samples, plasma pools obtained from fasting controls were used. Validation studies used pools prepared from plasma samples collected before and after a methionine loading test. Samples analyzed in the method comparison were obtained from participants before and after methionine loading, and from patients with homocystinuria.

The plasma samples were obtained by a standardized procedure. Blood was collected by venipuncture into EDTA tubes and cooled immediately in ice-water. Plasma was separated by centrifugation at 2000g for 15 min at 4 °C within 30 min of collection, and samples were immediately stored at −80 °C until analysis.

**SAMPLE PREPARATION**

The TBP method has been adapted from the method originally published by Araki and Sako (8); the TCEP method was a modification of the method of Ubbink and Vermaak (10) published by Gilfix et al. (16). Briefly, 100 μL of plasma sample was mixed with 75 μL of water and 25 μL of internal standard [IS; N-(2-mercaptopropionyl)-glycine, 16 mg/L] and incubated with either (a) 25 μL of TCEP (120 g in 1 L of PBS, pH 7.4) for 30 min at room temperature (24 ± 2 °C) or (b) 25 μL of 100 mL/L TBP in dimethylformamide for 30 min at 4 °C to reduce the disulfides and release protein-bound thiols. Deproteinization was achieved by the addition of 100 μL of 100 g/L trichloroacetic acid containing 1 mmol/L EDTA. Precipitated proteins were removed by centrifugation at 15 000g for 3 min, and 25 μL of supernatant was mixed with 100 μL of derivatization solution containing 70 μL of 0.125 mol/L borate–4 mmol/L EDTA (pH 9.5) and 30 μL of 1 g/L SBD-F in the borate-EDTA buffer. The sample was then incubated for 30 min at 60 °C in the dark. After derivatization, the samples were cooled on ice and protected from light until injection onto the column.

**APPARATUS AND CHROMATOGRAPHIC CONDITIONS**

HPLC analyses were performed on a Shimadzu LC-10A system consisting of a LC-10AT pump with a FCV-10AL low-pressure gradient flow control valve, a SIL-10AXL sample injector, a RF-10AXL fluorescence detector, and a CLASS-LC10 Workstation System. Aminothiols were separated on a Watrex 100 × 3.2 mm (i.d.) column (Watrex Prague) packed with Nucleosil 100-3 C18 (3 μm) particles. The linear gradient elution ran from 80% solvent A (50 mmol/L phosphate buffer, pH adjusted to 2.0 with o-phosphoric acid) to 70% solvent B (50 mmol/L phosphate buffer, pH 2.0, containing 300 mL/L acetonitrile) in 5 min at a flow rate of 0.7 mL/min. After elution of retained compounds with 70% B for 2 min, the column was reconditioned with 80% A for 5 min. Total analysis time, including equilibration, was 12 min. The analyses were performed at room temperature. More than 300 samples were analyzed on the column without loss of resolution. Typically, 10 μL of sample was injected onto the column. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm.

**VALIDATION**

Calibration samples covering a range of 0–400 μmol/L for total cysteine (tCys), 0–150 μmol/L for total cysteinyl-glycine (tCys-Gly) and tHcy, and 0–50 μmol/L for total glutathione (tGSH) were prepared by adding known aminothiol concentrations to eight plasma aliquots and eight PBS aliquots. The calibration curves were obtained by least-squares linear regression analysis of the aminothiol/IS peak area ratio vs the aminothiol concentration added to the calibration sample. The slopes of the calibration curves obtained by analysis of plasma matrix calibrators were used to calculate the total plasma aminothiol concentrations. The PBS and plasma calibration slopes were compared to evaluate the matrix effects.

The intraassay (within-day) imprecision (CV) of the methods was established by replicate analyses (n = 10) of samples containing both normal and post-methionine load increased tHcy concentrations. The interassay (be-
between-day) CV was established by replicate analyses of
the same samples on 10 separate days over the course of
10 weeks.

