Failure of Processed Quality-Control Materials to Detect Interference from Labile Form of Hemoglobin A1c

To the Editor:
Our laboratory uses the Bio-Rad Variant™ system to measure hemoglobin A1c. The procedure begins with an autodiluter aspirating 5 μL of blood and dispensing it with 1 mL of hemolyzing reagent. The hemolyzing reagent also contains chemicals to remove the Schiff base-labile form (pre-A1c) (1).

A technologist incorrectly connected wash solution to the autodiluter. Bio-Rad’s hemolysis reagent, wash solution, and buffer solutions all come in rectangular clear plastic bottles of identical shape and size. The bottles are labeled on only one side, and that side of the hemolysis reagent bottle lies next to the autodiluter (to minimize the distance the plastic tubing runs between the front of the autodiluter and the bottle cap, allowing the tubing to reach the bottom of the bottle). Therefore, no other technologists saw that the wrong solution was being used as hemolyzing reagent for 3 weeks. Calibration and quality-control results were acceptable on every run because those stabilized preparations contain no labile form.

After the error was discovered, we retested the 618 available specimens (specimens older than 14 days had been discarded) whose incorrect results (A1c plus pre-A1c) exceeded our A1c reference range. In the statistics that follow, results from specimens older than Bio-Rad’s recommended storage time of 7 days were excluded. The mean difference between the original results (range, 6.6–17.0%) and the corrected results (after exclusion of three outlier results >4.5 SD from mean) was 1.0% (n = 292; range, 0.2–2.1%; median, 0.9%; SD = 0.36%; near-gaussian distribution with a skewness of 0.81 and kurtosis of 0.24). These results contradict Bio-Rad’s claim that “Schiff base seldom exceeds 1.0% of total hemoglobin in nondiabetic and diabetic individuals” (1). The difference (presumed labile form) correlated linearly with the A1c percentage (presumed labile form = 0.116[A1c] + 0.049; r² = 0.327; ANOVA F-value = 25.7, P < 0.0001; power = 1.000).

To assess whether part of the difference between the original incorrect results and the repeated results with the appropriate hemolyzing reagent was attributable to loss of A1c with storage, I examined the relationship between the paired differences and the intervals between specimen receipt and the repeat assay. Blood tubes had been stored at 2–8 °C for 1–14 days. There was no significant correlation between storage times and A1c differences (n = 616, 3 outliers excluded; linear regression, r² = 0.03; ANOVA F-value = 0.80; P = 0.37; power = 0.139). This implies that the differences between the original incorrect results and the repeated A1c results were attributable to the removal of hemoglobin labile form and not to degradation of A1c over time. Differences in the pH and buffer composition of the hemolysate caused by use of wash solution instead of hemolyzing reagent could have affected the A1c results in ways unrelated to labile form. However, chromatography was not discernibly affected by the inadvertent use of wash solution: Retention times, peak widths, and baselines were unchanged.

Glycohemoglobin differences of 0.5% or more can be clinically significant. Only 6 of the 618 retested specimens had differences <0.5%. We delivered 612 corrected A1c reports. We offered free specimen recollection and A1c testing to patients with increased A1c results whose specimens had been discarded before discovering the error.

To avoid this problem in the future, we added a patient control at the beginning of each hemoglobin A1c run. The patient control is a repeat analysis of a specimen from a previous run. We require the repeat result to be within 0.4% of the original. We asked Bio-Rad to change its A1c reagent packaging by placing labels on two or three sides of the bottles and to color-code the bottles, bottle caps, or reagents. We also advised Bio-Rad to issue an addendum to the A1c instruction manual describing the inability of stabilized control materials to assay labile form removal and recommending the additional use a fresh or frozen patient control to assure that the hemolysis reagent removes the labile form.

References

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Sample Requirements for Plasma Renin Activity and Immunoreactive Renin

To the Editor:
Renin, an aspartic protease, is rate limiting in the generation of angiotensin (Ang) II, which is a key regulator in the maintenance of salt and water balance by regulating aldosterone secretion and blood pressure (1). Renin is synthesized and secreted into the blood exclusively by the juxtaglomerular cells of the kidney from prorenin, an inactive precursor, which is produced not only by the kidney, but by other tissues, including the adrenal glands, gonads, and the uteroplacental unit (2). Whereas the secretion of renin is tightly controlled, prorenin secretion is not and is ~10-fold higher than renin (3).

