Altered Distribution of Transferrin Isoforms According to Serum Storage Conditions, Brice M.R. Appenzeller* and Robert Wennig (Centre de Recherche Public–Santé, Laboratoire National de Santé, Toxicologie, Université du Luxembourg, 162A, Ave de la Faïencerie, L-1511 Luxembourg, * author for correspondence: fax 352-22-13-31, e-mail appenzel@cu.lu)

Measurements of carbohydrate-deficient transferrin (CDT) are used to detect alcohol abuse (1–6). Various patient factors (7–11) and comigration of analytes with transferrin (Tf) isoforms (12, 13) can affect the results of the measurements. Recent studies have questioned the influence of serum and blood storage conditions on CDT and yielded varying (and sometimes contradictory) findings (14–17). Moreover, the stability of CDT may vary among serum samples (14, 16).

We studied CDT and Tf isoforms in serum specimens stored at 25 °C, 4 °C, and −20 °C. We used 100 discarded serum samples; 8 were from alcohol abusers in treatment (4 with increased disialo-Tf and the presence of asialo-Tf; 4 with increased disialo-Tf but without asialo-Tf), and 6 were from moderate drinkers (CDT within reference values). For sera from alcohol abusers, the alcohol abuse diagnosis was based on psychological evaluation; CDT, γ-glycyltransferrase, and transaminase (aspartate aminotransferase and alanine aminotransferase) concentrations; and erythrocyte mean corpuscular volume. Each of the 14 specimens was divided into 3 samples and stored in glass vials (4 mL; Supelco 27137) at room temperature (25 °C in the dark), in a refrigerator (4 °C), or in a freezer (−20 °C). Samples were then analyzed periodically for 28–81 days [mean (SD), 51.6 (14.7) days]. The main reason for discontinuing follow-up was the appearance of solid particles in the serum. For frozen samples (−20 °C), the time between thawing and refreezing was <5 min. Serum Tf was analyzed (13) by use of capillary electrophoresis (P/ACE 5500) and a reagent set (CEoxif; ANALIS). Electrophrograms displayed all major Tf isoforms: asialo-Tf (when present), disialo-Tf, trisialo-Tf, tetrasialo-Tf, penta- sialo-Tf, and hexasialo-Tf. The signal was integrated in the valley-to-valley mode. Measurements were made in triplicate.

CDT was expressed as the percentage of total Tf:

\[
CDT = \frac{(0\text{asialo-Tf} + 2\text{asialo-Tf})}{(0\text{asialo-Tf} + 2\text{asialo-Tf} + 3\text{asialo-Tf} \quad + 4\text{asialo-Tf} + 5\text{asialo-Tf} + 6\text{asialo-Tf})}
\]

(1)

where 0asialo-Tf is asialo-Tf, 2asialo-Tf is disialo-Tf, 3asialo-Tf is trisialo-Tf, and so forth. Because CDT calculations included all Tf isoforms, relative error on the CDT calculation was expressed as AC/CDT, derived from Eq. 1 by the logarithmic differential method, giving:

\[
(\Delta CDT/CDT)_i = 2(\Delta 0\text{asialo-Tf}/0\text{asialo-Tf})_i + 2(\Delta 2\text{asialo-Tf}/2\text{asialo-Tf})_i
\]
where \( \Delta X_{\text{sialo-Tf}} = [X_{\text{sialo-Tf}}]_0 - X_{\text{sialo-Tf}} \).

Hexasialo-Tf was also expressed as percentage of total Tf, as was CDT in Eq. 1. The hexasialo-Tf/pentasialo-Tf ratio was calculated similarly. Anti-human Tf (Q0327; Dako) was used for confirmatory identification of Tf isoforms. Serum was added to undiluted anti-Tf at ratios of 3:1 and 1:1 (by volume) and mixed by 5 simple pipette aspirations/expressions.

The storage of serum at room temperature markedly changed the pattern of Tf isoforms (Fig. 1). In this sample, the decrease in asialo-Tf and disialo-Tf led to a 50% decrease in measured CDT (Table 1; also see Fig. 1 in the online Data Supplement). CDT was stable during the entire period stored at 4 °C and decreased below the positive cutoff of 2%. In all sera alcohol abusers displayed the most marked decrease in with lower CDT after storage at 25 °C (Table 1). Sera from content/vol51/issue11). All samples behaved similarly, of this Technical Brief at http://www.clinchem.org/ Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue11). All samples behaved similarly, with lower CDT after storage at 25 °C (Table 1). Sera from alcohol abusers displayed the most marked decrease in with lower CDT after storage at 25 °C (Table 1). Mean slopes of \( \Delta \text{CDT/CDT} \) vs time were higher for sera stored at room temperature [0.136 (0.070) for alcohol abusers and 0.080 (0.049) for moderate drinkers] than for sera stored at 4°C [0.027 (0.042) for alcohol abusers and 0.013 (0.012) for moderate drinkers] or -20°C [0.014 (0.011) for alcohol abusers and 0.012 (0.012) for moderate drinkers].

