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Carryover between Internal Standards in the Nova 2 Ionized Calcium Analyzer

To the Editor:

A Nova 2 ionized calcium analyzer (Nova Biomedical, Newton, MA 02164) with the new electrode system, which has a replaceable inner element, was evaluated before being introduced into the clinical laboratory. An evaluation of the instrument with a factory-sealed electrode has been published (1). An analytical problem was noted which, when corrected by the manufacturer, should improve both accuracy and precision.

All operations were carried out with the sampler in the “up” position for accepting samples from syringes or capillary tubes. Imprecision was assessed by use of aliquots of reconstituted Versatol control sera (General Diagnostics) which had been stored in the deep-freeze and were chosen because they contain no bicarbonate and therefore have an acceptable pH of about 7.3. Both within-day and between-day imprecision at high, medium, and low concentrations of ionized calcium were satisfactory by conventional standards (Table 1) and between-day imprecision was significantly less than that reported for a medium concentration in a modified human serum pool with the old electrode system (2).

A formal evaluation of carryover from a high to a low concentration in serum showed no measurable sample interaction. Accuracy was assessed by using the external aqueous standards supplied by the manufacturer. While most readings fell within the manufacturer’s “acceptable limits” of ±2% of the quoted values, the mean value was significantly low for the 0.5 mmol/L standard and significantly (p < 0.05) high for 1.5 mmol/L and 2.5 mmol/L standards. Internal standards containing 1.0 and 2.0 mmol/L calcium per liter are used during the automatic calibration cycle. Aliquots were removed from the fluids pack and measured as unknown external samples. The 1.0 mmol/L internal standard gave accurate readings, but 59 readings of the 2.0 mmol/L internal standard during nine days had a mean value of 2.014 mmol/L (CV 1.1%), a mean error of +0.7% (p < 0.001). The magnitude of the error varied from calibration to calibration. There was always a close linear relationship between the readings given by the 0.5, 1.0, 1.5, 2.0, and 2.5 mmol/L standards, but the slope of the line varied between calibrations, appearing to swing about 1.0 mmol/L.

To investigate the problem further, I made duplicate readings of a Versatol A control serum after each of 14 calibrations. The mean ionized calcium concentration was 1.183 mmol/L with a CV of 0.69%, nearly twice that found with 22 measurements of the same material made after a single calibration (Table 1). Analysis of variance showed that differences between calibrations made a highly significant contribution to analytical imprecision (F = 13.8; p < 0.001).

A colleague, John Thompson, suggested that the cause might be that the 2.0 mmol/L internal standard was variably contaminated with 1.0 mmol/L standard during calibration because too little 2.0 mmol/L standard was aspirated to adequately wash out the septum cavity, probe, sample line, and electrode system, all of which contain 1.0 mmol/L internal standard before the machine is operated. Carryover into the 2.0 mmol/L internal standard was confirmed visually by replacing the 1.0 mmol/L internal standard with a colored solution.

The effect of improving washout was shown by first making duplicate measurements of a Versatol A Alternate control serum after each of 12 calibrations and then repeating the measurements, but before each calibration manually washing out the septum space, probe, and electrode system with 2.0 mmol/L internal standard. As anticipated, the mean value after washout calibration was lower and the overall imprecision was significantly less (Table 2).

Clearly, the manufacturers should amend the software of the integral microprocessor to increase the volume of 2.0 mmol/L internal standard aspirated during calibration. The Nova 2 ionized calcium analyzer would then show improved accuracy and decreased imprecision for all ionized calcium concentrations, with a slightly lower and narrower reference range.

References

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\text{Table 1. Within-Calibration and Between-Day Imprecision} \\
\begin{array}{|c|c|c|c|}
\hline
\text{Within-day, within-calibration} & \text{IONIZED CALCIUM, mmol/L} & \text{Mean} & \text{SD} & \text{CV, %} \\
\hline
\text{Versatol A Alternate} & 18 & 2.090 & 0.0117 & 0.56 \\
\text{Versatol A} & 22 & 1.800 & 0.0044 & 0.37 \\
\text{Versatol Pediatric} & 18 & 0.594 & 0.0039 & 0.66 \\
\hline
\text{Between-day, between-calibration} & \text{IONIZED CALCIUM, mmol/L} & \text{Mean} & \text{SD} & \text{CV, %} \\
\hline
\text{Versatol A Alternate} & 15 & 1.916 & 0.0308 & 1.61 \\
\text{Versatol A} & 15 & 1.168 & 0.0107 & 0.92 \\
\text{Versatol Pediatric} & 15 & 0.625 & 0.0113 & 1.81 \\
\hline
\end{array}
\]

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\text{Table 2. Effect of Improved Wash-out before Calibration on} \\
\text{Contribution of Calibration to Imprecision} \\
\begin{array}{|c|c|c|c|}
\hline
\text{n} & \text{IONIZED CALCIUM, mmol/L} & \text{CV, %} & \text{Analysis of variance: between calibrations} \\
\hline
\text{With automatic wash-out} & \text{Mean} & \text{SD} & F & p \\
\hline
24 & 1.933^a & 0.0181^b & 0.94 & 12.1 & <0.001 \\
\hline
\text{With manual wash-out} & \text{Mean} & \text{SD} & F & p \\
\hline
24 & 1.910^a & 0.0082^b & 0.43 & 4.1 & 0.014 \\
\hline
\end{array}
\]

* t = 5.85; p < 0.001. \( \text{F} = 4.85; p < 0.001. \)
Relative Merits of One- and Two-Dimensional TLC of Phospholipids in Amniotic Fluid

To the Editor:

Gross et al. (1) recently compared one-dimensional (1-D) and two-dimensional (2-D) thin-layer chromatography (TLC) of amniotic fluid phosphatidylglycerol (PG). Several of their observations and conclusions do not agree with our experience. My greatest concern is with respect to PG quantitation. In addition, the usefulness of 1-D and 2-D TLC for meconium-contaminated or blood-contaminated specimens needs discussion.

