curves. The effects of applying different pressures for 10 min were as follows: At 70 MPa (10 000 lb./in.²), no pressure effect was evident; ~10% of maximal binding was observed at 140 MPa, 20% at 210 MPa, 50% at 280 MPa, and 80% at 420 MPa. Preincubation of the anti-p24 antibody alone (without the p24 antigen) at 420 MPa for 10 min did not result in any discernible decrease in absorbance, indicating that pressures as great as 420 MPa did not affect the ability of the antibody to subsequently bind to the antigen. Applying 420 MPa for different times enhanced the binding of antigen to antibody rapidly, with 50% binding in 5 min. Maximum binding was reached in 10 min, as indicated by binding of 79% in 10 min, 69% in 25 min, and 82% in 50 min (Fig. 1). As these data show, the binding achieved in overnight incubations at atmospheric pressure was not attained at the pressures used in this study. Higher pressures than those studied here or longer incubations at lower pressures may allow greater binding to occur.

From this preliminary study we conclude that high pressure can be used to accelerate the binding of an antibody to an antigen. This phenomenon could find practical application in shortening assay times and in greatly reducing the amount of reagents required in immunoassays and other binding assays, such as those used in clinical and high-throughput screening assays.

Acidic Citrate Stabilizes Blood Samples for Assay of Total Homocysteine, Huib P.J. Willems, Gerard M.J. Bos, Wim B.J. Gerrits, Martin den Heijer, Stephanie Vloet, and Henk J. Blom

Homocysteine is a sulfhydryl-containing amino acid, formed by demethylation of the essential amino acid methionine. Homocysteine is either transsulfurated to cysteine or is remethylated to methionine by methionine synthase. Excess intracellular homocysteine is likely to be transported to the extracellular compartment [1].

Increasing evidence indicates that homocysteine is implicated in the pathogenesis of thromboembolic diseases. Several case control studies have shown a relationship between increased total plasma homocysteine (tHcy) concentrations and an increased risk of arterial [2–4] and venous thrombosis [5–8]. An increase of the tHcy concentration of 5 μmol/L is associated with 1.5–1.9 times increased risk for coronary artery or cerebrovascular disease [9]. These values indicate that small differences might be of clinical importance. Therefore, practical standardized conditions for handling blood specimens for tHcy determination are required. In most studies, blood is drawn in tubes containing K₃EDTA. The whole-blood sample is immediately put on crushed ice and then centrifuged as soon as possible to prevent an increase of tHcy concentrations. This tHcy increase is caused by ongoing homocysteine metabolism in blood cells, the majority of which are red blood cells [10, 11]. This blood handling protocol is not practical, particularly when larger studies are conducted outside a hospital setting; even in a routine clinical setting, this protocol might be hard to put into practice. To find an alternative, more suitable blood-collection medium, we investigated the effect of different blood-collection media on tHcy production when whole blood is kept at room temperature for 6 h.

Blood was drawn by venipuncture of the antecubital vein from laboratory coworkers or from consecutive patients who visited the outpatient clinics of the Leyenburg Hospital in The Hague for various reasons, unknown to the authors. Informed consent was obtained in accordance with the current revision of the Helsinki declaration of 1975. Two studies were performed. A pilot study was done with blood from 11 patients and 11 laboratory coworkers (12 men and 10 women; ages 18–63 years). Blood was drawn in tubes with 1.8 g/L K₃EDTA (Vacutainer Tube; Becton Dickinson), in tubes with 2.5 g/L sodium fluoride and 2 g/L potassium oxalate as anticoagulant (Vacutainer Tube), in tubes with 0.5 mol/L acidic citrate (Biopool Stabilyte), and in tubes with a mixture of the sodium fluoride, potassium oxalate, and acidic citrate. Care was taken that all tubes were completely
filled by blood. The EDTA-treated blood was put on crushed ice immediately after venipuncture. The other blood samples were kept at room temperature.

