working day, in two separate assays. The out-of-range samples were reanalyzed on the final day, after appropriate dilution.

The within- and between-batch CVs for the control samples were <10% and the within-batch CV for the patient samples was <10%. The inaccuracy, against a value defined by HPLC–tandem mass spectrometric measurement using independently prepared calibrators (19), was <6% for the control samples and <13% for the three pooled patient samples (defined sirolimus concentrations were 8.3, 15.7, and 28.4 µg/L). The sirolimus-free samples were identified correctly and the inaccuracy for the out-of-range sample was 6.7%.

In summary, a HPLC-UV method for the quantification of sirolimus in human whole blood has been developed and validated. With the run time (<15 min) and the simplified extraction procedure described, 48 samples and the requisite calibrators and QC samples can be extracted and quantified in <20 h. The assay is linear over the range of 2.5–75 µg/L, based on a 0.5-mL sample. The data presented in this report demonstrate that the method provides rapid, sensitive, precise, and accurate measurements of sirolimus concentrations in human whole blood.

References

Determination of D-Mannose in Serum by Capillary Electrophoresis, Hubert A. Cardon and Jaak Jaqueen (Centre for Metabolic Disease, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium; * address correspondence to this author at: Centre for Metabolic Disease, Campus G asthma, Belgium; fax 32-16-347284, e-mail hubert.cardon@med.kuleuven.ac.be)

Congenital disorders of glycosylation (CDG) are a newly delineated group of inherited multisystem disorders associated with abnormal glycosylation of glycoproteins (1). In CDG group I, which includes all defects in N-glycan assembly (2), the subgroup CDG type Ib, attributable to phosphomannomannose isomerase (EC 5.3.1.8) deficiency, is treatable by mannose supplementation (3). Monitoring of this treatment necessitates the availability of methods to quantify D-mannose in serum.

The determination of mannose in serum is hampered by the presence of an ~100-fold excess of glucose. Jolley et al. (4) used high-resolution liquid chromatography, whereas Alol (5) used gas-liquid chromatography after treating the sera with glucose oxidase. Soyama (6) and Akazawa et al. (7) used enzymatic methods that involved treatment with glucose oxidase. In all of these studies, the presence or elimination of glucose remained critical. Pitkänen and Kanninen (8) were able to measure mannose using gas chromatography–mass spectrometry. However, this method is not suitable for routine purposes. The assay proposed by Etchison and Freeze (9) involves the elimination of glucose by glucokinase (EC 2.7.1.2), followed by the removal of anionic products by a subtle ion-exchange chromatography step. Finally, the mannose concentration is determined enzymatically.

We investigated whether capillary electrophoresis (CE) of fluorophore-labeled carbohydrates was an appropriate method. In the resulting procedure, D-mannose can be determined in small amounts of serum in the presence of...
p-glucose without loss of selectivity, accuracy, and sensitivity.

1-Aminopyrene-3,6,8-trisulfonate (APTS) was purchased from Lambda Fluoreszenz Technologie GmbH. Sodium cyanoborohydride (1.0 mol/L in tetrahydrofuran) was obtained from Aldrich Chemical. Boric acid and ethanol were from Merck; glucokinase and the carboxydrates were obtained from Sigma. All reagents used were of analytical grade. p-Mannose, r-rhamnose, and p-glucose are hereafter referred to as mannose, rhamnose, and glucose in the text.

CE separations were performed in fused-silica capillaries from Polymicro Technologies [20 cm effective length \( \times \) 20 \( \mu \)m (i.d.)] at 20 °C on a Beckman Instruments P/ACE 5000 system and monitored on column with a Beckman laser-induced fluorescence detector using an argon-ion laser with excitation at 488 nm and emission at 520 nm. Borate-NaOH (135 mmol/L, pH 10.2) was used as the running buffer (10). After a long or overnight period of inactivity, the capillary was washed sequentially with 0.1 mol/L NaOH, water, 10 mmol/L EDTA, water, and 1 mol/L phosphoric acid for 2 min and finally with water for 5 min. Before each analysis, the capillary was reconditioned for 3 min with running buffer followed by a sample injection for 3 s corresponding to a sample volume of 0.15 nL (0.2% of the effective capillary volume). Finally, water was injected for 1 s to rinse the capillary end and the electrode. Separation was performed at 25 kV for 10 min. At the end of each analysis, the capillary was rinsed with water, 1 mol/L phosphoric acid, and water for 30 s, 3 min and 1 min, respectively. Solutions were filtered through Millipore filters (0.45 nm membrane) before analysis. The rinsing steps before and between analyses to obtain a reproducible electroendosmotic flow are an essential part of the separation procedure.

