The authors of the cited article respond:

To the Editor:

We would like to thank Hoyer and Scheidt for their interest in our report (1), and we wish to comment on points raised in their letter.

Our report detailed the experience of a prospective study of more than 60,000 samples in a clinical laboratory, rather than a reference/research laboratory, with its use of HPLC. An algorithm involving retention time, percentage of hemoglobin fraction, and peak characteristics was proposed to aid in the processing of future samples. This algorithm has now been tested in more than 26,000 samples. Preliminary results have been presented (2), and a final manuscript is in preparation.

Hoyer and Scheidt state that the retention time can change with different lots of columns and reagents on the Variant system, which has also been reported elsewhere (3), and they give examples of retention time ranges for 3 variants from their 10-year experience. Our analysis of the retention times for hemoglobin (Hb A₀), Hb A₂, and Hb S showed no statistical difference over a 32-month period during which we used 3 different lots of columns and 10 different lots of reagents on the Variant II. Although we agree with Hoyer and Scheidt that the range in retention times of hemoglobin variants that they experienced may be attributable to manufacturing changes over a long time period, these changes also may be attributable to the different performance characteristics of the HPLC systems involved. Furthermore, it appears to us that the calculated SD for the retention time ranges for Hb D-Los Angeles, Hb Korle-Bu, and Hb D-Iran (0.035, 0.040, and 0.048 min, respectively) provided by Hoyer and Scheidt are nonetheless consistent with the range reported in our study (0.026 ± 0.016 min).

In Table 1 of their letter, Hoyer and Scheidt list examples of hemoglobin variants, not seen in our series, that eluted in the A₀, A₂, S, and D windows. There is no doubt that other hemoglobin variants elute in these and other windows, and it would be of considerable interest to know what the incidence and clinical and hematologic impacts of these other variants are. In fact, we urge Hoyer and Scheidt to publish their experience with such a large database of retention times of common and rare hemoglobin variants along with any algorithm used in their laboratory. We believe this would be a valuable contribution to the literature on the use of HPLC in the clinical laboratory.

Finally, Hoyer and Scheidt state that some of our data were published without their knowledge or consent. Although this is true, it was the result of an unfortunate misunderstanding and was not malicious in intent. Certain specimens were forwarded to the Mayo Medical Laboratories for confirmation, as stated in our publication, through Quest Diagnostics. Additional testing was performed by isoelectric focusing, globin chain electrophoresis, and screening for unstable hemoglobins where appropriate. DNA sequence analysis was performed on 8 specimens for the definitive identification of a difficult or rare hemoglobin. We assumed that because the tests were performed on a fee-for-service basis and we had not specifically requested the DNA sequence analysis, we were not obligated to acknowledge the Mayo Medical Laboratories more than what was done in our publication. Although the report did state that the DNA sequence analysis was performed on a “research basis”, we interpreted this statement, as many of our colleagues believe logically, to be a disclaimer that care should be taken in interpreting the results because of the research nature of the methodology used to ascertain the results. The Mayo Medical Laboratory report has subsequently been altered to clarify that DNA sequencing is performed under an Institutional Review Board-approved research protocol and that results may not be published in any form without the consent of the laboratory director.

We feel that there is a need for further dialogue on the proper use of HPLC in the clinical laboratory, possibly starting with the formation of local and national users’ groups. It is our hope that method-specific guidelines, recommendations, and standards can be developed to assist the clinical laboratory in the proper use of HPLC as a diagnostic tool. This can be achieved, however, only when the reliability and performance characteristics of this methodology have been fully determined.

References


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Limited Additional Release of Cardiac Troponin I and T in Isoproterenol-Treated Beagle Dogs with Cardiac Injury

To the Editor:

Many studies have shown that determination of the blood concentrations of cardiac troponin I and T (cTnI and cTnT) offers higher clinical sensitivity and specificity for the diagnosis of cardiac injury than do the standard cardiac protein markers such as creatine kinase (CK) (1, 2). However, the release of cardiac troponins into
the blood stream of laboratory animals in response to cardiac insults such as drugs is still not well defined. It was our objective to examine the release of cardiac troponins together with CK by repeat dosing in isoproterenol (ISO)-treated beagle dogs, a routinely used animal model in preclinical studies.

ISO is a sympathomimetic agent and β-adrenergic receptor agonist that causes acute cardiac injury at high doses (3). In the present study, 1 male and 1 female beagle dog (9 months old) were given a 1 mg/kg subcutaneous injection of ISO on each of 2 consecutive days. Abnormal pathologic alterations, including severe coagulative necrosis, were observed in both animals. Blood was collected from the jugular vein into collection tubes (Beckon Dickinson) containing EDTA for the analysis of cardiac troponins and into the same type tubes with no additive for CK. The blood concentration of cTnI was measured by a modified Access® CK activity was measured according to the manufacturer’s recommended experimental conditions. cTnT was determined by a chemiluminescent substrate, according to the manufacturer's recommended conditions (Pierce Biotechnology). CK activity was measured with an N-acetylcysteine-activated test at 37 °C on an ADVIA® 1650 Chemistry System (Bayer), as developed by Szasz et al. (4).

Both troponin assays were validated by use of purified human and dog troponin calibrators. The calibrators were linear on dilution up to 50 μg/L. The cTnI assay specifically measured purified dog cTnI, but not purified dog cTnT, up to 50 mg/L. Similarly, the cTnT assay specifically measured purified dog cTnT, but not purified dog cTnI, up to 50 mg/L. In addition, the blood concentrations of cardiac troponins in untreated healthy dogs were very low (<0.7 μg/L) or not detectable.

