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Possible Overestimation of Ultracentrifugally Isolated “Free” Apoprotein A-I in Serum

To the Editor:

We read with great interest the report by Duval et al. (1) describing the concentration of free apolipoprotein (apo) A-I in serum of patients with end-stage renal failure. They cited one of our studies (2) but, surprisingly, with an erroneous interpretation. Our patients showed normal serum apo A-I (or rather, not significantly different from controls) and a significantly greater ratio of apo A-I to HDL cholesterol than in the controls (2). Similar results have been obtained in a study of patients with chronic renal failure who are undergoing hemodialysis in the Seattle area (3). We wonder if such disagreement could be due to some of the characteristics of the control group studied by Duval et al. (1), these being significantly younger than the uremic patients and with most of the women taking oral contraceptives.

Some authors (4, 5) have demonstrated that ultracentrifugation has a deleterious effect on apo A-I-containing lipoproteins as well as on other particles normally bound to lipoproteins. If this is true, then ultracentrifugation doesn’t seem suitable for separating apolipoproteins apparently not bound to lipoproteins. A note of caution should be added to interpretation of results so obtained, at least to the quantitative aspects.

However, preliminary results of a project we are conducting in Seattle confirm some of the results reported by Duval et al. In plasma from uremic patients, particles containing A-I and A-II as well as particles containing A-I but no A-II, isolated by the method described by Cheung and Albers (6), showed a striking heterogeneity when compared with those in plasma from controls. Some of the particles found have an extremely low Stokes diameter; probably these account for the increased concentration of “free” apo A-I in serum. These small subpopulations were present irrespective of the concentration of hepatic lipase activity and the presence or not of triglyceride-rich lipoprotein remnants—evidence that is apparently against a substantial role for these particles in the metabolism of these lipoproteins. We also confirm the absence of immunoreactive apo A-I in plasma ultrafiltrates from these patients.

Finally, this comment on another point in the field of terminology: Unfortunately, “freedom” is a rare and ever-changing concept that deserves a more philosophical approach. In the case of a protein that is extraordinarily avid for lipids and is present in our biological “milieu,” freedom is only a utopian possibility. In the absence of a better expression, the adjective “free” should be written with quotation marks.

References

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Stability of AST and ALT Assays in Triis Buffers

To the Editor:

Wroblewski and LaDue (1) described the first kinetic assay for alanine aminotransferase (ALT, EC 2.6.1.2) in serum. Analyses for ALT and aspartate aminotransferase (AST, EC 2.6.1.1) are now done by the methods recommended by the International Federation of Clinical Chemistry (IFCC) (2–4). The principle of the methods is the same but there have been the following changes: (a) substrate concentrations were optimized; (b) Tris buffers are used instead of phosphate; (c) combined buffer and serum are pre-incubated to allow reactions with NADH; and (d) the reaction is measured after the substrate, alphaketoglutarate, is added.

Our clinical research requires us to re-analyze some patients’ sera, sometimes after several months during which the specimens have been stored at −20°C. To compare the performance of the IFCC reagents in this assay under these conditions, we analyzed aliquots of 50 different patients’ serum specimens (Set 1) for ALT and AST before and after freezing at −20°C. A Hitachi 736 analyzer and the BMD-system packs (cat. nos. 797359 and 798029; Boehringer Mannheim Diagnostics, Indianapolis, IN) were used for the analysis. Freezing did not alter the AST activity, but there was substantial loss of activity of ALT.

Tris buffers have been shown to inhibit recombination of the apoenzyme with the cofactor, pyridoxal 5-phosphate (P-5-P). We therefore assayed the frozen specimens after activation with this cofactor, which partly restores the original activity. Because the availability of reagents for the older (pre-IFCC) method was limited, we analyzed fewer (20) patients’ sera samples (Set 2) with these reagents before and after freezing at −20°C. Freezing did not affect measurements of AST or ALT activity when we used these reagents.

To determine whether this loss in activity is related to freezing and subsequent thawing or simply takes place slowly with time, we analyzed 15 serum samples (Set 3) that had been stored at 4°C for three weeks, performing the ALT and AST analyses after two weeks of storage and at the end of the three weeks. The results in Table 1 support the relationship of the freeze–thaw process to the loss of ALT activity.
In summary, whereas AST analysis with the IFCC reagents (in the absence of exogenous pyridoxal phosphate) is unaffected by freezing, the same is not true for ALT analysis. The only difference between the reagents used is the Tris buffer in the IFCC method, which suggests a possible change in the conformation of ALT in this buffer.

