The results for unmanipulated samples and for samples at pH 6.5, 7.3, and 8.0 are summarized in Fig. 1. Intermediate results were seen at pH 7.0 and 7.6 (not shown). Immediately after the pH manipulation, the β-crosslaps increased 0.2–6.6% (P >0.05). After 2 days, all samples exhibited a significant decrease of the β-crosslaps concentrations. In the control samples, β-crosslaps decreased by 12% at 37 °C and by 5% at room temperature on the second day of storage. After 5 days at room temperature, the decreases in the β-crosslaps concentrations in the samples were even more pronounced. The β-crosslaps concentrations in the samples stored in the refrigerator remained unchanged.

In the pH-manipulated aliquots, we observed a positive correlation between the pH and the β-crosslaps concentration (Fig. 1). The lower the serum pH, the lower was the measured concentration of β-crosslaps. This pH-dependent decrease in the β-crosslaps concentration occurred more rapidly at higher storage temperatures. In the 37 °C samples, the decreases at pH 6.5 and 8.0 were 57% and 13%, respectively. The changes in the room temperature samples were similar, but less pronounced, after 2 days. After 5 days at room temperature, the decreases at pH 6.5 and 8.0 were 64% and 7%, respectively. At 4 °C, the decreases in β-crosslaps were 6–36% after 5 days and 8–54% after 10 days. The pH-induced effects were in addition to the changes caused by the various storage procedures.

The explanation for the observed decreases in β-crosslaps is not clear. It may, in part, reflect a pH-dependent change of conformation in the octapeptide, from the β-form into the isomeric α-form, which is not detected by the antibodies of the test. Furthermore, it is not clear whether a switch from the β- to the α-enantiomeric form is of any importance in this assay. Another explanation could be a degradation of the octapeptide or the detecting antibody by proteolytic enzymes in serum with different pH and temperature optima. We conclude that inappropriate preanalytical treatment can significantly complicate clinical interpretation and that standardized preanalytical treatment is necessary. At a serum pH >8.0, the β-crosslaps concentration remains relatively stable for some days if stored in a refrigerator. We recommend centrifugation of the blood samples immediately after their arrival in the laboratory and storage at 4 °C (refrigerator) for no more than 5 days.

This study was supported by Roche Diagnostics (Mannheim, Germany). We thank Dr. Ebert (Roche Diagnostics, Penzberg, Germany) for stimulating this work and for helpful scientific discussion of the results, and Kathrin Beyersdorf for constructive corrections.

References

Agreement of Owren and Quick Prothrombin Times: Effects of Citrate and Calcium Concentrations and International Sensitivity Index Correction, Juhás Horsti (Valkeakoski District Hospital, Tampere University Hospital Laboratory, 37600 Valkeakoski, Finland; fax 358-3-586-7435, e-mail juha.horsti@tays.fi)

The prothrombin time (PT) is commonly measured by either the “Quick method”, which is based on the technique described by Quick and co-workers in 1935 (1, 2), or by the Owren method (3) (combined thromboplastin reagent). The latter is the predominant approach used in the Nordic countries, Benelux, and Japan. These two methods are rarely mentioned or noted in the scientific literature (4, 5), and more attention is paid to the thromboplastin in the reagent and to its origin (6, 7).

The Owren method is sensitive to coagulation factors II, VII, and X. Fibrinogen (factor I) and factor V are in the reagent. The Quick method is affected by deficiencies of fibrinogen and factors II, V, VII, and X. Both methods are suitable for the control of anticoagulant treatment. The most important technical difference between the two is the sample volume in the reaction mixture: 5% in the Owren method, and 33% in the Quick method. This has the effect of making the coagulation reaction in the Quick method more sensitive to many preanalytical variables, e.g., citrate concentration in the sample (8, 9). The small sample volume in the reaction mixture with the Owren method makes it possible to use even EDTA plasma (10).