The results of this pilot study indicated that tHcy concentrations remained stable in acidic citrate. We conducted a second study to explore this phenomenon more extensively. This main study was done in 30 laboratory coworkers (17 men and 13 women; ages 18–52). Blood was taken in tubes with EDTA and acidic citrate (as described above). From every volunteer, one-half volumes of the tubes containing EDTA were kept at room temperature; the other half were put on crushed ice immediately after sampling. From the tubes with acidic citrate, one-half volumes were kept at room temperature, and the other half were stored in water of 37 °C.

In both study groups, the blood was centrifuged for 10 min at 2000g as soon as possible (within 15 min) after sampling (“0 h”) and 2, 4, and 6 h after the venipuncture. After separation, the plasma was stored at −20 °C until determination of the tHcy concentration at the Laboratory of Pediatrics and Neurology of the University Hospital Nijmegen (by H.J.B. and S.V.) by automated HPLC with reversed-phase separation and fluorescent detection [Gilson 232–401 sample processor (Gilson Medical Electronics, Inc.), Spectra-Physics 8800 solvent delivery system, and Spectra-Physics LC 304 fluorometer], according to the method described by Fiskerstrand et al. [12] with some modifications [13]. The tHcy concentrations from the tubes containing the acidic citrate were corrected for the dilution caused by the fluid already present in the tube before blood collection.

Paired-sample t-tests were used to calculate the significance of the increase of tHcy concentrations in the collection media. Paired-sample t-tests were also used to calculate the significance of the differences between the tHcy concentrations in the different collection media at baseline. Results of the t-tests are given as the intervals that show P < 0.05 significance [95% confidence intervals (CI)].

Results of the pilot study are shown in Table 1. tHcy concentrations in blood taken in tubes containing sodium fluoride rose markedly after 2 h (0.9 μmol/L; 95% CI, 0.5–1.3 μmol/L), whereas tHcy concentrations in blood taken in tubes containing acidic citrate rose markedly for 6 h (0.9 μmol/L; 95% CI, −0.2 to 0.7 μmol/L). tHcy concentrations in the EDTA-containing tubes that were stored at room temperature rose 2.0 μmol/L after 2 h (95% CI, 1.6–2.4 μmol/L) and up to 4.7 μmol/L (95% CI, 4.1–5.3 μmol/L) after 6 h.

At room temperature, the tHcy concentrations in acidic citrate did not rise markedly for 6 h after collection (0.3 μmol/L; 95% CI, −0.2 to 0.7 μmol/L), whereas tHcy concentrations in blood collected in acidic citrate stored at 37 °C increased markedly after 4 h (0.9 μmol/L; 95% CI, 0.5–1.3 μmol/L).

We found a difference of 1.3 μmol/L (95% CI, 0.9–1.6 μmol/L) between tHcy concentrations measured in blood sampled in tubes with EDTA and stored at 0 °C and tHcy concentrations measured in blood sampled in tubes with acidic citrate kept at room temperature. Such significant differences were present at all measurement times. When the results of the pilot study and the main study were combined, this difference decreased but was still significant (0.8 μmol/L; 95% CI, 0.4–1.1 μmol/L; range, −3.1 to 2.2 μmol/L).

Blood cells produce homocysteine, which can lead to falsely increased plasma homocysteine concentrations. Thus, in the ideal setting, blood cells should be separated from plasma immediately after collection. In EDTA-containing blood, we found that tHcy concentrations increased at room temperature, which has been demonstrated before [10,14]. Even storage at 4 °C has been associated with a smaller but steady increase of the tHcy concentration [10]. However, we found that tHcy concentrations remained stable for 6 h when EDTA-containing blood was stored at 0 °C, which confirms the findings of Kittner et al. [15].

In a study by Möller and Rasmussen [16], heparin-containing tubes with sodium fluoride added to a concentration of 2 or 4 g/L of blood prevented the increase of tHcy for only 2 h. In our pilot study and also in the study by Ubbink et al. [10], comparable results of increasing

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**Table 1.** Mean (±SD) increase in homocysteine (μmol/L) in whole blood in different collection media.