METHODOLOGY COMPARISONS
The TBP and TCEP methods were compared on 45
samples from clinical practice, which covered a wide
range of tHcy and tCys concentrations. On 9 separate
days within 5 weeks, aminothiol concentrations were
measured in plasma samples in side-by-side assays using
both methods.

Because none of the evaluated methods could be
considered a reference procedure, we used Deming re-
gression to assess agreement between the TBP and TCEP
methods. The regression was carried out based on the
assumption that the random errors of both methods were
proportional to the measured aminothiol concentration,
i.e., the CVs of both methods were constant over the
aminothiol measurement range. The CV ratio of the two
methods was set to 1:1 for regression calculations
(19).

Bland–Altman plots of the difference between the meth-
ods vs their mean were also obtained. Limits of agreement
were assessed by calculating the central 0.95 interval
(mean of the differences ± 2 SD). Using the SE, we
computed 95% confidence intervals (mean of the differ-
ences ± 2 SE) to estimate possible systematic bias. Subse-
quently, the mean difference and the limits of agreement
were calculated on the log-transformed data as described
by Bland and Altman (20). Antilogs of the mean differ-
ences were calculated to assess the mean proportional
biases of the TCEP method with respect to the TBP
method. Antilogs of the limits of agreement were calcu-
lated to express the intervals (ranges of percentages) by
which 95% of the determinations measured by the TCEP
method were expected to differ from the TBP method.
Comparisons of peak areas and calibration slopes were
performed by the Student paired t-test, with P <0.05
regarded as statistically significant.

REDUCTION STEP
The plasma and calibration samples were processed un-
der different conditions to evaluate the reduction step.

The samples (n = 15) were incubated at 4°C or room
temperature (24 ± 2°C) with TBP or TCEP. The tested
concentrations of the reduction mixtures used for sample
processing (see “SAMPLE PREPARATION” above) were 50,
100, and 200 mL/L TBP in dimethylformamide and 60,
120, and 180 g/L TCEP in PBS. Three incubation times for
samples (n = 15) with reducing agents were examined: 15,
30, and 60 min.

Results

LINEARITY AND IMPRECISION
The analyses of calibration samples prepared in PBS or
plasma matrix produced linear calibration curves for all
aminothiols determined by both the TBP and TCEP meth-
ods (r² >0.99). The results of the intraassay (within-day)
and interassay (between-day) precision studies are sum-
mORIZED in Table 1. The intraassay CV of both methods
was 1.2–2.6% for all determined aminothiols (Table 1).
The interassay CVs for tCys, tCys-Gly, and tHcy deter-
mined by the TBP method (3.5–7.9%) were higher than the
corresponding CVs for the TCEP method (2.6–3.9%). The
highest CVs were observed for tGSH using both methods
(>12%).

COMPARISON OF TBP AND TCEP METHODS
The Deming regression correlations (Fig. 1) showed ap-
parent positive proportional biases for the TCEP method
determinations with respect to the TBP method. The
wider scatter of data points along the identity line and the
lower correlation coefficient for the comparison of tCys
determinations might be caused by the higher interassay
CV of the tCys determination using the TBP method. The
Bland–Altman difference plots are shown in Fig. 2. The
difference plots showed a wider scatter of difference data
points for tCys, tCys-Gly, and tGSH than for tHcy deter-
minations. The difference plots also revealed an apparent
relationship between the method difference and the ami-
nothiol concentrations: with increasing aminothiol con-
centrations, the difference between the methods also
increased. The data were therefore log-transformed, and
the mean difference and limits of agreement between the
methods were calculated on the log-transformed data,

Table 1. Results of precision studies.