Fig. 1. β-Crosslaps concentrations during storage of control samples. Samples were stored at the temperatures indicated either without manipulation or after adjustment of pH (+, P <0.05).
The International Normalized Ratio (INR) is recommended to harmonize PT results for oral anticoagulant therapy (11). The recommendation conforms with those made by WHO using reference thromboplastins (12). Regardless of the reagent, instrument, or method used, the results should be the same when the INR system is used.

Because these two major PT methods have not previously been compared, the aim of this study was to compare the PT results obtained with two Owren reagents as INR units in samples containing 0.129 mol/L (3.8%) citrate solution and to compare the PT results and clinical significance with the same calibration between one Owren reagent and one Quick reagent as INR units in samples containing 0.129 mol/L (3.8%) and 0.109 mol/L (3.2%) citrate solution. Because calcium (factor IV) is in a key position in coagulation reactions as a cofactor in several steps, we sought to ascertain the ionized calcium (Ca$^{2+}$) concentration in the reaction mixtures in both methods and to study the effect of sample citrate concentration on the Ca$^{2+}$ concentration in the reaction mixtures by both methods.

All procedures were approved by our institution's responsible committee in accordance with the Helsinki Declaration of 1975. We studied patient samples chosen without conscious bias from among hospital and health center patients; 145 patients were sampled with a citrate coagulation tube (Vacutainer cat. no. 367702, 9NC; Becton Dickinson) containing 0.35 mL of 0.129 mol/L (3.8%) citrate solution (blood volume, 3.15 mL). The sample needle (Precision Glide, cat. no. 360213; Becton Dickinson) was 0.8 × 38 mm. Sample tubes were centrifuged at 1560 g for 10 min at 20 °C to separate the plasma.

PT was measured in samples collected in both kinds of sample tubes and reported in INR units. Measurement commenced within 2 h of blood collection. In 30 of the same patients, Ca$^{2+}$ concentrations were measured in the reaction mixtures for both sample types (0.129 and 0.109 mol/L citrate) and for both methods.

The ACL 1000 and ACL 7000 [Instrumentation Laboratory (IL)] are automatic microcentrifugal analyzers. We measured PT on the ACL 1000 analyzer using Nycotest PT coagulation reagent [cat. no. 1002834; International sensitivity Index (ISI), 1.10; rabbit tissue thromboplastin] and citrate-barbiturate buffer as diluent (cat. no. GHI 155; both from Nycomed Pharma), with a 10-μL sample, 50 μL of diluent, 140 μL of reagent, and IL Test Reference (cat. no. 97569-00).

We measured PT on the ACL 7000 analyzer using Owren’s PT coagulation reagent (cat. no. GHI 131-10; ISI, 1.22; rabbit brain thromboplastin), 25 mmol/L CaCl$_2$ (cat. no. GHI 155) as diluent, Owren buffer [cat. no. GHI 150; all from the Global Hemostasis Institute (GHI)], with a 10-μL sample, 50 μL of diluent, and 140 μL of reagent. We also measured PT on the ACL 7000 analyzer using PT-Fibrinogen Recombinant coagulation reagent (cat. no. 20005000; ISI, 1.12; rabbit tissue thromboplastin) and sample diluent (cat. no. 09756800; both from IL), with a 50-μL sample and 100 μL of reagent. All three methods (reagents) were calibrated with the same calibrators: calibration plasma (cat. no. 08467300; IL) and ISI calibrators (Etaloc, quick, cat. no. 00496; Diagnostica Stago).

ACL calibration is needed for INR units. The ACL 1000 calibration data with Nycotest PT reagents were as follows (n = 6 at every point): 100%, 19.8 s, CV = 0.00%; 50%, 26.4 s, CV = 0.04%; 25%, 40.87 s, CV = 0.75% (R$^2$ = 1.00). The ACL 7000 PT calibration data with the Owren’s PT method were as follows (n = 4 at every point): 100%, 20.0 s, CV = 0.00%; 50%, 27.6 s, CV = 0.00%; 25%, 38.80 s, CV = 0.00% (R$^2$ = 0.99). The ACL 7000 PT calibration data with the PT-Fibrinogen Recombinant method were as follows (n = 4 at every point): 100%, 11.4 s, CV = 0.73%; 50%, 20.5 s, CV = 0.53%; 25%, 34.2 s, CV = 1.7% (R$^2$ = 1.00).

