Thus the ideal sample volume to maximize FAEE detection was 799.740 for FAEE ethyl 16:0. Although the FAEEs were in less abundance in the 0.1-mL sample, they were still clearly detectable in a sample with a blood ethanol concentration at the lower legal limit for intoxication. Therefore, in the solvent-extraction step, 0.5 mL of serum and 4 mL of acetone are recommended.

To evaluate smaller serum sample sizes of 0.25 and 0.1 mL, a third series of experiments was performed. The samples in these studies had a blood alcohol concentration of 850 mg/L (18.5 mmol/L), which we purposely selected because it approximates the minimum blood alcohol concentration at which a person is considered legally intoxicated. The serum-to-acetone ratio of 2 mL of acetone (a ratio of 1:8) showed a lower FAEE abundance than in Ratio 3 with the same sample-to-acetone ratio of 1:8. This suggests the need for a minimum sample volume of 0.5 mL. However, even at 850 mg/L (18.5 mmol/L) blood alcohol, a sample size of 0.10 mL or possibly lower could be used for FAEE analysis.

In summary, the sensitivity for FAEE detection is improved by use of a smaller sample volume of 0.5 mL and a serum-to-acetone ratio of 1:8.

<table>
<thead>
<tr>
<th>FAEE</th>
<th>Ratio 1 (1 mL/4 mL relative to 1 mL/2 mL)</th>
<th>Ratio 2 (0.5 mL/2 mL relative to 1 mL/2 mL)</th>
<th>Ratio 3 (0.5 mL/4 mL relative to 1 mL/2 mL)</th>
<th>Ratio 4 (0.25 mL/2 mL relative to 0.5 mL/2 mL)</th>
<th>Ratio 5 (0.10 mL/2 mL relative to 0.25 mL/2 mL)</th>
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</thead>
<tbody>
<tr>
<td>E14:0</td>
<td>3.35</td>
<td>4.82</td>
<td>0.69</td>
<td>0.40</td>
<td>0.40</td>
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<tr>
<td>E16:0</td>
<td>5.19</td>
<td>3.38</td>
<td>2.92</td>
<td>0.59</td>
<td>0.55</td>
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<tr>
<td>E16:1</td>
<td>5.01</td>
<td>2.12</td>
<td>1.39</td>
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<tr>
<td>E17:0</td>
<td>7.27</td>
<td>7.21</td>
<td>8.26</td>
<td>0.71</td>
<td>0.34</td>
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<tr>
<td>E18:0</td>
<td>8.57</td>
<td>5.43</td>
<td>5.32</td>
<td>0.74</td>
<td>0.35</td>
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<tr>
<td>E18:1</td>
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<td>3.67</td>
<td>0.70</td>
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<tr>
<td>E18:2</td>
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<td>3.70</td>
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<tr>
<td>E20:4</td>
<td>4.35</td>
<td>2.11</td>
<td>3.22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For each ratio, such as 1 mL/4 mL, the numerator represents the volume of sample and the denominator represents the volume of solvent.

b E, ethyl.

c Internal standard.

References


Relationships among Plasma Homocysteine, Cysteine, and Albumin Concentrations: Potential Utility of Assessing the Cysteine/Homocysteine Ratio, Glen L. Hortin,* Patricia Sullivan, and Gyorgy Csako (Department of Laboratory Medicine, Warren Magnuson Clinical Center, NIH, Bethesda, MD 20892; * address correspondence to this author at: Department of Laboratory Medicine, NIH, Building 10, Room 2C-407, Bethesda, MD 20892-1508; fax 301-402-1885, e-mail ghorton@mail.cc.nih.gov)

McCully (1) initially observed that patients with extremely increased plasma concentrations of homocysteine (Hcy) attributable to homocystinuria have accelerated
Atherosclerosis. Subsequently, even moderate hyperhomocysteinemia became recognized as a risk factor for atherosclerosis and thrombosis (2–4), although the mechanism by which increased concentrations of Hcy produce these effects is uncertain. Hcy occurs in the circulation in multiple forms, including Hcy linked via disulfides to albumin (70%), as a mixed disulfide with Cys (25%), as a disulfide-linked dimer (5%), as the free reduced amino acid (5%), and as a thiolactone (trace) (5–6). It is not clear which of these components are physiologically active, and efforts to measure individual components have been technically challenging because of the interconversion of different forms during specimen processing. As a result, measurement of total Hcy (tHcy), including all forms except the trace amount of thiolactone, has become the standard clinical test (5–7).