The CDT decrease occurring at 25 °C was accompanied by other changes in the Tf isoform pattern (Fig. 1). All isoforms decreased with time except for pentasialo-Tf, which remained stable or decreased slightly, and hexasialo-Tf, which increased (Fig. 1; also see Fig. 1 in the online Data Supplement). Serum treatment with anti-Tf confirmed that the apparent increase in hexasialo-Tf was not attributable to comigration of other substances and also demonstrated the presence of another anti-Tf–reactive substance visible just after hexasialo-Tf (probably heptasialo-Tf; Fig. 1; see also Fig. 3 in the online Data Supplement). We observed signals in lines \( t = 2 \) days, \( t = 4 \) days, and \( t = 16 \) days (Fig. 1) for all sera at time points between 6.4 and 6.6 min but not later. Although the peak distributions seemed to indicate Tf isoforms, they were neither cancelled by treating serum with anti-Tf nor correlated with an increase or decrease in any Tf isoforms. We attributed this signal to unidentified serum degradation products having no direct relationship to Tf. The increase in hexasialo-Tf in serum samples stored at 25 °C led to values ranging from 4.4% to 26% (see Table 1 in the online Data Supplement). In comparison, the mean (SD) value for hexasialo-Tf determined in 100 fresh sera was 3.4 (0.6)%. The increases in hexasialo-Tf concentration were significantly correlated with time for all sera investigated here, but slopes were highly variable among sera (see Table 1 in the online Data Supplement). Increases in hexasialo-Tf also correlated with decreases in CDT (see Fig. 4 in the online Data Supplement). Correlation was more significant for serum samples from alcohol abusers (see Table 1 in the online Data Supplement), but slopes varied markedly among samples. It is notable that the serum samples with the smallest increase in hexasialo-Tf (samples J and L) also displayed more stable CDT (Table 1). The hexasialo-Tf/pentasialo-Tf ratio also correlated with both time and a decrease in CDT (see Fig. 4 and Table 2 in the online Data Supplement), and the correlation was more significant in serum samples from alcohol abusers. After storage at 25 °C, some samples displayed hexasialo-Tf/pentasialo-Tf ratios up to 1.5 (see Table 2 in the online Data Supplement). In comparison, the mean (SD) value observed in 100 fresh sera was 0.256 (0.046). For samples stored at 4 °C and -20 °C, as observed for CDT, the hexasialo-Tf concentration and the hexasialo-

\[
\begin{align*}
\Delta \text{X}_{\text{sialo-Tf}} &= \frac{1}{[X_{\text{sialo-Tf}}]} - X_{\text{sialo-Tf}} \\
&= \frac{1}{X_{\text{sialo-Tf}}} - X_{\text{sialo-Tf}}
\end{align*}
\]

\[
(2)
\]

\[
\begin{align*}
&\Delta \text{hexasialo-Tf} = \frac{1}{[X_{\text{hexasialo-Tf}}]} - X_{\text{hexasialo-Tf}} \\
&= \frac{1}{X_{\text{hexasialo-Tf}}} - X_{\text{hexasialo-Tf}}
\end{align*}
\]

\[
(3)
\]

\[
\begin{align*}
&\Delta \text{pentasialo-Tf} = \frac{1}{[X_{\text{pentasialo-Tf}}]} - X_{\text{pentasialo-Tf}} \\
&= \frac{1}{X_{\text{pentasialo-Tf}}} - X_{\text{pentasialo-Tf}}
\end{align*}
\]

\[
(4)
\]

\[
\begin{align*}
&\Delta \text{CDT} = \frac{1}{[X_{\text{CDT}}]} - X_{\text{CDT}} \\
&= \frac{1}{X_{\text{CDT}}} - X_{\text{CDT}}
\end{align*}
\]

