Diagnostic Accuracy of an Agarose Gel Electrophoretic Method in Multiple Sclerosis

To the Editor:

In >95% of patients suffering from clinically unambiguous multiple sclerosis (MS), oligoclonal IgG can be detected in cerebrospinal fluid (CSF) (1, 2). Isoelectric focusing is the most sensitive method for detecting oligoclonal bands (OCBs) (1), whereas agarose gel electrophoresis is reported to detect OCBs in ~80% of cases (2). A commercial electrophoresis unit (Beckman Instruments) is commonly used in clinical laboratories. Laboratory support of a clinically probable diagnosis of MS in combination with brain magnetic resonance imaging is important to allow initiation of appropriate treatment as early as possible.

We evaluated the sensitivity of the Beckman modified agarose gel electrophoresis to detect OCB and estimated its diagnostic accuracy. We examined paired CSF and sera from 168 patients (122 females) with clinically unambiguous MS (3) for the presence of oligoclonal IgG.

We followed the manufacturer’s instructions for application of samples to the buffered agarose gel (SPE-II gel). Electrophoresis was performed at 100 V for 40 min, followed by immobilization in a fixative solution and staining. All tests were done by the same technician and evaluated by the same experienced hematologist, both unaware of clinical or magnetic resonance imaging data.

OCB was detectable in 90 of the 168 patients (53%; 95% confidence interval, 52.9–53.1%). The mean IgG index [(CSF-IgG/serum-IgG)/(CSF-albumin/serum-albumin)] of OCB-negative patients was 0.87. Patients with detectable OCB had a mean IgG index of 1.28 (reference value <0.7).

This electrophoretic method does not appear to provide adequate clinical sensitivity because false-negative results in the detection of OCB may delay diagnosis and the start of treatment.

References


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More on Troponin Assays and Heparin

To the Editor:

A recent article [Troponin T and I Assays Show Decreased Concentrations in Heparin Plasma Compared with Serum: Lower Recoveries in Early than in Late Phases of Myocardial Injury (Clin Chem 2000;46:817–21)] concluded that “Until such methods [which are resistant to interference by both heparin and EDTA] are available, the sample of choice for cardiac troponin determinations is serum collected in tubes with or without gel, or in thrombin tubes with and without gel”. We believe that the conclusion is not supported by the data presented in the article.

It is clear from the data presented in Table 1 of the article that a bias exists between plasma and serum measurements for the Elecsys 2010 and the Immulite systems. No data in the article, however, suggest that the measured bias is related to heparin. With specific reference to Table 1, the authors state that the heparin concentrations in the tubes range from 40 to 70 IU/mL. In their experimental design, they added heparin to serum tubes at concentrations of 50–450 IU/mL. The authors have erroneously calculated the concentrations of heparin in the plasma tubes as listed in Table 1. In fact, the total heparin in the tubes ranges from 40 to 70 IU to achieve a final concentration of 14–15 IU/mL (1). In calculating the heparin concentration in the tube, the manufacturer does not consider that blood consists of red blood cells, which presumably may not take up heparin. Therefore, recalulation of the manufacturer’s stated concentration, assuming a 40% hematocrit, gives final heparin concentrations in the plasma of between 24 and 25 IU/mL. The association made by the authors comparing troponin concentrations measured in serum with added heparin (50–450 IU/mL) and in plasma from heparin tubes with whole blood (24–25 IU/mL) should not be made because the heparin concentrations are very different and the sample matrix (serum vs plasma) is different. The authors state that “Therapeutic concen-
tions of heparin at AMI [acute myocardial infarction] and at cardiac surgery have been estimated to [be] 1 IU/mL and 5 IU/mL, respectively” and that “These correspond to 1.5% and 7% of the concentrations in heparin tubes and do probably not cause significant in vivo losses of cardiac troponins”. The heparin concentrations during cardiac surgery, ignoring hematocrit because both matrices are blood, can be up to ~35% of the heparin concentrations in the blood collection tubes (5 IU/mL/14 IU/mL). If one believes that heparin associates with troponin, then in vivo therapeutic heparin concentration becomes significant with respect to heparin in blood tubes.

Assuming that heparin does interact with one or more troponin forms at concentrations significantly higher than in heparin tubes, the affinity of the association must be very weak, although the affinity appears to be higher for troponin T than for troponin I. This conclusion is supported by the data in the article, which show that a nearly 10-fold increase in heparin concentration (from 50 to 450 IU/mL) changes the plasma/serum ratios for troponin T only from 86–109% to 51–78%. The article also shows that a nearly 5-fold increase in heparin (from 98 to 450 IU/mL) gives plasma/serum ratios for troponin I of 77% and 69%, respectively. This finding is consistent with heparin affecting the troponin T assay and not consistent with heparin affecting the troponin I assay, as there does not appear to be a significant heparin-dose-dependent decrease in the ratio for the troponin I assay. With reference to Fig. 3 in the article, the authors fail to provide a statistical analysis of the data at each time point, which is necessary to determine whether the indicated changes in the plasma/serum ratio are actually significant. At the earliest time points, the concentrations measured will be the lowest with the greatest error. The error in the ratio is derived from the error of the two measurements. In addition, the authors fail to show whether the plasma/serum ratio changes as a function of time at relevant heparin concentrations (24 IU/mL rather than 98 IU/mL). A hypothesis that was not suggested by

the authors, which we would like to put forth, is that a matrix effect is being measured, at least for the troponin I assay. Clearly, plasma and serum are very different, and matrix effects in immunoassays are well known.