<table>
<thead>
<tr>
<th></th>
<th>TBP method</th>
<th></th>
<th>TCEP method</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean, µmol/L</td>
<td>CV, %</td>
<td>Mean, µmol/L</td>
<td>CV, %</td>
</tr>
<tr>
<td></td>
<td>Intraassay</td>
<td>Interassay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCys</td>
<td>116.2</td>
<td>1.9</td>
<td>7.9</td>
<td>210.4</td>
</tr>
<tr>
<td>tCys-Gly</td>
<td>29.4</td>
<td>2.0</td>
<td>6.4</td>
<td>40.3</td>
</tr>
<tr>
<td>tHcy</td>
<td>8.2</td>
<td>1.7</td>
<td>4.9</td>
<td>8.9</td>
</tr>
<tr>
<td>tGSH</td>
<td>49.7</td>
<td>1.6</td>
<td>3.5</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td>2.6</td>
<td>17</td>
<td>9.2</td>
</tr>
</tbody>
</table>

* n = 10; the differences between the mean values of aminothiols determined by the TBP and TCEP methods are the result of method differences.

* Plasma samples with normal and increased concentrations of tHcy were obtained from individuals before and after the methionine loading test. Samples with
increased or decreased concentrations of other aminothiols were not available.
according to the method of Bland and Altman (20). The results of the method comparisons by Bland–Altman analysis are summarized in Table 2. The 95% confidence intervals (mean ± 2 SE) indicated an apparent positive bias for all aminothiols determined by the TCEP method. The mean proportional bias (antilog of the mean difference calculated from log-transformed data) was largest for tCys and tGSH determinations: 65.5% and 59.6%, respectively. The narrowest interval for the limits of agreement and the lowest mean proportional bias were observed for tHcy determinations.

We evaluated possible factors leading to differences between plasma aminothiol concentrations determined by the compared methods. The plasma aminothiol concentrations were calculated by comparing the aminothiol/IS peak area ratio with the slope of appropriate aminothiol calibration curve. Variations in the components used for quantitative calculations (i.e., aminothiol and IS peak areas, calibration slopes) were therefore evaluated to better understand the differences between methods.

Aminothiol peak areas (i.e., fluorescence intensities) were higher in plasma samples reduced with TCEP than when TBP was used as a reducing agent. We found, however, that the difference between the areas was not constant for all studied endogenous aminothiols. In our experiments, the peak areas obtained for tCys, tCys-Gly, tHcy, tGSH, and the IS were 77%, 36%, 34%, 56%, and 12% higher, respectively, with the TCEP method than with the TBP method.

We prepared two different sets of calibration samples by adding the reduced aminothiols to plasma or PBS. The resulting calibration slopes were compared to assess the

Fig. 1. Comparisons of the TBP and TCEP methods by Deming regression. Clinical samples (n = 45) were analyzed in side-by-side assays using the TCEP and TBP methods. The results, expressed in μmol/L, were analyzed by Deming regression. The insets show the resulting regression equations; SDs of the slope and intercept are indicated in parentheses.
effects of calibrator matrix on aminothiol determinations. The TBP method yielded significantly ($P<0.05$) higher aminothiol calibration slopes for tHcy, tCys-Gly, and tCys with plasma matrix calibrators than with PBS matrix calibration (Table 3). When we used TCEP as a reducing agent, however, the differences between calibration slopes in the plasma and PBS matrices were not statistically significant ($P>0.1$) for all aminothiols, with the exception of tCys. In the latter, we observed lower calibration slopes in the plasma matrix than in PBS. Thus, the matrix of the calibration sample did not significantly affect quantification when the samples were reduced with TCEP. On the other hand, the results of aminothiol determination were dependent on the matrix used for the calibration samples when TBP was the reductant.

Table 3 also shows the relatively small differences ($<13\%$) between the means of plasma calibration slopes from which the aminothiol concentrations for the TBP and TCEP method comparisons were calculated. The mean proportional biases between the TBP and TCEP determinations of tCys, tCys-Gly, and tGSH (Table 2) therefore originated in the differences between the aminothiol peak areas in the clinical plasma samples (see above) rather than from the differences between calibration slopes.