\[
(5)
\]
Table 1. Stability of CDT with respect to storage conditions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CDT value at $t_0^{*}$</th>
<th>Last CDT value measured$^{*\ast}$</th>
<th>Period of follow-up, days</th>
<th>Slope of $\Delta$CDT/CDT vs time (correlation coefficient; significance$^\dagger$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol abusers</td>
<td></td>
<td></td>
<td></td>
<td>Ambient (25 °C)</td>
</tr>
<tr>
<td>A$^{\dagger\ast}$</td>
<td>11.64</td>
<td>6.45</td>
<td>73</td>
<td>0.223 (0.99; P &lt; 0.001)</td>
</tr>
<tr>
<td>B$^{\dagger\ast}$</td>
<td>13.71</td>
<td>10.89</td>
<td>56</td>
<td>0.066 (0.97; P &lt; 0.02)</td>
</tr>
<tr>
<td>C$^{\dagger\ast}$</td>
<td>9.04</td>
<td>6.19</td>
<td>47</td>
<td>0.184 (0.98; P &lt; 0.001)</td>
</tr>
<tr>
<td>D$^{\dagger\ast}$</td>
<td>5.88</td>
<td>4.26</td>
<td>60</td>
<td>0.137 (0.93; P &lt; 0.01)</td>
</tr>
<tr>
<td>E$^{\ast}$</td>
<td>2.81</td>
<td>1.48</td>
<td>70</td>
<td>0.094 (0.99; P &lt; 0.001)</td>
</tr>
<tr>
<td>F$^{\ast}$</td>
<td>2.26</td>
<td>1.72</td>
<td>41</td>
<td>0.210 (0.98; P &lt; 0.001)</td>
</tr>
<tr>
<td>G$^{\ast}$</td>
<td>2.62</td>
<td>2.31</td>
<td>47</td>
<td>0.027 (0.99; P &lt; 0.01)</td>
</tr>
<tr>
<td>H$^{\ast}$</td>
<td>2.37</td>
<td>1.71</td>
<td>43</td>
<td>0.150 (0.99; P &lt; 0.01)</td>
</tr>
</tbody>
</table>

Moderate drinkers

<table>
<thead>
<tr>
<th>Sample</th>
<th>CDT value at $t_0^{*}$</th>
<th>Last CDT value measured$^{*\ast}$</th>
<th>Period of follow-up, days</th>
<th>Slope of $\Delta$CDT/CDT vs time (correlation coefficient; significance$^\dagger$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.30</td>
<td>0.47</td>
<td>81</td>
<td>0.103 (0.88; NS)</td>
</tr>
<tr>
<td>J</td>
<td>1.12</td>
<td>0.99</td>
<td>41</td>
<td>0.048 (0.99; P &lt; 0.05)</td>
</tr>
<tr>
<td>K</td>
<td>1.36</td>
<td>0.86</td>
<td>28</td>
<td>0.169 (0.93; P &lt; 0.001)</td>
</tr>
<tr>
<td>L</td>
<td>0.83</td>
<td>0.81</td>
<td>46</td>
<td>0.065 (0.93; P &lt; 0.001)</td>
</tr>
<tr>
<td>M</td>
<td>1.15</td>
<td>0.85</td>
<td>47</td>
<td>0.058 (0.99; P &lt; 0.02)</td>
</tr>
<tr>
<td>N</td>
<td>1.42</td>
<td>1.28</td>
<td>42</td>
<td>0.036 (0.78; NS)</td>
</tr>
</tbody>
</table>

$^a$ As percentage of total Tf.

$^b$ In the ambient stored sample.

$^c$ Bilateral Student test: NS, nonsignificant ($P > 0.05$).

$^d$ Asialo-Tf present.

$^e$ No asialo-Tf.

Tf/pentasialo-Tf ratio remained stable over the whole follow-up period (see Fig. 1 in the online Data Supplement).

Two mechanisms may explain the observed serum impairment: degradation of Tf molecules (attributable to endogenous enzymes and/or bacterial contamination), leading to a decrease in total Tf, and fixation of free sialic acids [naturally present in blood in considerable amounts (18)] on Tf isoforms having N-glycans but without terminal sialic acid, causing dominance of forms with a higher number of fixed sialic acid residues. The intensity of these mechanisms is dependent, however, on proper serum characteristics, because impairment kinetics were highly variable among sera. Because of this interserum variability, it is not practical to propose a time limit for room storage; therefore, validity of the CDT measurement may be assessed by use of indicators such as the hexasialo-Tf concentration and the hexasialo-Tf/pentasialo-Tf ratio, because both are correlated with a decrease in CDT. Because laboratories use various procedures and methods to quantify Tf in serum, definite values cannot be proposed for assessing serum impairment with these indicators, and adoption of a universal CDT cutoff value for alcohol abuse remains a point of contention. It may be more advantageous for laboratories to compare suspicious samples with reference values observed for hexasialo-Tf and the hexasialo-Tf/pentasialo-Tf ratio in fresh sera and to consider that a serum with abnormally increased indicator values (for example, 2-fold above reference values, corresponding to −6% for 6sialo-Tf and 0.35 for the 6sialo-Tf/6sialo-Tf ratio, respectively, in our experiments) is inappropriate for CDT analysis. According to such criteria, all sera tested here that were stored at 25 °C were still fit for CDT analysis within 5 days, 21% were unfit after 7 days, 36% were unfit after 10 days, and 64% were unfit after 15 days. An analysis performed in parallel with the Bio-Rad %CDT immunologic test showed comparable decreases in CDT for sera stored at 25 °C (data not shown), confirming that serum degradation also interferes with immunologic tests. Nevertheless, evaluation of serum degradation with hexasialo-Tf and the penta/hexasialo-Tf ratio is possible only with techniques giving access to all Tf isoforms (e.g., capillary electrophoresis and HPLC) and hence is not applicable to CDT analysis performed with current immunoassay tests.