We agree with the recent National Academy of Clinical Biochemistry recommendations that suggest using plasma or anticoagulated whole blood for the stat analysis of cardiac markers (2). The calibration of immunoassays must be specific to the sample type when matrix effects are known to exist. Therefore, immunoassays that are recommended by the manufacturer for use with heparin plasma should be calibrated with heparin plasma to minimize the bias.

The intention of our short report was not to completely elucidate the mechanism by which these factors may interact. Below are our detailed responses to Buechler and Nakamura (1) (quotes in italics).

1. “No data in the article, however, suggest that the measured bias is related to heparin.”

(a) Our documentation of decreasing troponin concentrations as a function of increasing heparin concentrations in our titration experiments (2) strongly suggests that heparin does interfere with the respective analytical systems.

(b) Stiegler et al. (3) recently carried out a study of 100 patients. Despite late sampling in 50 patients after cardiac surgery and having lower heparin concentrations in sampling tubes (calculated as 25 IU/mL of plasma at a hematocrit of 0.40) they, like us, found great individual variations among individual plasma/serum (P/S) ratios, e.g., 50–120% for cardiac troponin I (cTnI) on the ACS:Centaur; 76–123% for cTnI on the Abbott AxSYM; and 34–129% for cardiac troponin T (cTnT) on the Roche Elecsys. Table 4 in their article identified two samples (samples 2 and 8) from the acute phase of myocardial infarction showing losses >40% (3). Treatment of heparin samples with heparinase reversed the decreases in cTnT but not cTnI (ACS:Centaur). This would not have been possible if troponin loss in plasma was attributable to a matrix effect (plasma vs serum) as alleged by Buechler and Nakamura (1). Although mean P/S values (untreated) for all three assays ranged from 94% to 101%, Stiegler et al. (3) did conclude that they could not recommend the use of heparin plasma for determination of cardiac troponins for two of the investigated assays (Abbott AxSYM was the possible exception).

Heparin interference with TnI assays is not a new observation. Inserts from several TnI assays describe up
to 30% losses with heparin plasma relative to serum. The losses vary according to the assay methodology used. The one common factor in these different assays is the TnI molecule itself. We (2) and Stiegler et al. (3) suggested that heparin binds to troponin forms, reducing their immunoreactivities, an effect shown by Katrukha et al. (4). For example, the insert for Beckman Access TnI (1998) warns: “Do not collect samples in heparin”. A recent poster (5) described the development of “an improved cardiac troponin I (cTnI) immunoassay for Beckman Coulter’s Access system using a new pair of cTnI specific antibodies” and that “Heparinized plasma is the preferred sample type”.

2. “The authors have erroneously calculated the concentrations of heparin in the plasma tubes as listed in Table 1. In fact, the total heparin in the tubes ranges from 40 to 70 IU to achieve a final concentration of 14–15 IU/mL. In calculating the heparin concentration in the tube, the concentration of heparin in some tubes does not vary from 72 to 108 IU in plastic and up to 143 IU in glass tubes. For simplicity, we calculated plasma concentrations for a hematocrit of 0.5. As long as we do not know the actual content of heparin in each type of sampling tube, only the minimum content, this must be a fair approximation. We now understand from the responses of Buechler and Nakamura and others to our report (2) that there seems to be rather great confusion around actual tube heparin concentrations. Accordingly, in Table 1 we have compared the values from various international recommendations and the most recent information on the BD tubes used in our study. We want to emphasize that the estimates concern minimum heparin concentrations. Incomplete filling of, e.g., BD tube 367793 containing 143 IU to only 3 mL of whole blood will produce a heparin concentration of 60 IU/mL of plasma, or ~60% of that used in our addition experiments. The current confusion around actual tube heparin contents confirms the importance of a point we made in our discussion (2): Sample tubes for troponin determinations should be validated and specified by manufacturer and catalog numbers in assay inserts and studies.

3. “The heparin concentrations during cardiac surgery, ignoring hematocrit because both matrices are blood, can be up to ~35% of the heparin concentrations in the blood collection tubes (5 IU/mL/14 IU/mL).”

In view of the above, our comparisons between in vitro tube (Table 1) and in vivo heparin concentrations are reasonably correct.

4. “Assuming that heparin does interact with one or more troponin forms at concentrations significantly higher than in heparin tubes, the affinity of the association must be very weak ... a nearly 10-fold increase in heparin concentration (from 50 to 450 IU/mL) changes the plasma/serum ratios for troponin T only from 86–109% to 51–78%.”

Our study was not designed to estimate molecular affinities, and we do not see any problem in that troponin losses by heparin are not linear. In our view, the observation of a larger decrease of troponin concentrations with an increase of heparin concentration from 50 to 450 IU/mL in two completely different assays justified the suggestion of a binding phenomenon rather than an unspecific matrix effect.