**Table 2. Results of the TBP-vs-TCEP method comparisons by Bland–Altman analysis.**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean (SD)$^b$</th>
<th>95% limit of agreement$^b$</th>
<th>95% CI$^c$ of the mean</th>
<th>Mean proportional bias,$^d$ %</th>
<th>Upper limit of agreement,$^d$ %</th>
<th>Lower limit of agreement,$^d$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>tCys</td>
<td>78.7 (27.2)</td>
<td>24.3 to 133.0</td>
<td>70.4–86.9</td>
<td>65.5</td>
<td>25.1</td>
<td>118.7</td>
</tr>
<tr>
<td>tCys-Gly</td>
<td>6.2 (3.4)</td>
<td>–0.6 to 13.0</td>
<td>5.2–7.2</td>
<td>27.2</td>
<td>1.9</td>
<td>58.8</td>
</tr>
<tr>
<td>tHcy</td>
<td>2.0 (3.5)</td>
<td>–5.1 to 9.0</td>
<td>0.9–3.0</td>
<td>6.3</td>
<td>–10.0</td>
<td>25.7</td>
</tr>
<tr>
<td>tGSH</td>
<td>2.2 (1.4)</td>
<td>–0.6 to 5.0</td>
<td>1.8–2.6</td>
<td>59.6</td>
<td>22.0</td>
<td>108.8</td>
</tr>
</tbody>
</table>

$^a$ $n=45$ (5 samples on 9 separate days).

$^b$ Values in μmol/L; 95% limits of agreement were calculated as mean ± 2 SD.

$^c$ CI, confidence interval; 95% CIs were calculated as mean ± 2 SE.

$^d$ Calculated from log-transformed data (see text).
**Reduction Step Evaluation**

The compared methods differed in the temperature of the reduction step. We therefore evaluated the effect of temperature on reduction efficiency with both reductants. Two other factors that may influence the reduction were also examined: time of reduction and concentration of reducing agent. We evaluated the influence of these factors on the aminothiol peak areas in plasma samples, the peak area of the IS, and the calibration slopes.

**Temperature.** The effect of temperature on the aminothiol peak areas in plasma samples reduced with TBP was significant for all determined aminothiols. The areas of the tCys, tCys-Gly, and tGSH peaks were higher, on average, by 20%, 10%, and 40%, respectively, when the plasma samples were reduced with TBP at room temperature compared with reduction at 4 °C. In contrast, the areas for tHcy were 10% lower, on average, when reduction with TBP was performed at room temperature. The IS areas were not significantly influenced by the temperature of reduction. The calibration slopes for tCys and tCys-Gly were significantly lower when calibration samples were reduced with TBP at room temperature rather than at 4 °C (Table 3).

Taken together, reduction with TBP at room temperature yielded higher areas for the tCys and tCys-Gly peaks in plasma samples, whereas in calibration samples the areas were lower than they were at 4 °C. This demonstrates a major difference in the properties of the calibration samples to which aminothiols had been added and real plasma samples during the reduction process. The effects of temperature on the aminothiol peak areas as well as on the calibration slopes led to significant differences between the calculated aminothiol concentrations in plasma samples reduced with TBP at 4 °C or room temperature. The concentrations of tCys, tCys-Gly, and tGSH determined after reduction with TBP at room temperature were higher, on average, by 65%, 32%, and 37%, respectively, than when reduction was performed at 4 °C. The calculated concentrations of tHcy were not significantly affected by the changes in temperature of reduction.

With the TCEP method, the differences between aminothiol peak areas in samples reduced at room temperature or 4 °C were <10%. Furthermore, the calibration slopes were less influenced by the temperature of reduction (Table 3), and the differences between concentrations determined after TCEP reduction at room temperature or 4 °C were <10% for all aminothiols.

**Concentration of reductant.** It was proposed previously that a reaction between the reductant and the fluorogenic reagent may have been responsible for the decrease in the fluorescence intensities of the determined aminothiols (14). We tested whether this effect was dependent on the nature and concentration of the reductant in different matrices.

The decreases in the tCys, tCys-Gly, and tHcy peak areas in plasma matrix samples were 10–25% when the concentration of TBP increased from 100 to 200 mL/L. Concentrations of TBP between 50 and 100 mL/L had no significant effect on the aminothiol peak areas in plasma samples. In PBS calibration samples, the decrease in the peak area in the tested TBP concentration range was even higher (25–30% decreases in tCys, tCys-Gly, and tHcy peak areas) than in plasma samples. In our experiments, the effects of changes in TBP concentration on the peak areas and calibration slopes produced nonsystematic variations of calculated aminothiol concentrations in the range of 10–20%.