The present study examined the relationships among concentrations of tHcy, plasma albumin, and tCys (tCys; includes protein-bound, disulfide-linked forms, and reduced). Considering that albumin and Cys serve as covalent carriers of most of the Hcy in circulation, these components may affect circulating tHcy and the physiological action of Hcy. Cys has the potential for multiple interactions with Hcy because Cys is not only a covalent carrier but also a competitor for binding sites on proteins, a potential competitor for uptake into cells, and a metabolite of Hcy via the transulfuration pathway (8, 9).

tHcy and tCys in EDTA plasma were analyzed simultaneously by HPLC after the reduction of plasma disulfides with tris(2-carboxyethyl)phosphine, precipitation of proteins with trichloroacetic acid, derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), and fluorescent detection as described previously (10) using cystamine as an internal standard. Performance characteristics of the assay have been described previously (10). HPLC analysis was performed on 151 unselected plasma specimens submitted to the laboratory for tHcy determination. Patients included 75 males and 76 females (median age, 54 years; <20 years, n = 2; 20–39 years, n = 30; 40–59 years, n = 72; 60–79 years, n = 42; ≥80 years, n = 4; the age of 1 subject was unknown). Albumin was measured for 142 specimens using a Hitachi 917 analyzer with bromcresol green reagent from Roche. Our nonparametric reference interval for albumin was 37–47 g/L.
use of specimens was approved by our Institutional Review Board.

We anticipated that an increased quantity of the major binding protein (albumin) might relate to increases in tHcy or tCys, but we found little evidence for this. Neither tHcy nor tCys correlated with albumin (range, 25–48 g/L) in plasma (Fig. 1, A and B). It may be that the binding capacity of plasma proteins for Hcy far exceeds normal plasma tHcy (8), and factors controlling the interchange between protein-linked and soluble forms of Hcy and Cys are not fully understood.

In this study, tHcy concentration was ~28-fold lower than tCys: mean tHcy concentration, 9.0 μmol/L; range, 4.4–17.7 μmol/L (most within reference interval); mean tCys concentration, 238 μmol/L; range, 141–368 μmol/L). There was a highly significant ($P <0.0001$) positive correlation between tHcy and tCys (Fig. 1C). This agrees with the observations of El-Khairy et al. (11) for subjects with tHcy concentrations <15 μmol/L. The observed linear relationship between tHcy and tCys is consistent with the proposition that part of the Cys is derived from Hcy metabolism via the transsulfuration pathway in an amount proportional to the Hcy concentration.

Subjects with tHcy <13 μmol/L, which is the upper reference limit assigned in our laboratory, had higher tCys/tHcy ratios (mean, 29; range, 17–40; n = 138) than subjects with tHcy concentrations ≥13 μmol/L (mean ratio, 20; range, 15–25; n = 13). There was an overall negative correlation of the tCys/tHcy ratio with tHcy concentration (Fig. 1D). The relationship had substantial curvature; therefore, it could not be represented well by a simple linear fit.

Individuals with homocystinuria attributable to defects in the transsulfuration pathway provide further evidence for the importance of this pathway in the relationship between tCys and tHcy; these individuals have low plasma tCys despite severe hyperhomocysteinemia (9). The relationship between tHcy and tCys appears to be more complex than a simple precursor–product relationship, however. The linear relationship between tHcy and tCys is reported to break down at tHcy >15 μmol/L, with tCys decreasing as tHcy increases above this concentration (11). In addition, slight decreases in tCys occur after methionine or Hcy loading at times when there is transient hyperhomocysteinemia (12, 13). In these situations, there would be a progressive decline in tCys/tHcy ratios as tHcy increases. Renal clearance of amino acids may have a role in lowering tCys concentrations because both tCys and tHcy are increased in patients with renal failure (14).

As shown here and in earlier reports (10,11), dual analysis of tHcy and tCys can be readily provided by a chromatographic method. Evaluation of the tCys/tHcy ratio potentially has several practical consequences. One consequence is that the tCys/tHcy ratio may reflect abnormalities in the function of the transsulfuration pathway. It is estimated that 1% of the population is heterozygous for deficiency of cystathionine-β-synthase, the enzyme for which homozygous deficiency leads to homocystinuria (15). tHcy is increased and tCys is decreased in heterozygous deficiency of this enzyme, and a low tCys/tHcy ratio assists in identifying heterozygotes (15). The other consequence is that the tCys/tHcy ratio may have physiologic significance in that some of the causes of hyperhomocysteinemia, such as homocystinuria (9) and renal failure (14), that are strongly associated with cardiovascular disease produce low tCys/tHcy ratios, and Cys may affect bioavailability of Hcy by serving as a carrier or competitor of Hcy. Further investigation is warranted to explore whether this ratio serves as an indicator of cardiovascular risk that supplements tHcy measurements. The third consequence is that low tCys/tHcy serves as an indicator of preanalytical errors. tCys decreases (10–20% over 1 day) if anticoagulated whole blood samples are left at room temperature, whereas tHcy increases (30–90%) (16, 17). Thus, unusually low tCys/tHcy ratios should trigger a review of whether there is a physiological explanation or a preanalytical problem such as delayed separation of plasma from blood cells and/or storage of whole blood at inappropriately high ambient temperature. Finally, evaluation of the tCys/tHcy ratio may serve as an internal quality check of each analysis. In the absence of other causes (see above), unexpectedly high or low tCys/tHcy ratios would identify samples with increased probability of analytical errors. For the 138 plasma specimens with tHcy <13 μmol/L, the nonparametric and parametric reference intervals for the tCys/tHcy ratio would be 19–42 and 17–40, respectively.