<table>
<thead>
<tr>
<th>Storage medium</th>
<th>Temp. °C</th>
<th>Baseline</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0</td>
<td>12.5 (2.4)</td>
<td>0.1 (1.1)</td>
<td>0.4 (0.9)</td>
<td>0.1 (1.2)</td>
<td>12.7 (3.3)</td>
<td>0.1 (1.3)</td>
<td>0.1 (1.3)</td>
</tr>
<tr>
<td>Acidic citrate</td>
<td>23</td>
<td>12.6 (2.6)</td>
<td>0.1 (1.1)</td>
<td>0.4 (0.8)</td>
<td>0.4 (1.1)</td>
<td>14.0 (3.6)</td>
<td>0.0 (1.1)</td>
<td>0.0 (1.1)</td>
</tr>
<tr>
<td>NaF</td>
<td>21</td>
<td>11.3 (1.8)</td>
<td>0.9 (0.9)</td>
<td>1.6 (0.8)</td>
<td>1.7 (1.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic citrate and NaF</td>
<td>21</td>
<td>11.6 (1.9)</td>
<td>0.1 (0.9)</td>
<td>−0.1 (1.0)</td>
<td>0.6 (1.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>21</td>
<td>13.3 (3.4)</td>
<td>2.0 (1.0)</td>
<td>3.5 (1.4)</td>
<td>4.7 (1.7)</td>
<td>13.9 (3.5)</td>
<td>0.0 (1.0)</td>
<td>0.9 (1.1)</td>
</tr>
<tr>
<td>Acidic citrate</td>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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concentrations. However, is temperature dependent because tHcy concentrations rise when blood is stored at 37 °C. This blockage, which the acidic citrate keeps tHcy concentrations stable.

Baseline tHcy concentrations in the EDTA-containing tubes kept at room temperature and centrifuged at \( t = 0 \) h were higher than the concentrations in EDTA tubes put on ice and centrifuged at 0 h. As mentioned earlier, there was a time interval between the venipuncture and the centrifugation of the blood at \( t = 0 \) of, at most, 15 min. When cooling of the blood instantly stops the export of homocysteine, tHcy concentrations increase in blood that is kept at room temperature from the time between the blood samples being taken and the separation of plasma from blood cells because of ongoing export, explaining the difference at \( t = 0 \).

We also found a difference between the mean baseline concentrations of tHcy measured in EDTA-treated blood that was stored at 0 °C and tHcy concentrations in blood taken in acidic citrate. This difference can only partly be explained in the same way as above (i.e., a rise of tHcy concentrations in the time interval between blood sampling and separation). Other factors, such as a higher osmolarity of EDTA than acidic citrate, leading to higher plasma volumes, could be responsible for the difference. This, however, needs further investigation.

This study was conducted to find an alternative collection medium for the determination of tHcy concentrations in plasma. When blood is collected outside a hospital or laboratory setting (e.g., in a large epidemiological study), it is not always possible to put blood tubes on crushed ice. Even within hospitals, it can easily be forgotten or found impractical to put the tubes on crushed ice before processing. Therefore, this method can be prone to inappropriate handling of blood for determination of tHcy concentrations. This study shows that acidic citrate is a good alternative when screening patients in an epidemiological field study because tHcy concentrations stay stable for 6 h. However, because of the differences found between the mean baseline tHcy concentrations in EDTA on ice and in acidic citrate at room temperature, new reference values need to be established before tHcy concentrations obtained in EDTA tubes and stored on crushed ice can be replaced by acidic citrate tubes for determination of tHcy concentrations in individual patients.

This work was supported by grant 94141 from the Dutch Heart Foundation.