Within the same run (n = 6), the CVs were as follows: for Nycotest PT, CV = 0.5% (mean, 21.1 s) and 0.4% (mean, 38.8 s); for Owren’s PT, CV = 0.4% (mean, 21.2 s) and 0.9% (mean, 35.3 s); for PT-Fibrinogen Recombinant, CV = 0.8% (mean, 12.0 s) and 1.0% (mean, 23.3 s).

The day-to-day CVs were as follows: for Nycotest PT, CV = 1.5% (mean, 20.0 s; n = 14); for Owren’s PT, CV = 1.7% (mean, 20.3 s; n = 14); for PT-Fibrinogen Recombinant, CV = 2.0% (mean, 11.7 s; n = 10).

Ionized calcium was measured with an IL 1640 blood gas analyzer, using IL Test contrIL PLUS (cat. no. 98321-30, lot. no. N0208038). The CV was 0.62% at a mean calcium concentration of 1.47 mmol/L (n = 9), 0.71% at 1.09 mmol/L (n = 10), and 1.4% at 0.62 mmol/L (n = 6).

For Ca$^{2+}$, we used IL Test Cal 1 (cat. no. 98318-00), Test Cal 2 (cat. no. 98342-00), and Test BGE Flush (cat. no. 98320-00). For Ca$^{2+}$ measurements, a reaction mixture was prepared with volumes two times those for the ACL (for Owren’s PT, 20 μL + 100 μL + 280 μL; for Quick PT, 100 μL + 200 μL); after coagulation the solution was centrifuged at 12,700 g for 10 min, and Ca$^{2+}$ was measured in the supernatant by an IL 1640. The regression equations for calculating the patient data were as follows:

\[
\text{INR} = 0.98 \sim 1.0 \times [(\text{sample}/\text{normal})^{1.06}]^{1.20}
= (\text{sample}/\text{normal})^{1.21}
\]

Owren ISI × 1.20 for 0.129 mol/L citrate.

\[
\text{INR} = 1.007 \sim 1.0 \times [(\text{sample}/\text{normal})^{0.06}]^{1.14}
= (\text{sample}/\text{normal})^{1.15}
\]

Owren ISI × 1.14 for 0.109 mol/L citrate (Fig. 1B).

These regression models were applied to convert the results obtained by the Quick and Owren methods to ISI, making its routine use easy.

The regression equations for PT estimation from citrate plasma (0.129 mol/L) were as follows for results obtained as INR: for two Owren reagents, Nycotest PT (x) vs Owren’s PT (y), y = 1.08x − 0.15 (R$^2$ = 0.99); for the
Owren (x; Nycotest PT) and Quick (y) methods, $y = 0.98x^{1.20}$ ($R^2 = 0.95$).

For patient results obtained by the Owren (Nycotest PT) and Quick methods using 0.129 and 0.109 mol/L citrate samples, for results $<$2.0 INR, the differences between the methods were not clinically significant with both sample types (Table 1). For results $>$2.0 INR, however, the means of the differences were clinically high and thus significant with both sample types. The Bland and Altman method (13) was not suitable for analyzing results in the range $>$2 INR before correction because the distribution was not gaussian (Table 1 and Fig. 1A). In the therapeutic range 2–4 INR, the difference in patient results was $>$0.5 INR for 31 of 83 samples (37%) in 0.129 mol/L citrate and 28 of 103 (27%) samples in 0.109 mol/L citrate.

When the Owren ISI was corrected according to the correlation equation, INR = (sample/normal)\(^{1.21}\), between the methods from 1.006 to 1.21 (1.21 = 1.006 $\times$ 1.20), eight results were outside 2 SD in the range 2–4 and $>$4 INR (mean difference, 0.07 INR; limits, −0.55 to 0.69 INR). According to Bland and Altman (13), 7 results of 145 could be outside the limits. With a probability of 94%, the clinical significance of the results is the same.