Although peripheral conversion of prorenin to renin is limited and does not typically occur in vivo, in vitro conversion of prorenin to renin can occur by cryoactivation, so sample handling is critical (4). Historically, samples for renin activity have been collected on ice, frozen, and assayed at 2–8 °C. Cryoactivation occurs when samples are exposed to refrigerated temperatures (4 °C) for ex-
tended periods of time (4–8). Because of the high concentration of prorenin relative to renin, cryoactivation can lead to a marked increase in renin concentration. Therefore, to avoid cryoactivation, samples should be collected and assayed at room temperature. Recently, IRMA assays have been developed to directly measure renin in the circulation.

For many years, plasma renin activity (PRA) has been used to assess the in vivo activity of the renin-angiotensin system. With the PRA assay, plasma is incubated at 37 °C, and renin acts on angiotensinogen to generate Ang I, which is quantified by RIA. Because plasma angiotensinogen concentrations may vary, PRA is not always a good measure of circulating renin concentrations.

We compared renin results from two commercially available kits to determine the influence of sample handling on assay results, using samples collected at room temperature and on ice. The Active Renin kit (Nichols Institute Diagnostics) measures immunoreactive renin. The Rainen kit by DuPont measures PRA by quantification of generated Ang I.

EDTA-plasma samples were collected from 41 healthy adults. Two tubes were immediately placed on ice, and two tubes were kept at room temperature. Iced tubes were centrifuged at 4 °C for 20 min, whereas room temperature tubes were centrifuged at 25 °C. Samples were aliquoted and stored at −70 °C until testing. Before testing, iced tubes were allowed to thaw in the refrigerator for ~2 h, whereas room temperature tubes were quick-thawed at room temperature using a fan. Samples were then assayed by both renin kits following the manufacturers’ directions.

To measure active renin, 200 μL of calibrator, control, or sample was pipetted into plastic tubes, and 100 μL of 125I-labeled renin antibody solution was added to each tube. The tubes were vortex-mixed, and an avidin-coated bead was added to each tube. The tubes were incubated at room temperature for 24 h. Beads were washed three times with 2 mL of working wash solution. Liquid was aspirated, and tubes were counted in a gamma counter for 2 min. The calibration curve was calculated using a four-parameter logistics program.

In the assay of PRA, Ang I generation was performed in room temperature samples at ambient temperature and in iced tubes in an ice bath. Control or sample (0.5 mL) was pipetted into plastic tubes, and 10 μL of dimeracrol solution and 10 μL of 8-hydroxyquinoline solution were added to each tube and mixed. Malate buffer (1.0 mL) was then added to each tube. After mixing, 0.75 mL of each control or sample mixture was transferred to a second plastic tube. The tubes with the 0.75-mL aliquot were incubated at 37 °C for 1 h. At the end of the 1-h incubation, the 0.75-mL aliquot tubes were transferred to an ice bath.

For the Ang I RIA, 100 μL of calibrator, generated or nongenerated control, or generated or nongenerated sample was pipetted into plastic tubes, and 100 μL of tracer solution was added to all tubes. Blank antiserum (100 μL) was then added to the blank tubes, and 100 μL of antiserum was added to all tubes except the blank. Tubes were vortex-mixed and incubated for 2 h. At the end of the first incubation, 0.5 mL of second antibody was added. The tubes were vortex-mixed and incubated for 30 min. At the end of the second incubation, the tubes were centrifuged and then decanted. Tubes were counted for 1 min. The data were calculated using a four-parameter logistics program. Results were multiplied by 3 to correct for dilution factor, and generated was subtracted from nongenerated to give the final result in μg·L⁻¹·h⁻¹.

Samples from 41 healthy volunteers were assayed for active renin and PRA. Correlation between the refrigerated and room temperature sample sets was very good for active renin: \( y = 0.9681x + 0.5441; r^2 = 0.9895 \). The average room temperature result was 100% of the average iced result (Fig. 1A).

For PRA, correlation between the refrigerated and room temperature sample sets was good: \( y = 1.1896x - 0.7892; r^2 = 0.8838 \). The average room temperature result was 75% of the average iced result (Fig. 1B).