The TCEP concentration also affected the aminothiol peak areas in PBS calibration samples. An increase of the TCEP concentration from 60 to 180 g/L, however, produced a decrease of <10% of the peak area. The PBS matrix calibration slopes were therefore less affected by changes in the TCEP concentration than when different concentrations of TBP were tested. The aminothiol peak areas in plasma samples, plasma matrix calibration slopes, and the calculated aminothiol concentrations were

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### Table 3. Calibration slopes for the TBP and TCEP methods: Effects of calibrator matrix and temperature of reduction.

<table>
<thead>
<tr>
<th>Aminothiol</th>
<th>Plasma (Room temperature 4 °C)</th>
<th>PBS (Room temperature 4 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCEP reduction</strong> (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCys</td>
<td>0.0077 ± 0.0006</td>
<td>0.0079 (0.0005)</td>
</tr>
<tr>
<td>tCys-Gly</td>
<td>0.0380 ± 0.0021</td>
<td>0.0393 (0.0025)</td>
</tr>
<tr>
<td>tHcy</td>
<td>0.0334 ± 0.0016</td>
<td>0.0342 (0.0009)</td>
</tr>
<tr>
<td>tGSH</td>
<td>0.0207 ± 0.0016</td>
<td>0.0207 (0.0011)</td>
</tr>
<tr>
<td><strong>TBP reduction</strong> (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCys</td>
<td>0.0058 ± 0.0006</td>
<td>0.0081 ± 0.0009</td>
</tr>
<tr>
<td>tCys-Gly</td>
<td>0.0334 ± 0.0024</td>
<td>0.0393 ± 0.0036</td>
</tr>
<tr>
<td>tHcy</td>
<td>0.0282 ± 0.0013</td>
<td>0.0294 ± 0.0016</td>
</tr>
<tr>
<td>tGSH</td>
<td>0.0205 ± 0.0017</td>
<td>0.0213 ± 0.0018</td>
</tr>
</tbody>
</table>

*Mean of slopes used for calculation of aminothiol concentrations in method comparisons (Figs. 1 and 2).*
not influenced by the tested concentrations of the TCEP reductant.

Time of reduction. Changes in reduction time did not influence plasma and PBS calibration slopes. The peak areas in plasma samples reduced with TBP for 15 min were, however, substantially lower than the aminothiol peak areas in samples reduced for 30 or 60 min. None of the tested reduction times produced changes in aminothiol peak areas or calculated aminothiol concentrations when plasma samples were reduced with TCEP.

Discussion

The reduction of the disulfide bonds between aminothiols and plasma proteins and the reduction of aminothiol disulfides probably represent one of the most delicate steps in the determination of total plasma aminothiols. The efficiency of the reduction step may significantly influence the performance of the entire method; this also applies to highly sensitive and selective mass spectrometry, which is often chosen as the reference method in comparison studies.

The TCEP and TBP methods have been compared several times in the literature (16–18). These comparisons, however, always focused on the tHcy determination, and the results showed good agreement between the methods. Similarly, in our study we found acceptable agreement of calculated homocysteine concentrations when calibration was performed in plasma: the 35% higher peak areas for tHcy observed in plasma samples reduced with TCEP were compensated for, in the concentration calculations, by a higher calibration slope obtained with TCEP-reduced calibrators. We found significant differences, however, between concentrations of the other aminothiols (tCys, tCys-Gly, and tGSH) determined by the TBP and TCEP methods, the latter method yielding higher values (Figs. 1 and 2). Although higher does not necessarily mean better, we prefer the TCEP method for the following reasons: (a) the interassay CVs were lower for all aminothiols determined with the TCEP method; (b) the TCEP method was less sensitive to changes of reduction temperature and reductant concentration; (c) TCEP is soluble in water, whereas TBP must be dissolved in toxic dimethylformamide; and (d) TBP is volatile, flammable, corrosive, and has an unpleasant odor. Furthermore, TBP is a compound that is sensitive to humidity and air and must be stored under argon or nitrogen; improper storage conditions may cause decomposition of the reductant and thus a decrease in TBP concentration in the assay. As described in the Results, the concentration of TBP used in the assay may significantly influence the PBS calibration slope. These arguments should be sufficient to justify the choice of TCEP as a reductant rather than TBP.