The regression equations for PT estimation from citrate plasma (0.109 mol/L) were as follows: for the Owren (x; Nycotest PT) and Quick (y) methods, with the results obtained as INR, $y = 1.007x^{1.14}$ ($R^2 = 0.97$). The difference plot (13) before correction (mean difference, $-$0.33 INR; limits, −0.99 INR to 0.33 INR) is shown in Fig. 1A.

When the ISI of the Owren method was corrected according to the correlation equation, INR = (sample/normal\(^{1.15}\), between the methods from 1.006 to 1.15 (1.15 = 1.006 $\times$ 1.14), nine results were outside 2 SD in the range 2–4 and $>$4 INR (mean difference, $-$0.01 INR; limits, −0.49 INR to 0.47 INR). According to Bland and

![Fig. 1. Differences between patient results obtained by the Owren and Quick methods as INR in 0.109 mol/L (3.2%) citrate before (A) and after (B) ISI correction.](image-url)
Altman (13), 8 results of 157 could be beyond the limits (Fig. 1B). With a probability of 94%, the clinical significance of the results is the same.

The mean Ca\(^{2+}\) concentrations in the reaction mixtures for the Owren and Quick methods using both citrate samples (0.129 or 0.109 mol/L) are given in Table 1.

In the comparison of one Owren and the Quick method, we obtained fairly good nonlinear correlation using both citrate samples (\(R^2 = 0.95\) for 0.129 mol/L; \(R^2 = 0.97\) for 0.109 mol/L). The correlation equations were the power functions \((y = 0.98x^{1.20}\) and \(y = 1.007x^{1.14}\)). The difference in exponents is 0.06, and this change depends on the change in sample citrate concentration (0.02 mol/L). Because the citrate concentration in the sample affects the Ca\(^{2+}\) concentration in reaction mixtures with the Quick method but not the Owren method (Table 1), we may conclude that the correlation equation formula derives from the Ca\(^{2+}\) concentration. The correlation equation shows that by changing ISI, we can improve the comparability of results between the Owren and Quick methods. The correlation between the methods is somewhat better when 0.109 mol/L citrate samples are used rather than 0.129 mol/L citrate samples. The correlation improves as the citrate concentration decreases.

Clinically, there was no significant difference between the Owren and Quick methods when INR results were <2 INR. In the important therapeutic range 2–4 INR and >4 INR, Quick results were markedly higher (mean differences, 0.44 and 0.39 for 2–4 INR, and 1.48 and 1.26 for >4 INR), and the differences were clinically significant for both citrate concentrations (Table 1). The patients whose samples were analyzed by Owren PT methods received too much medication, and this should be reduced. Oral anticoagulant therapy recommendations are based on Quick PT (5, 11). In published scientific reports, the situation was the same in that the results were not equal.

Using ISI correction (Owren ISI × 1.20 for 0.129 mol/L; Owren ISI × 1.14 for 0.109 mol/L), we can unify the results clinically with a probability of 94%.

In a previous study (10), the correlation equation for INR units was: \(y = 1.20x - 0.162\) (\(R^2 = 1.00\)) for EDTA (x) and citrate (y) samples using the Owren method. The equation for results obtained by the Quick method (y) in INR units vs results obtained for EDTA samples (x) by the Owren method was: \(y = (1.20x - 0.162)\) (Owren ISI × 1.20 or Owren ISI × 1.14).

The two different Owren reagents show good correlation (\(R^2 = 0.99\)), and the results involve no clinically significant difference.

The physiological concentration of Ca\(^{2+}\) in plasma is

### Table 1. Comparison between Owren and Quick methods with 0.129 and 0.109 mol/L citrate samples, and mean Ca\(^{2+}\) concentration in the reaction mixtures with Owren and Quick methods.