The correlation \( (r^2) \) between PRA and AR was 0.83; \( n = 41; P < 0.01 \).

Sample results for active renin showed little variation between re-
fribered and ambient samples. This indicates that limited exposure to 4°C, such as in this evaluation, has minimal or no affect on active renin results. However, the manufacturers’ data show a definite effect on sam-

These results are consistent with previous observations (2, 3).

The lower values for the ambient sample set for PRA indicates that sample collection and handling does affect assay results. Our findings suggest that the enzyme activity of renin is affected by sample handling, but its immunoassay detection is not influenced by ambient temperature. The apparent increase in renin activity from the refrigerated samples, therefore, does not suggest that 2 h on ice increases renin by cryoactivation of prorenin. Rather, renin enzyme activity appears to be decreased by processing at room temperature.

In addition, our results show a good correlation between PRA and immunoreactive renin. However, immunoreactive renin has advantages of less assay variation than PRA and is not limited by substrate concentrations.

In conclusion, prolonged exposure to refrigerator temperatures should be avoided to prevent cryoactivation of prorenin to renin, which leads to falsely increased renin results. Samples for measurement of immunoreactive renin may be collected at either 4°C for 2 h or at ambient temperature before assay without affecting assay results. However, collection of samples for assay of PRA should be avoided because it will lead to lower assay values.

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Rapid Enantiomeric Differentiation of Urinary Metabolites in a Patient with Bacterial Overgrowth Syndrome

To the Editor:

Patients with short bowel syndrome (SBS) are prone to periods of acute life-threatening metabolic acidosis attributable to the accumulation of D-lactate caused by bacterial overgrowth (1–3). The malabsorption of carbohydrates in the gut leads to large amounts of D- and L-lactate produced by intestinal flora (3). Because only L-lactate is routinely ana-
lyzed in most laboratories, an unrecognized D-lactic acidosis can have severe neurological consequences if not diagnosed and treated promptly.

A 4-year-old boy with SBS after surgical correction for a small intestinal volvulus was admitted with a history of periodic metabolic acido-
sis. During a bout of fever, he developed a severe metabolic acidosis (pH 7.19; reference range, 7.35–7.45; Pco2, 21.8 mmHg; reference range, 27–40 mmHg; base excess, –20.0; reference range, –4 to 2 mmol/L), was drowsy with indistinct speech, and was hy-
perventilating. Initially, he was treated orally (40 mL) and sub-
sequently intravenously (40 mL) with buffered bicarbonate, and glucose (20 mL of a 200 g/L solution), after which he could be aroused. Serum t-lactate was 2.8 mmol/L. The possibility of bacterial overgrowth was considered, and routine qualitative urinary organic acid analysis (gas chromatography–mass spectrometry) (4) revealed increased excretion of lactate, 3-hydroxypropionate, phe-
nyllactate, and 4-hydroxyphenylacetate, a pattern characteristic of bac-
terial overgrowth syndrome. A subsequent enantiomeric analysis of urinary organic acids using enantio-
selective multidimensional capillary gas chromatography–mass spec-
trometry (enantio-MDGC-MS) (5) demonstrated a D:L-lactate ratio of 99:5, indicating D-lactic acidosis. We also found increased concentrations of 2R,3S- and 2S,3S-2-hydroxy-
3-methylpentanoic acids in the same chromatographic run. Increased D-phenyllactate and 4-hydroxyphenyl-
lactate were also found, confirming the results of others (6). Enantio-
MDGC-MS also detected D-alloisole-
ucine and D- and L-alanine with D:L ratios of ~50:50, also confirming the bacterial origin of the D-form (7). The high concentration (9.1 mmol/L) of D-lactate in plasma supported the diagnosis, and large numbers of Lacto-
bacillus were cultured from stool samples. The patient was treated with a course of antibiotics (isocillin; 300 000 units, three times a day) and subsequent intestinal wash-out. Analysis of urine samples when the patient was clinically well showed a D:L-lactate ratio of 50:50.

D-Lactate is a very rare metabolite in healthy individuals, but it is de-
tected frequently in patients with SBS and bacterial overgrowth. Most routine clinical chemistry laborato-
ries do not assay L-lactic acid; thus, a diagnosis of D-lactic acidosis may be delayed. In this patient, routine uri-
inary organic acid analysis provided