One-dimensional TLC with their solvent system (1) gives complete separation of lecithin, sphingomyelin, phosphatidylinositol, and phosphatidylglycerol—the compounds of interest for fetal lung maturity testing. This agrees with our experience, although we use a different 1-D solvent system (2). Separation of phosphatidylethanolamine (PE) and phosphatidylserine (PS) is achieved by 2-D TLC. It is uncertain at this time what role, if any, PE and PS play in the mature surfactant. Therefore, although separation of these compounds may be noteworthy, it does not offer information relevant to the prediction of fetal lung maturity.

To compare 1-D and 2-D TLC, we determined the amount of PG (expressed as the percentage of total phospholipid) for four amniotic fluid specimens by our 1-D method and their 2-D method. The 1-D method gives higher percentages of PG than does the 2-D method (Table 1). Attempting to explain this difference, we refined this comparison by doing 1-D and 2-D TLC of known amounts of pure PG. One microgram of PG is detectable after 1-D TLC, but not after 2-D TLC (Figure 1). In a range from 1 to 5 μg, the amount of PG detectable by the densitometer (expressed as integrator units) after 2-D TLC is always significantly less (37% to 100% less) than that measured by the 1-D method (Table 2). Thus, the higher PG values obtained by 1-D TLC are unlikely to be due to co-migrating substances. Rather, we attribute this difference to the increased diffusion after the second dimension of chromatography. In contrast to the results reported by Gross et al. and as shown in Figure 1, we find that 1-D TLC results in a better, less-diffuse PG spot. This is expected, as any diffusion resulting from the PG spot traveling in the first dimension is unlikely to be corrected by chromatography at a right angle in the second dimension. In fact, the spot will be further diffused in the second dimension in part as a function of the distance traveled by the compound (3), and PG in low concentrations will become indistinguishable from the background of the TLC plate after charring. The fact that we can observe PG at low concentrations by 1-D TLC and not by 2-D TLC is an important point. The possibility exists that the 2-D method would miss small quantities of PG in amniotic fluid, which may result in unnecessarily prolonging a pregnancy.

The basis for the statement by Gross et al. that ammonium sulfate in the silica gel H matrix inhibits phospholipid charring after 1-D TLC is unclear. We have not observed this inhibition. With silica gel H plates containing ammonium sulfate, phospholipids are charred after 1-D or 2-D TLC by heating the plate at 300 °C, much like the charring observed after sulfuric acid spraying and heating.

Table 1. Amniotic Fluid Phosphatidylglycerol (PG)

<table>
<thead>
<tr>
<th>Patient</th>
<th>1-D TLC</th>
<th>2-D TLC</th>
<th>2-D TLC/1-D TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>L</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>2.5</td>
<td>0.3</td>
<td>0.12</td>
</tr>
<tr>
<td>H</td>
<td>4.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Expressed as the percentage of total phospholipid.

Even though spraying these plates is not essential for visualization, we find that spot intensity increases and reproducibility of quantitation improves when phospholipids are charred after a sulfuric acid spray. This is true whether the plates are subjected to 1-D or 2-D chromatography, so long as the plates are dried. With 2-D chromatography, however, complete drying of the plates subjected to chromatography in a solvent system containing tetrahydrofuran, methyal, methanol, and ammonium hydroxide takes a relatively long time (over 1 h at 100 °C) vs <2 min for the complete drying of plates subjected to 1-D chromatography in chloroform/methanol/water. If after 2-D chromatography an incompletely dry plate is sprayed with sulfuric acid, then the entire plate is charred. The high variation (CV 21%) Gross et al. see for PG may result from not spraying their 1-D plates. In fact, when we did repetitive L/S ratios and PG determinations on a pooled amniotic fluid sample with acid-sprayed plates, we observed L/S ratios of 5.23 ± 0.49 (CV 9.4%, n = 21) and PG percentages of 4.87 ± 0.55 (CV 11.3%, n = 19).

Amniotic fluid contaminated with blood or meconium presents a problem. Gross et al. suggest that 2-D TLC may be better than 1-D TLC when samples are so contaminated. The L/S ratio of

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**Fig. 1. Comparison of 1-D TLC and 2-D TLC for detection of phosphatidylglycerol**

1, 2, and 3 μg of PG were chromatographed by our 1-D method (2) (A), and their 2-D method (1) (B-D). In addition to PG, standard mixtures of phospholipids (PG and PL, PS, PE, PS, PC, PG, and PL) were applied to the TLC plates for 1-D TLC. For the second dimension of TLC, PG and PL were also applied. Charring for each plate was done according to the respective methods (1, 2).

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**Table 2. Phosphatidylglycerol Quantitation**

<table>
<thead>
<tr>
<th>PG, μg</th>
<th>1-D TLC</th>
<th>2-D TLC</th>
<th>2-D TLC/1-D TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.8</td>
<td>0.5</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>0.9</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>4.0</td>
<td>0.63</td>
</tr>
</tbody>
</table>

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**Table 3. Phosphatidylglycerol Quantitation**

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<tr>
<th>PG, μg</th>
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<th>2-D TLC/1-D TLC</th>
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**Table 4. Phosphatidylglycerol Quantitation**

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