Unfortunately, in the tGSH determinations, both the TBP and the TCEP methods have unacceptably high interassay CVs (17% and 13%, respectively). For this reason, we cannot recommend either the TBP or the TCEP method for plasma tGSH determination. Other authors, however, have adopted methods using TBP or TCEP as the reductant for analysis of plasma tGSH (21–23). The different chemical and physical properties of the disulfide bonds of protein-bound aminothiols (1) may explain the variable performance of both methods for individual aminothiols. The different polarities of both reductants may also influence their reactivities with protein-bound aminothiols.

Another controversial point is the use of proper calibrators for aminothiol determination. Because matrix effects cause differences between plasma and PBS calibration slopes (Table 3), plasma is often preferred as a matrix for calibration samples. It is also assumed that plasma calibrators will behave similarly to the real plasma samples in the assay (24). Some aminothiols, however, are not stable when added to a plasma matrix. For example, a 10% decrease in the added glutathione concentration (50 μmol/L) was observed when the plasma to which it had been added was incubated at room temperature for 30 min, probably because of enzymatic conversion to Cys-Gly by γ-glutamyltranspeptidase (data not shown). The limited stability of plasma glutathione might also contribute to the higher interassay CVs for tGSH determinations observed in our study. The possible conversions of aminothiols added to plasma matrix favors the use of PBS calibrators, especially with the TCEP method (where the matrix effects are less significant, as shown in Table 3).

In the preparation of homocysteine calibrators, homocysteine has often been preferred as a calibrator because of its chemical stability, the purity of commercially available calibrators, and the possibility of checking the reduction step (24, 25). In our study, we prepared plasma calibration samples by adding the reduced forms of aminothiols to the plasma aliquots. The reduced aminothiols were used for the following reasons: (a) the solubility of disulfide calibrators in water is low, and this complicated the preparation of concentrated calibrator; and (b) we have tested the ability of added aminothiols to form protein-bound disulfides. In agreement with the results of Ueland et al. (26), we found that plasma proteins have a limited capacity to bind exogenously added aminothiols (data not shown). The ratio between free reduced, oxidized, and protein-bound disulfides is, then, clearly different in real plasma and calibration samples to which aminothiol calibrators have been added. The performance of the reduction step may be significantly different when real plasma or plasma calibration samples are processed. This was demonstrated, for example, in the evaluation of the effects of temperature on TBP reduction, where a temperature increase produced an increase in peak areas for the plasma samples and a decrease in peak areas for the calibration samples.

A possible explanation for the decrease in peak areas can be an interference of the reductant with aminothiols during the sample preparation process; this effect may be manifested more in calibration samples reduced with TBP.
at room temperature than in physiologic plasma samples. In the latter, the reduction efficiency for protein-bound aminothiols may be improved with a temperature increase. Similar phenomena may be the cause of the above-mentioned matrix effects. In previous studies, the matrix effects were attributed to the presence of plasma proteins or other plasma species, which may have increased the fluorescent yield of the determined aminothiols (24, 27). In our study, we showed that the matrix effects are dependent on the type of reductant (Table 3), the reduction temperature, and the concentration of reductant. These results suggest that the matrix effect is probably caused by decreased fluorescence attributable to interference of the reductant with aminothiols, which may be manifested in PBS more than in the plasma matrix samples.

In conclusion, the agreement between the TCEP and TBP methods for aminothiol determination is too low to allow the methods to be used interchangeably. Because of its better reproducibility and robustness, we recommend the TCEP method for research and routine aminothiol determinations in human plasma.

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References