References
14. Mansoor MA, Guttorpsen AB, Fiskerstrand T, Refsum H, Ueland PM,
Fully Automated Nucleic Acid Extraction: MagNA Pure LC, Harald H. Kessler,1* Gerhard Mühlbauer,2 Evelyn Stelzl,1 Elisabeth Daighofer,1 Brigitte I. Santner,1 and Egon Marth1 (1 Institute of Hygiene, Karl-Franzens-University Graz, A-8010 Graz, Austria, and 2 Roche Diagnostics GmbH, A-1211 Wien, Austria; * address correspondence to this author at: Molecular Diagnostics Laboratory, Institute of Hygiene, KF-University Graz, Universitätsplatz 4, A-8010 Graz, Austria; fax 43-316-380-9649, e-mail harald.kessler@uni-graz.at)

Kinetic PCR analysis by real-time monitoring of DNA amplification was first described 8 years ago (1). Recently, the LightCycler™ instrument (Roche Molecular Biochemicals, Mannheim, Germany), which allows high-speed thermal cycling by use of air instead of thermal blocks and on-line real-time fluorescence monitoring, was introduced (2). Several reports have been published with regard to utilization of the LightCycler technology for detection of pathogens (3-11).

Real-time PCR has greatly decreased the amount of hands-on time needed to generate and detect amplification products. Before amplification, pathogen-specific DNA or RNA must be extracted from the specimen. This procedure, also called sample preparation, remains the most labor-intensive and time-consuming part of widely automated molecular assays and may be considered the major weakness in most molecular assays today (12, 13). Therefore, a fully automated sample preparation system is urgently needed for the routine diagnostic laboratory.

In this study, a fully automated specimen preparation instrument, the MagNA Pure LC™ (Roche) was evaluated. The new instrument was used for extraction of herpes simplex virus (HSV) DNA in combination with real-time PCR on the LightCycler instrument.

In the first experiment, the interassay variation and the detection limit were tested. The Second European Union Concerted Action HSV Proficiency Panel, which consists of 12 vials with different concentrations of HSV type 1 (HSV-1), strain MacIntyre (American Type Culture Collection), HSV type 2 (HSV-2), strain MS (American Type Culture Collection), varicella-zoster virus, and negative samples, was used. Samples were analyzed three times on different days. Results were compared with a molecular assay, which consisted of an in-house DNA extraction protocol and real-time PCR.

In the second experiment, intra-assay variation of the new molecular assay was tested. Plasma was collected from a patient without clinical presentations compatible with HSV infection. Aliquots were supplemented with dilutions of a culture supernatant of commercially available HSV-1-infected Vero cells (VR-260; American Type Culture Collection). Each dilution was analyzed eight times. Each assay contained five positive and three negative controls (water).

In the third experiment, precision and the influence of different sample materials were tested. Whole blood (3-mL EDTA tubes), serum, and plasma were collected from a patient without clinical presentations compatible with HSV infection. Aliquots were supplemented with a culture supernatant of HSV-1 as described above. Different sample volumes were analyzed three times with the new molecular assay. Each assay contained three negative controls (blank reagent and water). For experiments 2 and 3, each sample was processed independently through both the MagNA Pure LC and LightCycler stages.

The MagNA Pure LC is a benchtop instrument that can extract 32 samples in parallel. The extraction protocol “Total NA Serum, Plasma, Blood” was used. We used a 200-μL sample volume for experiments 1 and 2 and sample volumes of 50, 100, 150, or 200 μL for the third experiment. An elution volume of 100 μL and a dilution volume of 0 μL were chosen for each analytical run. Other details, such as reagent volumes and number of reaction tips needed for the run, were automatically calculated by the software. The MagNA Pure LC automatically performed all steps of the procedure.

After DNA extraction was completed, the MagNA Pure LC Cooling Block, which included a sample carousel with the correct number of LightCycler capillaries, and the reaction vessel, including the master mixture, were placed into the postelution area. After the start of the postelution protocol, which had been programmed before the start of the first run, the MagNA Pure LC automatically pipetted 15 μL of the master mixture and 5 μL of the processed sample into each of the LightCycler capillaries.

For the in-house DNA extraction method, a rapid DNA extraction protocol, which has recently been described in detail, was used (6). After completion, 15 μL of the master mixture and 5 μL of the processed sample were manually pipetted into each of the LightCycler capillaries.

For the real-time PCR assay, oligonucleotides deduced from the published sequence of the DNA polymerase gene-coding region of HSV were used as described previously (6). The LightCycler FastStart DNA Master Hybridization Probes reagent set (Roche) with the TaqMan™ probe described previously was used (6).

After completion of the postelution protocol, the LightCycler capillaries were sealed. The sample carousel with the capillaries was then centrifuged in the LightCycler Carousel Centrifuge and placed into the LightCycler. After denaturation for 10 min at 95 °C, a total of 55 PCR cycles were run. Each cycle consisted of 10 s at 95 °C and...