Błonski et al. (10) of 2.0 μg/L and Holt et al. (12) of <3.0 μg/L. HPLC-MS has no detectable interference with respect to endogenous compounds. The quantification limits based on acceptable imprecision, recovery, and ability to discriminate from potential interferences within the calibration range of the MEIA are 3.0 μg/L (the lower limit of quantification) to 22.0 μg/L. We have demonstrated that a higher limit of quantification by MEIA (150.0 μg/L) can be achieved through dilution, which hedges the HPLC-MS advantage of a wider quantification range.

The sirolimus concentrations from a total of 125 blood samples obtained from 25 renal transplant patients receiving sirolimus therapy were measured by both methods and are shown in Fig. 1A. The range of whole blood sirolimus concentrations measured by HPLC-MS was 2.1–25.6 μg/L; by MEIA the range was 3.2–41.6 μg/L. Regression analysis of the data yielded the equation for the line of best fit as: MEIA = 1.39 (± 0.04) × HPLC-MS + 1.30 (± 0.46) μg/L (r = 0.951; S_{\text{Sy|x}} = 2.46; n = 125). These data were further analyzed by the method described by Bland and Altman (13). A plot of the difference in sirolimus concentrations (MEIA minus HPLC-MS) against the mean sirolimus concentration as measured by both methods is shown in Fig. 1B. The line of best fit and 95% confidence intervals have been shown. The equation for the line of best fit is: MEIA – HPLC-MS = 0.38 (± 0.03) × mean concentration + 0.36 (± 0.39) μg/L (r = 0.779; S_{\text{Sy|x}} = 2.02). Based on the 95% confidence intervals, it can be determined that at mean concentrations of 10 and 30 μg/L, MEIA will give corresponding estimates of 10.1–18.6 and 37.8–46.2 μg/L, respectively. The mean bias expressed as a percentage ± the SD between the two methods for renal transplant patient samples was 41.9% ± 15.3%.

Unlike HPLC-mass spectrometers, the IMx analyzer is a common instrument in clinical laboratories and is less labor intensive, with shorter sample preparation and analysis time. Thus, if the analytical performance in relation to sirolimus patient samples was similar between the methods, then MEIA could be used as an alternative to HPLC-MS. The acceptable imprecision and recovery data for HPLC-MS for the three quality controls confirm our previous validation (4). Similarly, MEIA had acceptable quality control imprecision and recovery data, which is consistent with reported MEIA studies that were based on a smaller number of batches (10, 12). The positive mean bias of 41.9% is potentially attributable to the cross-reactivity of the MEIA antibody with circulating sirolimus metabolites. The overestimation we observed was greater than that cited by Holt et al. (12), who reported a mean bias of 19.8%. The differences in the observed overestimation between these studies may be attributable to variations in the metabolite patterns in the renal transplant groups investigated.

We conclude that both HPLC-MS and MEIA (Mode 1A) have suitable analytical performance, based on weighed-in quality control samples, within the clinically relevant range (14). However, as with other immunoas-

Enzymatic Assay of Calcium in Serum with Phospholipase D

Yoshitaka Morishita,1 Yoshitsugu Iinuma,1 Nobuo Nakashima,1 Akira Kadota,2 Akira Mïke,2 and Toshio Tanade2

(1) Department of Clinical Laboratory, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan; (2) Research Laboratories, Kyowa Medex Co., Ltd., 600-1 Minamimishiki, Nagaiizu-cho, Suno-gun, Shizuoka 411-0932, Japan; * author for correspondence: fax 81-52-744-2613, e-mail yinuma@tsuru.med.nagoya-u.ac.jp)

Various methods for determining calcium in body fluids have been reported. Atomic absorption spectrophotome-
try (AAS) (1) is a most reliable method; however, because the AAS instrument is expensive and maintenance is difficult, it is unsuitable for routine assay. Spectrophotometric methods by use of α-cresolphthalein complexone (CPC) (2–6), which is based on chelation, is now widely available to assay calcium in clinical laboratories. Several enzymatic methods that use porcine pancreatic α-amylase (EC 3.2.1.1) (7) or phospholipase D (PLD; EC 3.1.4.4) (8) to measure calcium have also been developed. These methods are based on the activation of the enzyme by calcium. On the other hand, Kimura et al. (9) reported a kinetic assay for calcium in serum that uses urea amidolyase (EC 6.3.4.6) based on the inhibition of the enzyme by calcium. Previously reported method for determining free calcium ions in serum using PLD required appropriate ionic strength for its reaction (8). We have established a new and stable enzymatic method for determining calcium in serum that uses PLD, stabilizing calcium status by adding a large excess of bovine albumin to the reaction system.