<table>
<thead>
<tr>
<th></th>
<th>&lt;2.0 INR</th>
<th>2–4 INR</th>
<th>&gt;4 INR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Patient results with 0.129 mol/L citrate sample (n = 145)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nycotest PT</td>
<td>55 patients</td>
<td>85 patients</td>
<td>5 patients</td>
</tr>
<tr>
<td>PT-Fibrinogen Recombinant</td>
<td>42 patients</td>
<td>83 patients</td>
<td>20 patients</td>
</tr>
<tr>
<td>Mean difference (Nycotest PT – PT-Fibrinogen Recombinant)</td>
<td>−0.07 INRa</td>
<td>−0.44 INR</td>
<td>−1.48 INR</td>
</tr>
<tr>
<td>Differences out of 2 SD (−0.31 to 0.17)</td>
<td>1/48</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. Patient results with 0.109 mol/L citrate sampling (n = 157)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nycotest PT</td>
<td>56 patients</td>
<td>98 patients</td>
<td>3 patients</td>
</tr>
<tr>
<td>PT-Fibrinogen Recombinant</td>
<td>43 patients</td>
<td>103 patients</td>
<td>11 patients</td>
</tr>
<tr>
<td>Mean difference (Nycotest PT – PT-Fibrinogen Recombinant)</td>
<td>−0.07 INRa</td>
<td>−0.39 INR</td>
<td>−1.26 INR</td>
</tr>
<tr>
<td>Differences out of 2 SD (−0.33 to 0.19)</td>
<td>2/48</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. Mean Ca(^{2+}) concentration in the reaction mixtures (n = 30)</strong></td>
<td>Owren’s PT</td>
<td>PT-Fibrinogen Recombinant</td>
<td></td>
</tr>
<tr>
<td>0.129 mol/L citrate</td>
<td>2.43</td>
<td>1.48</td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+}), mmol/L</td>
<td>0.12</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>4.9</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>CV, %</td>
<td>2.21–2.75</td>
<td>1.07–1.79</td>
<td></td>
</tr>
<tr>
<td>Range, mmol/L</td>
<td>0.129 mol/L citrate</td>
<td>2.44</td>
<td>1.69</td>
</tr>
<tr>
<td>0.109 mol/L citrate</td>
<td>0.07</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>3.0</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>CV, %</td>
<td>2.26–2.61</td>
<td>1.30–2.02</td>
<td></td>
</tr>
<tr>
<td>Mean difference, mmol/L (%)</td>
<td>0.01 (0.4)</td>
<td>0.21 (14)</td>
<td></td>
</tr>
</tbody>
</table>

a No clinically significant difference.
when PT (ISI, 1.0; Quick method) and activated partial
plasmin for patient groups designated as
partial thromboplastin time measurements are longer
citrate samples, the times for PT coagulation and activated
reaction mixture in the Quick method.

In addition, in the Quick method, the Ca\(^{2+}\) concen-
tration varies widely (1.07–1.79 mmol/L in 0.129 mol/L
citrate samples), and the CV is 13%. In the 0.129 mol/L
reaction mixtures, in 5 of 30 samples, the Ca\(^{2+}\) concen-
tration was <1.3 mmol/L. When 0.109 mol/L citrate
samples were used in the Quick method, the lowest Ca\(^{2+}\)
concentration in the reaction mixture was 1.3 mmol. The
Ca\(^{2+}\) concentrations in the coagulation mixtures are
differently between two sample citrate concentra-
tions with the Quick method.

When a sample is diluted (1 + 9) with citrate solution,
the dilution occurs in plasma, not in whole blood, and
depending on the hematocrit, the plasma volume will
differ from sample to sample. The situation is similar if
too little blood is drawn into the collection tube and the
tube is underfilled. The Ca\(^{2+}\) concentration in patient
plasma can sometimes be low. The citrate concentration is
markedly variable in plasma, as well as in the coagulation
reaction mixture in the Quick method.