Cold ethanol (90 \( \mu \)L; −20 °C) was added to a mixture of 5 \( \mu \)L of 1 mmol/L rhamnose and 25 \( \mu \)L of serum. After 30 min at −20 °C, samples were centrifuged at 13,000 \( g \) (15 min; 4 °C), and the supernatant was evaporated. The derivatization procedure was essentially adapted from the method first described by Jackson (11) and discussed by Guttman et al. (12). Briefly, 2 \( \mu \)L of 0.1 mol/L APTS and 0.6 mol/L citric acid (13) in water and 2 \( \mu \)L of 1.0 mol/L sodium cyanoborohydride in tetrahydrofuran were added to the dried sample. The reductive amination was allowed to proceed for 90 min at 55 °C. Finally, the mixture was diluted with water to 400 \( \mu \)L for CE analysis. Data are represented by their estimated value ± SE.

The migration process in CE analysis, using fused-silica capillaries at alkaline pH, is dominated by the velocity of the electroendosmotic flow. Therefore, constant migration times require stable and reproducible electroendosmotic flows. By adding a rinsing step with 1 mol/L phosphoric acid to the separation procedure, we reduced the CV of the migration time (MT) for mannose to 2.5% \(( n = 13)\). Residual variation of the MT was further decreased by expressing the MT of the analytes in terms of the MT of the internal standard (IS). Consequently, the CV of mannose was further reduced to 0.07%. This relative migration time was therefore used as an identification parameter.

For the determination of mannose, rhamnose can be used as IS because both compounds are well separated (Fig. 1A) and serum does not contain contaminating analytes. In the presence of 5 mmol/L glucose, mannose remains completely separated in the range from 5 to at least 500 \( \mu \)mol/L. Under the present assay conditions, the lower detection limit for mannose can be defined at 2.5 \( \mu \)mol/L.

A linear relationship was obtained between mannose at concentrations of 5–500 \( \mu \)mol/L, in the presence of 200 \( \mu \)mol/L IS and 5 mmol/L glucose, and the corresponding peak area expressed relative to the peak area of the IS. For \( n = 12 \), the intercept, slope, and correlation coefficient were −0.003 ± 0.006, 0.005 ± 0.00003, and 0.999 ± 0.015, respectively.

The recovery of mannose added to the sample at different concentrations is shown in Table 1. The results indicate that the protein precipitation step does not significantly alter the mannose concentration.

The separation of mannose and glucose in the calibra-

Fig. 1. Electropherograms obtained in the mannose assay for serum without (A) and with (B) glucose elimination according to the method of Etchison and Freeze (9).

Peaks: 1, r-rhamnose (200 \( \mu \)mol/L); 2, p-mannose; 3, p-glucose. p-Glucose is not completely removed. CE conditions: fused-silica capillary [20 cm effective length \( \times \) 20 \( \mu \)m (i.d.); light source, argon-ion laser (excitation at 488 nm and emission at 520 nm); running buffer, 135 mmol/L sodium borate (pH 10.2); outlet, cathode; applied potential, 25 kV.
tion mixtures indicated that no preliminary elimination of glucose was required. Fig. 1B shows the electropherogram after glucose elimination according to the method of Etchison and Freeze (9). Glucose is largely removed but not completely absent. The mannose concentrations in this sample with and without glucose elimination were 44.7 ± 0.2 and 40.2 ± 0.3 μmol/L, respectively. The glucose concentration obtained after glucose elimination was 20.4 ± 0.5 μmol/L. A small but significantly higher mannose concentration was found when the glucose elimination procedure was included (n = 5; P < 0.001). We determined the serum mannose concentration in 26 pediatric controls and obtained a mean concentration of 30 ± 10 μmol/L (range, 7–45 μmol/L), whereas Panneerselvam et al. (14), using the enzymatic procedure, reported a mean mannose concentration of 54.5 ± 14.5 μmol/L (n = 32).

The electropherogram obtained during CE analysis is very simple and reflects the selectivity of the derivatization step. The number of reducing carbohydrates in serum seems to be limited to mannose and glucose, but we do not know why. Fructose, which immediately follows glucose in the electropherogram and is present in serum at about 0.5% of the blood content, whereas Panneerselvam et al. (14), using the enzymatic procedure, reported a mean mannose concentration of 54.5 ± 14.5 μmol/L (n = 32).

The present work was supported by the Funds for Scientific Research–Flanders (Grant G.0305.98).

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References

Table 1. Recovery of mannose added to the serum sample at various concentrations.

<table>
<thead>
<tr>
<th>Mannose concentration, μmol/L</th>
<th>Mean recovery, % (n = 3)</th>
<th>SE, %</th>
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<tbody>
<tr>
<td>10</td>
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