The proposed enzymatic method for determining calcium in serum is based on the following sequence of reactions:

\[
\text{Diacylphosphatidylcholine} + H_2O \xrightarrow{\text{Phospholipase D}} \text{Calcium} \rightarrow \text{Choline + diacylphosphatidic acid}
\]

\[
\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{Choline oxidase}} \text{Betaine + 2H}_2\text{O}_2
\]

\[
2H_2O_2 + 4\text{-aminoantipyrine} + \text{EMSE} \xrightarrow{\text{Peroxidase}} \text{Quinone dye (purple)}
\]

Diacylphosphatidylcholine (DAPC) is used for the PLD reaction substrate. The reaction rate at which PLD hydrolyzes DAPC into choline and diacylphosphatidic acid depends on the amount of calcium in serum. Choline oxidase (COD) produces betaine and H$_2$O$_2$ from choline and O$_2$. Peroxidase (POD) produces quinone from H$_2$O$_2$ with 4-aminoantipyrine (4-AA) and N-ethyl-N-(3-methylphenyl)-N’-succinyl-ethylendiamine (EMSE). The absorbance at 546 nm for the quinone dye is measured.

This proposed method and the conventional methods were performed with the Hitachi Model 7170 automated analyzer.

PLD (10.7 kU/g, from Streptomyces scabies) was purchased from Kyowa Hakko Kogyo; the Determiner PL kit was from Kyowa Medex; EMSE was from Daito Chemix; acetonitrile and silica gel 60-F254 thin-layer chromatography plates were from Kanto Chemical; and glyceroxophosphatidylcholine was from Nippon Oil Fats. COD (18.1 kU/g, from Alcaligenes sp.) and POD (110 kU/g, from horseradish) were purchased from Toyobo. HEPES was purchased from Dojindo Laboratories; 4-AA, ascorbic acid, acetic anhydride, MgCl$_2$, CaCl$_2$, KH$_2$PO$_4$, ZnCl$_2$, FeCl$_3$, CuSO$_4$·5 H$_2$O, sodium citrate, dipotassium EDTA, and silica gel 60-80 were from Wako Pure Chemical Industries; bilirubin and bovine albumin were from Sigma; and Intralipid 10% was from Kabé Vitrum AB. Chelex 100 resin was purchased from Bio-Rad Laboratories.

Serum specimens were collected from patients in Nagoya University Hospital. We obtained informed consent from patients to measure their serum calcium for this study.

The synthesis and purification of DAPC was as follows: Glycerophosphatidylcholine (12 g) was dissolved with 120 mL of acetic anhydride and allowed to stand at 80 °C for 4 h. The 120-mL reaction mixture was applied to a silica gel column (80 × 800 mm; gel volume, 4 L), and eluted with 600 mL/L acetonitrile at 20 mL/min as 100-mL fractions. The DAPC-rich fraction was collected and concentrated by evaporator to yield 11.8 g of DAPC. The detection of DAPC was as followed: 20 µL of every fraction was applied to silica gel thin-layer chromatography plates, and the chromatogram was developed with 600 mL/L acetonitrile. Determiner PL kit was added to the silica gel thin-layer chromatography plates for the detection of DAPC, with reaction at 37 °C for 10 min.

PLD solution, bovine albumin solution, and pooled human serum without calcium were prepared as follows: calcium and other metals were removed from the 30 kU/L PLD solution, 20 g/L bovine albumin solution, and pooled human serum by Chelex 100 resin batch method, with ~1 g of resin added to ~10 mL of the PLD, the bovine albumin solution, and the pooled human serum. The solution was stirred gently for ~30 min and decanted from the resin. Each supernatant was repeatedly treated with Chelex 100 resin in the same way. We confirmed the removal of the calcium in this treated solution by AAS, the CPC method, and another enzymatic method.

Reagent 1 contained 3.0 kU/L PLD (calcium free), 3.0 kU/L COD, 40 kU/L POD, 2.5 mmol/L EMSE, and 2.0 g/L bovine albumin (calcium free) in 100 mmol/L HEPES buffer (pH 7.5). Reagent 2 contained 3.0 mmol/L DAPC and 6.4 mmol/L 4-AA in 100 mmol/L HEPES buffer (pH 7.5).