5. “In addition, the authors fail to show whether the plasma/serum ratio changes as a function of time at relevant heparin concentrations (24 IU/mL rather than 98 IU/mL).”

If Buechler and Nakamura (1) mean time after sampling, it is cor-

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### Table 1. Heparin concentrations in recommendations and tubes.

<table>
<thead>
<tr>
<th>Recommendations</th>
<th>Amount of heparin per tube, IU</th>
<th>Blood vol. in filled tubes, mL</th>
<th>Heparin conc., IU or USP per mL of blood</th>
<th>Heparin conc., IU or USP per mL of plasma at hematocrit 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO 6710 (7)</td>
<td>12–30 IU</td>
<td>4.0</td>
<td>35 (23) IU</td>
<td>70 (46) IU</td>
</tr>
<tr>
<td>NCLLS (8)</td>
<td>10–30 USP</td>
<td>3.0</td>
<td>24 IU</td>
<td>48 IU</td>
</tr>
<tr>
<td>Plastic tubes</td>
<td>15 USP</td>
<td>2.5</td>
<td>32 IU</td>
<td>64 IU</td>
</tr>
<tr>
<td>WHO 1999 (9)</td>
<td>40–60 IU</td>
<td>3.0</td>
<td>24 IU</td>
<td>48 IU</td>
</tr>
<tr>
<td>IFCC 1995 (10)</td>
<td>40–60 IU</td>
<td>3.0</td>
<td>24 IU</td>
<td>48 IU</td>
</tr>
<tr>
<td>Glass tubes</td>
<td>12–50 IU</td>
<td>2.5</td>
<td>32 IU</td>
<td>64 IU</td>
</tr>
<tr>
<td>Plastic tubes</td>
<td>108 IU</td>
<td>2.5</td>
<td>44 IU</td>
<td>88 IU</td>
</tr>
</tbody>
</table>


Values in parentheses are revised according to new BD information (see text).
rect. We did not even try to answer that question; we believed that troponins are used as stat tests. The data in Table 2 of our article (2) clearly show the change of mean values of P/S ratios ± 95% confidence intervals for three time intervals after onset of chest pain (1–12, 13–48, and >48 h) calculated from the eight cases shown in the online supplement.

6. “With reference to Fig. 3 in the article, the authors fail to provide a statistical analysis of the data at each time point.”

Statistical treatment of serial data for individual patients is an interesting issue (6), but it is far beyond the scope of our short report. A t-test based on a day-to-day imprecision for TnT of 5.6% yields a least significant difference of 16% between two individual measurements at a concentration of 0.1 μg/L. If the imprecision in the measurement of ratio is estimated to 8%, the least significant difference between two samples is 22%. We omitted a discussion of these data in the short report in favor of the group mean values in Table 2.

7. “We agree with the recent National Academy of Clinical Biochemistry Recommendations that suggest using plasma or anticoagulated whole blood for the stat analysis of cardiac markers. The calibration of immunoassays must be specific to the sample type when matrix effects are known to exist. Therefore, immunoassays that are recommended by the manufacturer for use with heparin plasma should be calibrated with heparin plasma to minimize the bias.”

We think this approach is highly questionable. An average difference between serum and plasma troponins can easily be calculated, but given the great variability of P/S ratios during myocardial infarction, it seems to be a dangerous oversimplification to use a mean difference as a bias correction.

References

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Cerebrospinal Fluid Xanthochromia Testing Simplified

To the Editor:
We previously published a method that determines xanthochromia in cerebrospinal fluid (CSF) to detect subarachnoid hemorrhage (1). The method involved a spectrophotometric scan of the CSF in which the absorbances of hemoglobin (Hb; 415 nm) and bilirubin (440 nm) were determined by subtracting a background obtained by drawing a tangent from 360 to ~530 nm. The net absorbance of bilirubin was then determined from an equation that compensated for Hb absorbance. A closer examination of this method showed that the Hb spectrum gave zero absorbance at 476 nm even at Hb absorbances up to 1.5 at 415 nm. We then tested 476 nm to measure net bilirubin absorbance because, although it is not the $\lambda_{max}$ for bilirubin, the mathematics for the calculation are much simpler. We compared results for 112 CSF samples submitted for CSF xanthochromia analyses by the older method (1), which compensates for Hb interference in the bilirubin estimation, with results of the new method, which measures the absorbance of bilirubin at 476 nm and therefore does not use compensation for Hb absorbance. The new method, like the old one, uses the tangent approach to measure the net absorbance at 476 nm.

Linear regression of the absorbances yielded:

$$Xantho \ (new) = 0.87 \times Xantho \ (old) + 0.001$$

with $r^2 = 0.93$ ($P <0.001$) and mean absorbances (SD) of 0.0145 (0.0311) and 0.0136 (0.0199) for the old and new methods, respectively. In this group of samples, there was one pair of discrepant results with a marginally positive absorbance of 0.017 (reference >0.015) by the old method and borderline positive (0.013; reference interval, 0.01–0.014) by the new. In general, patients who are either positive or borderline positive have the same clinical follow-up, namely,