In summary, serum may be stored for several weeks at 4 °C or −20 °C before CDT analysis. Storage at 25 °C is acceptable for 5 days but not >7 days. After prolonged exposure to room temperature, Tf degradation may be assessed by measuring the hexasialo-Tf concentration and the hexasialo-Tf/pentasialo-Tf ratio.

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References


Riboflavin deficiency is common among chronic alcoholics, the elderly, and vegetarians (1–4); but intake in the United States is generally adequate (5, 6), unlike the widespread deficiency in regions of the world with limited animal food sources (7, 8). Riboflavin status has been assessed from measurements in urine, plasma, and erythrocytes (9–11). The erythrocyte glutathione reductase activity coefficient (EGRAC) is the commonly used test and reflects the adequacy of riboflavin to support enzyme function (10). In this assay, the stimulation of erythrocyte glutathione reductase by FAD is measured in vitro, and a higher activity coefficient reflects a larger amount of unsaturated glutathione reductase apo-enzyme resulting from lack of FAD. The EGRAC cutoffs (12) are based on observational studies in well-nourished American school children (n = 431) and adult men (n = 6) and a depletion–repletion study in Indian adults (n = 8) (13–15). These cutoffs are usually ≥1.4 for deficiency status, 1.2 to <1.4 for marginal status, and <1.2 for acceptable status.

Although erythrocyte riboflavin concentration is seldom used to assess riboflavin status, Hustad et al. (16) showed that the sum of erythrocyte flavin mononucleotide and FAD is correlated with the EGRAC and might be a useful indicator of riboflavin status in population studies. The elderly Irish persons (n = 122) in their study had marginal riboflavin status (mean EGRAC, 1.26), but no erythrocyte riboflavin cutoffs were evaluated. In healthy Californian adults (n = 22), we previously determined the 5th percentile of erythrocyte riboflavin concentration to be 170 nmol/L (range, 169–289 nmol/L) but did not measure the EGRAC in the same samples (L.H. Allen, unpublished data).

The purpose of our study was to compare erythrocyte riboflavin concentrations against the EGRAC in a group of pregnant women at risk for riboflavin deficiency. Because iron status can affect hemoglobin synthesis and erythrocyte production, the association of markers of riboflavin status with hemoglobin and plasma ferritin concentrations was compared. The erythrocyte riboflavin cutoff with the greatest sensitivity and specificity for detecting deficiency was determined by ROC analysis, with the EGRAC as the reference value.

The participants were women (n = 84) in their first to seventh month of pregnancy who had self-reported night blindness before participating in a large food-based vitamin A treatment trial (17). They were reportedly healthy and had no signs of xerophthalmia. The research was conducted from August to October 2000 in 52 village development communities of the Saptari District in southeastern Nepal. Approval was obtained from the ethics review committees at the University of California, Davis, and the Nepal Health Research Council, and informed consent was obtained from each participant.

Venous blood samples (7.5 mL) were collected into S-Monovette® (Sarstedt) tubes containing lithium heparin as anticoagulant. Hemoglobin was measured immediately by a portable hemoglobinometer (HemoCue®). Erythrocytes were processed under dim light by washing 3 times with cold saline (9 g/L NaCl) followed by centrifugation for 10 min at 1500g. Washed erythrocytes were stored at −20 °C in duplicate 1.5-mL amber-glass microcentrifuge tubes to minimize riboflavin degradation. Plasma ferritin was quantified by a 2-site immunoradiometric assay (Coat-A-Count IRMA; Diagnostic Products Corporation). EGRAC and erythrocyte riboflavin measurements were conducted within 2 months of each