Calibrators were sera containing 1.5, 2.0, 2.5, 3.0, and 4.0 mmol/L calcium, which were prepared by dissolving CaCl$_2$ in the calcium-free pooled human serum. We confirmed that the calcium values of calibrators were exactly the same as those examined by AAS, the CPC method, and another enzymatic method.

The assay was performed with the Rate A mode of the Hitachi 7170 automated analyzer. Three microliters of calcium calibrators (0–4.0 mmol/L) or patient serum and 240 µL of reagent 1 were poured into the reaction cuvettes; after the mixtures were incubated for 5 min at 37 °C, 60 µL of reagent 2 was added. The reaction rate was
measured at 25–34 time points (7.35–10.0 min) at 546 nm. The calcium values of sera from patients were calculated from the calibration curve obtained with 0–4.0 mmol/L calcium calibrators. The six-point calibration curve was a sigmoid curve that corresponded to the calcium values (Fig. 1A). However, the color development of the calcium aqueous solution was impaired; therefore, calcium aqueous solution could not be used for the calibrators.

We also measured the concentrations of calcium in sera from patients by a CPC method (Calcium-HR II kit from Wako Pure Chemical Industries), and another enzymatic method (Diacolor Ca kit from Ono Pharmaceutical) based on the activities of porcine pancreatic \( \alpha \)-amylase. The measurement of calcium by AAS (1) was carried out on the Hitachi Z-6100 Polarized Zeeman Atomic Absorption Spectrophotometer.

Optimization studies of this proposed method were carried out with the calibrator (2.50 mmol/L calcium), and two patient sera (1.50 and 2.10 mmol/L calcium). The effects of pH on the calcium determination were examined in 100 mmol/L HEPES buffer at various pH values (6.5, 7.0, 7.5, 8.0, and 8.5). The maximum reaction rate \( (A_{10 \text{ min}} - A_{7.35 \text{ min}}) \) of the calibrator was observed at pH 7.5, and the calcium values of the patients’ sera were almost stable at various pHs; therefore, pH 7.5 HEPES buffer was used. The reaction rate of the calibrator increased with increasing PLD activity and DAPC concentration. We chose the lowest PLD activity and DAPC concentration, 2.4 kU/L and 0.6 mmol/L, respectively, in the range of minimum variations of calcium values of the patients’ sera.

We next added a large excess of bovine albumin to the reaction system to avoid the effects of albumin contained in specimens. With increasing bovine albumin concentration, the reaction rate of the calibrator gradually decreased. Bovine albumin (1.6 g/L) was added to the reaction system because this concentration brought about minimum variation of the calcium values of the patients’ sera. A decrease of calcium values caused by albumin (20–100 g/L) added to calcium aqueous solution was therefore prevented.

We also determined the optimal concentrations of COD, POD, 4-AA, and EMSE in the same way; the optimal concentrations were 2.4 kU/L for COD, 32 kU/L for POD, 1.28 mmol/L for 4-AA, and 2.0 mmol/L for EMSE.

Typical time courses of reactions were carried out for the calibrator (2.50 mmol/L calcium), a patient’s serum (2.32 mmol/L calcium), and reagent blank by this proposed kinetic calcium assay. When reagent 2 was added to the mixtures of serum and reagent 1, the color for produced quinone dye was developed. A lag phase up to \( \sim 60 \text{ s} \) was observed, but after that the absorbance of color development was linear. The slight increase of absorbance for the reagent blank was recognized. We chose to measure between 7.35 and 10.0 min.

The precision of our assay were determined by using of pooled serum L, pooled serum M, and pooled serum H, which contained 1.92, 2.25, and 2.65 mmol/L calcium, respectively. Within-assay CVs were determined with 20 replicates of each sample, and day-to-day CVs were

<table>
<thead>
<tr>
<th>Table 1. Precision of the proposed calcium assay.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pooled serum L</strong></td>
</tr>
<tr>
<td>Within-assay (n = 20)</td>
</tr>
<tr>
<td>Mean, mmol/L</td>
</tr>
<tr>
<td>SD, mmol/L</td>
</tr>
<tr>
<td>CV, %</td>
</tr>
<tr>
<td>Day-to-day (n = 10)</td>
</tr>
<tr>
<td>Mean, mmol/L</td>
</tr>
<tr>
<td>SD, mmol/L</td>
</tr>
<tr>
<td>CV, %</td>
</tr>
</tbody>
</table>
determined from assays performed on 10 different days. As shown in Table 1, the within-assay CV was 0.45–0.55%, and the day-to-day CV was 1.2–1.8%.