References

Simultaneous Fluorescence Detection of Fecal Urobilins and Porphyrins by Reversed-Phase High-Performance Thin-Layer Chromatography, Ching-Wan Lam, Chi-Kong Lai, and Yan-Wo Chan

Determination of fecal urobilins is useful for the biochemical identification of feces in forensic samples [1] and as an indicator of fecal pollution in rivers [2]. Application of fecal urobilin determination in clinical situations has been limited, however, because methods used for determining fecal urobilins require extensive sample pretreatment, such as solvent extraction and chemical derivatization [3]. These methods are time-consuming and labor intensive and require special expertise. Interestingly, Kerkhoff and Peters [4] developed a simple and convenient assay for the quantification of urinary urobilins. The assay principle is based on the Schlesinger’s reaction in which a urobilinogen–zinc chelation complex exhibits a characteristic green fluorescence under ultraviolet light. In this study, we have applied this fluorescence detection method for urinary urobilins in our previous reversed-phase high-performance thin-layer chromatography (RP-HPTLC) method [5] for the detection of fecal urobilins.

Fecal samples from patients with porphyria cutanea tarda (PCT) were obtained from a previously described population [5]. Fecal samples from patients with variegate porphyria (VP) were obtained from two porphyria reference laboratories. The external quality-control (QC) sample taken from a VP patient was obtained from an external QC program run by the Australian Association of Clinical Biochemists Working Party on porphyria. The commercial urine control, Lyphochek Quantitative Urine Control, Level 2, was purchased from Bio-Rad ECS Div. The abnormal urine sample was obtained from a patient with acute hepatitis and a positive Urobilistix® result (Bayer Diagnostics). All reagents were analytical or chromatographic grade: acetonitrile, dioxane, ethanol, N-acetyl-N,N,N-trimethyl ammonium bromide, and paraffin (viscous, DAB8), and RP-18 HPTLC precoated plates were from Merck. Mixed calibrators of free porphyrins (uro- porphyrin I, heptacarboxylic porphyrin I, hexacarboxylic porphyrin I, pentacarboxylic porphyrin I, coproporphyrin I, and mesoporphyrin IX), deuteroporphyrin, i-urobilin, and stercobilin were purchased from Porphyrin Products.

Solid-phase extraction minicolumns (C_{18}, 0.5 g) were developed a simple and convenient assay for the detection of fecal urobilins. Interestingly, one of the VP fecal samples (lane 2 in Fig. 1, left) showed an additional 5-carboxyl porphyrin. However, the importance of this band was not known. In addition, a green fluorescent band migrating just above the 5-carboxyl porphyrin was observed in feces from all porphyric patients. This band was orange in color under visible light and was also found in normal feces (data not shown). The color and fluorescent characteristics were consistent with that of urobilins [3]. Furthermore, this band migrated to the same position as the urobinin calibrator in our RP-HPTLC system (Fig. 1, middle). Although no green band can be detected in the urine sample with a negative Urobilistix result, a green fluorescent band migrating at the position of 5-carboxyl porphyrin was detected in the urine of the patient with hepatitis and a positive Urobilistix result (Fig. 1, right). The appearance of this urobinin band is compatible with this condition.

A number of chromatographic techniques have been developed for the identification of urobilins in biological fluids [7, 8]. However, most of these methods rely on normal-phase separation, requiring tedious solvent extraction and time-consuming chemical derivatization. In addition, none of these systems permit the simultaneous identification of porphyrins and urobilins. To our knowledge, this is the first time that simultaneous fluorescence detection of porphyrins and urobilins has been reported. This technique can be used for distinguishing uroporphyrinuria because of the in vitro polymerization of porphobilinogen and congenital erythropoietic porphryia; only the latter is complicated by hemolytic anemia and urobilinuria. In addition to a direct identification of urobilins, our method also provides important corroborative evidence for fecal identification by demonstrating the prevalence of dicarboxylic porphyrins, which are the bacterial degradation products of protoporphyrin [9]. The simplicity of the present method for simultaneous detection of...