References

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Nonlinearity of Measurements of a Tumor Marker for Breast Cancer

To the Editor:
Mucin-like carcinoma-associated antigen (MCA) is a glycoprotein antigen related to human milk-fat globulin defined by the murine monoclonal antibody b12 (1). MCA has a high specificity for breast cancer. It is widely used to monitor therapy, to detect relapses, and to exclude bone metastases in conjunction with bone scintigraphy. Reports from various centers on the measurement of MCA in serum of patients with primary breast cancer and in serum of tumor-free patients after treatment of the primary cancer and benign breast diseases are in general agreement (2–4).

So far, there are few reports on the utility of MCA for monitoring patients with metastatic disease (5, 6). During a study of the change in MCA concentrations in serum in metastatic breast cancer, some sera showed a nonlinear response with dilution. Such results would lead to underestimations of MCA in serum.

To ensure maximal sensitivity of the assay, we investigated the phenomenon and now propose a simple modification of the enzyme immunoassay test procedure used (Roche Diagnostics; 1), to obviate the observed nonlinear response of results on dilution of samples.

In a preliminary experiment, 40 of 45 sera from patients with metastatic breast cancer, chosen because of their unexpectedly low values, reached a plateau only upon dilution. For samples from patients with nonmetastatic breast cancer, the dilution-corrected values for MCA were not altered by dilution.

In a second experiment, a further 206 samples from patients with metastatic breast cancer, having values for apparent MCA between 14 and 50 arbitrary units/mL, were retested after being diluted 10-fold, to investigate the extent of this anomaly. Upon dilution, 113 of the 206 samples showed an MCA increase of ≥50%. Although the initial results had exceeded the upper limit of normal, the change in value upon dilution could still alter the clinical significance of the results.

Because the use of a 10-fold dilution could result in loss of sensitivity in the lower end of the measurement scale, we investigated the effects of reducing the volume of the sample and standards from 50 to 20 μL and using 25 arb. units/mL instead of 50 arb. units/mL as the highest-concentration standard. Decreasing the sample volume resulted in dilution linearity up to 25 arb. units/mL, clearly identifying those sera that needed further dilution. We examined the effect of this change in procedure with 42 sera from patients with metastatic breast cancer whose first-measured values had been <50 arb. units/mL, which would not have received special comment. The nominal median MCA concentration of these samples was 21.6 arb. units/mL (range 10.1–44.7) when first measured; this increased to a median of 38 arb. units/mL (range 10.5–260) when measured by the modified procedure (P < 0.001). We therefore found this modified procedure suitable for quantifying MCA in metastatic disease.

This change in procedure had no effect on MCA concentrations measured in sera from 25 patients with primary tumors, 20 with benign breast disease, nine each of tumor-free patients with or without adjuvant chemotherapy, and 10 with local recurrence.

The cause of the nonlinearity with dilution of certain sera in the MCA test is not clear. It is interesting that this effect appears to be associated with metastatic breast cancer but is not seen in all patients. Moreover, it is not influenced by freezing and thawing.

Adopting the proposed procedure virtually eliminates underestimating MCA values due to nonlinearity. In our experience with 150 patients with metastatic breast cancer, only 7.5% had values in excess of 250 arb. units/mL. Four sera did not reach a plateau even after 100-fold dilution (>2500 arb. units/mL). The clinical significance of such increased concentrations of MCA remains uncertain.

References
2. Cooper EH, Forbes MA, Hancock AK, Price JJ, Parker D. An evaluation of mucin-like carcinoma associated antigen

Table 1. Effect of Freezing on ALT and AST in Serum

<table>
<thead>
<tr>
<th>Method</th>
<th>Test</th>
<th>Activity range, U/L</th>
<th>Fresh vs frozen 2 wks</th>
<th>Fresh vs refrigerate 3 wks</th>
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<td>Sample Set 1</td>
<td>IFCC</td>
<td>ALT 13–135</td>
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<td>IFCC (P-5-P-treated)</td>
<td>ALT 13–135</td>
<td>33</td>
<td>12</td>
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<td>IFCC</td>
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<td>ALT 7–69</td>
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<td>12</td>
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<td></td>
<td>IFCC</td>
<td>AST 11–69</td>
<td>8*</td>
<td>7</td>
</tr>
</tbody>
</table>

* After three weeks at 4 °C.