The recoveries were determined using pooled human serum (2.27 mmol/L calcium) supplemented with calcium (0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 mmol/L). Recoveries were 92.0–108.0% (mean, 102.6%).

The influences of various substances on this calcium determination method were examined with a pooled human serum (2.00 mmol/L calcium). The interference [interference (%) = (calcium value of test – calcium value of control)/calcium value of control × 100] was calculated. We found no interference with the calcium determination from ascorbic acid up to 1.14 mmol/L, bilirubin up to 0.342 mmol/L, Intralipid up to 200 mmol/L, KI up to 10.0 mmol/L, FeCl3 up to 1.0 mmol/L, CuSO4 up to 1.0 mmol/L, ZnCl2 up to 0.1 mmol/L, all in serum (less than ±3%). On the other hand, the calcium value decreased with calcium-chelating agents such as 1.0 g/L sodium citrate (−8.7%) or 1.0 g/L EDTA (−92.3%) used for banked blood, and with 1.0 g/L hemoglobin (−7.0%); calcium increased with 5.0 mmol/L KH2PO4 (8.5%) in serum. However, plasma from 20 patients prepared with heparin showed no interference. We next measured the calcium values of sera from patients with multiple myeloma. Only 1 of the 11 sera showed a decreased value (−7.6%).

The color developments of calibrators and patient serum decreased ~20% by use of reagents 1 and 2 that were kept at 4 °C for 10 days; however, the calcium values of sera from patients were not changed.

We examined the correlation between this proposed method and AAS for 42 patient serum samples (Fig. 1B). The correlation between calcium values obtained with this proposed method (y) and those obtained with AAS (x) was: y = 1.029x − 0.035 mmol/L; r = 0.976; Sy|x = 0.051 mmol/L. We also determined the correlation independently for 68 patient sera samples between this proposed method (y) and the CPC method [Calcium-HRII kit (x); y = 1.036x − 0.031 mmol/L; r = 0.959; Sy|x = 0.067 mmol/L] and the enzymatic method [Diacolor Ca kit (x); y = 1.018x + 0.001 mmol/L; r = 0.957; Sy|x = 0.068 mmol/L].

We investigated the enzymatic kinetic assay for measuring calcium in serum with the Hitachi 7170 automated analyzer by PLD, which requires calcium for activation. The amount of choline produced is proportional to that of the calcium and is determined with COD and POD in the presence of 4-AA and EMSE as the increase in absorbance at 546 nm for the quinone dye.

Among the several substances, hemoglobin, KH2PO4, sodium citrate, and EDTA influenced the results. Calcium values decreased with hemoglobin concentrations >0.5 g/L; therefore, hemolyzed serum is not a suitable specimen. On the other hand, calcium values increased with KH2PO4 concentration >3.0 mmol/L; therefore, in the case of high concentration of phosphorus in serum, this assay system indicates a slightly higher serum calcium value. Furthermore, calcium values decreased with sodium citrate or EDTA used for anticoagulant. Heparin-treated plasma was suitable for this calcium assay because heparin caused no significant interference. Only 1 of the 11 sera from patients with multiple myeloma showed a decreased value (−7.6%). Calcium-binding immunoglobulins, sometimes found in patients with multiple myeloma, may interfere this proposed calcium assay.

Calcium exists in normal serum in three forms: free calcium ions (calcium, −45–50%), albumin-bound calcium (−45–50%), and calcium complexed to anions (−5–10%). Tabata et al. (8) demonstrated a Ca2+ assay system based on the same principle that we investigated for the calcium assay in this report. They used phosphatidylcholine as substrate for PLD. DAPC is suitable for a kinetic assay because its Km value, 2.80 mmol/L, is large at pH 7.5 compared with the Km value of 0.053 mmol/L for phosphatidylcholine. Calcium ions are easily combined or separated with albumin and anions; therefore, the ionic strength is easily altered. This alteration may make the Ca2+ assay system described by Tabata et al. (8) unsuitable for routine use. In our assay system for calcium, a large excess of bovine albumin was added to the reaction system to maintain the calcium values. This added bovine albumin successfully prevent calcium values variation related to the albumin concentration in the serum.

This method had good precision, good analytical recovery, and good correlation with AAS and the conventional methods. This new enzymatic method can be rapidly and easily performed, which makes it suitable as a routine procedure for serum calcium determination.

We thank K. Kubono of SRL, Inc. (Tokyo, Japan) for measuring the calcium in the patient sera by AAS.

References