Because the Ca\(^{2+}\) concentration is lower in 0.129 mol/L
citrate samples, the times for PT coagulation and activated
partial thromboplastin time measurements are longer
than the corresponding times for patient groups contain-
ing 0.109 mol/L citrate for patient groups designated as
“no anticoagulant therapy” and “anticoagulant therapy”
when PT (ISI, 1.0; Quick method) and activated partial
thromboplastin time reagents are used (9). Duncan et al.
(8) showed that the concentration of citrate in the sample
affects the results and ISI calibration (Quick methods).
The citrate concentration exerts an ~10% effect on the ISI
of reagents. The above-mentioned results of two studies
agree well with the PT and calcium results in our study.

With Owren’s PT, the mean difference in the Ca\(^{2+}\)
concentration in the reaction mixture was 0.01 mmol/L
(0.4%) and the CVs were 4.9% for 0.129 mol/L citrate
and 3.0% for 0.109 mol/L. The sample citrate concentra-
tion has no effect on the Owren method, and this method is
also less sensitive to other preanalytical variables by
reason of the small sample volume (5%) in the reaction
mixture. Because the Owren and Quick methods depend
differently on sample citrate concentrations (Ca\(^{2+}\) in
the reaction mixture), the correlation is not so good.

We subscribe to the demand for unification of recom-
mendations of a sample citrate concentration of 0.109
mol/L to improve results for PT and activated partial
thromboplastin times (8, 9). For results obtained by the
Owren method, ISI correction should be used to standard-
ize patient INR results and anticoagulant therapy to be
the same as the results obtained by the Quick method
because the recommendations for care are made accord-
ing to the Quick method. The patients whose samples
were analyzed by the Owren method were receiving too
much medication and were in greater danger of bleeding.

I thank the staff of the Laboratory at the Valkeakoski
District Hospital for their assistance.

References
1. Quick AJ, Stanley-Brown M, Bancroft FW. A study of the coagulation defect in
2. Quick AJ. The prothrombin time in haemophilia and in obstructive jaundice.
4. van den Besselaar AM. International standardisation of laboratory control of
oral anticoagulant therapy: A survey of thromboplastin reagents used for
anticoagulants mechanism of action, clinical effectiveness, and optimal
6. Kirkwood TB. Calibration of reference thromboplastins and standardisation
7. Hermans J, van den Besselaar AM, Loeliger EA, van der Velde EA. A
collaborative calibration study of reference materials for thromboplastins.
8. Duncan EM, Casey CR, Duncan BM, Lloyd JV. Effect of concentration of
trisodium citrate anticoagulant on the calculation of the International
Normalized Ratio and the International Sensitivity Index of thromboplastin.
9. Adcock DM, Kressin DC, Marlar RA. Effect of 3.2% vs 3.8% sodium citrate
concentration on routine coagulation testing. Am J Clin Pathol 1997;107:
105–10.
10. Horst J. Measurement of prothrombin time in EDTA plasma with combined
11. International Committee on Standardisation in Haematology. International
Committee on Thrombosis and Haemostasis. ICSH/ICTH recommenda-
tions for reporting prothrombin time in oral anticoagulant control. Thromb

Assay for Hexosamine Pathway Intermediates (Uridine
Diphosphate-N-Acetyl Amino Sugars) in Small Samples
of Human Muscle Tissue, Paul N. Span,1,2 Marie-José J.M.
Pouwels,2 André J. Ollilhaar,1 Renko R. Bosch,1,2 Ad R.M.M.
Hermus,2 and C.G.J. (Fred) Sweep2 (Departments of 1
Chemical Endocrinology and 2 Internal Medicine, Division of
Endocrinology, University Medical Centre Nijmegen,
6500 HB Nijmegen, The Netherlands; * address corre-
spondence to this author at: Department of Chemical
Endocrinology 530, University Medical Centre Nijmegen,
PO Box 9101, 6500 HB Nijmegen, The Netherlands; fax
31-24-3541484, e-mail p.span@ace.azn.nl)

It has been suggested that the hexosamine biosynthetic
pathway is involved in the pathogenesis of insulin resist-
tance in patients with type 2 diabetes mellitus because the