The blood samples analyzed in the present study were collected before administration of ISO and at 1, 4, 8, 10, and 24 h after each daily dose. After the initial ISO administration, the blood concentrations of the cardiac troponins in both dogs increased quickly by 4 h after the first ISO administration, then peaked at ~8 h, followed by a slow disappearance of the cardiac troponins (panels A and B in Fig. 1 for the male and female dog, respectively). The blood concentrations of cTnI and cTnT were highly correlated in the male ($r = 0.9326; P <0.0001$) and female dogs ($r = 0.9189; P <0.0001$). The cTnI concentration remained increased at 24 h after the first ISO administration, whereas cTnT remained increased only in the male dog. Nevertheless, the blood content of cTnI and cTnT appeared to correlate with the pathology finding that the cardiac injury in the male dog was more severe than in the female dog. After the first administration, for example, the peak blood concentration of cTnI [mean (SD), 17-fold] in the male dog was ~17-fold higher than the blood concentration [3.2 (0.2) μg/L] in the female dog. Similarly, the peak blood concentration of cTnT [19.1 (6.0) μg/L] in the male dog was ~16-fold higher than that in the female dog [1.2 (0.1) μg/L].

Comparing the release of cardiac troponins after each of 2 administrations of ISO, the second administration triggered only a slight increase, but the concentrations still peaked at 8 h after the second administration (Fig. 1). The mean (SD) cTnI and cTnT concentrations at the second peak [31.3 (7.6) μg/L cTnI and 12.4 (0.5) μg/L cTnT in the male dog, and 2.5 (0.1) μg/L cTnI and 0.7 (0.1) μg/L cTnT in the female dog] were markedly lower than the concentrations at the first cardiac troponin peak, which might be attributable to β-adrenergic receptor internalization and desensitization (5).

To examine the possible relationship of CK with cardiac troponins, we used the collected blood samples from the same animals to determine

![Fig. 1. Release profiles of cTnI (■), cTnT (▲), and CK (●) in male (A) and female (B) beagle dogs treated with 1 mg · kg⁻¹ · day⁻¹ ISO. The arrows indicate the time points when ISO was administered.](image)
total CK activity, a frequently used cardiac protein marker (6). As shown in Fig. 1, the blood CK concentrations quickly increased in both ISO-treated dogs. Similar to the release of cardiac troponins, the increase started at 4 h after the initial administration. Similarly, the initial increase in CK was much more dramatic than the second increase. Differently, CK in the female dog showed maximal activity at 4 h, which was earlier than the release of cardiac troponins (Fig. 1B) with a faster clearance as well. The correlation of CK activity with the release of cTnI differed between the male and female dogs, mainly in the times of their peak blood concentrations. In the male dog, cTnI release was correlated with CK activity \((r = 0.7165; P = 0.0131)\), but in the female dog, we found no correlation of CK activity with the release of cTnI \((r = 0.2748; P = 0.4135)\).

The possible relationship of cardiac troponins and CK has been examined in ISO-treated rats (7, 8). Similar to our findings in beagle dogs, the release of cardiac troponins in rats occurred within 4 h after ISO administration, but it peaked at 4 h and returned to baseline by 24 h. However, no significant increase in CK was observed in ISO-treated rats, suggesting that beagle dogs might represent a better experimental model for the investigation of drug-induced cardiac injury, at least for therapeutic agents with a mechanism of action to similar that of ISO.

In conclusion, the release of cardiac troponins and CK exhibited time-dependent and monophasic increases in ISO-treated beagle dogs. The increases in these protein markers on the second day was markedly lower, indicating limited additional release. Most importantly, CK decreased from peak concentrations faster than both cTnI and cTnT, indicating a narrower diagnostic window. The return to basal values of cardiac troponins and CK was delayed by repeat dosing, but was not inhibited. Thus, the limited additional release of cardiac troponins and CK does not prevent their use as indicators for cardiac injury. On the basis of the dynamic increases seen in the present study and the cardiac-specific origin, cardiac troponin concentrations in beagle dogs provide an ideal experimental measurement for cardiac injury, bridging preclinical and clinical studies to evaluate new therapeutic agents (2).

References

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Quality Specifications for Imprecision of B-Type Natriuretic Peptide Assays

To the Editor:

Apple et al. (1), on behalf of the Committee on Standardization of Markers of Cardiac Damage of the IFCC, recently reviewed and abstracted the scientific literature to provide recommendations pertaining to the quality specifications for B-type natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) assays (together abbreviated here as NPs). They stated that a decision concerning what is acceptable imprecision is needed and concurred with the goals derived using the model proposed by Cotlove et al. (2) based on the concept that analytical imprecision \(CV_A\) should not significantly affect clinical use. This model suggests that this desired negligible clinical impact can be obtained when \(CV_A\) is lower than or equal to one-half the intrindividual biological variation \(CV_I\). The mathematical explanation of this and the many other models available for the setting of quality specifications for analytical performance characteristics have been described in detail (3). The authors state that, because of high biological variation for NPs \((CV_B \approx 30\%-50\%)\), very low \(CV_A\) may be unnecessary: however, for monitoring of therapy with serial BNP measurements in clinical cases, it may be desirable to minimize \(CV_A\) (1).

Furthermore, Apple et al. (1) state that a desirable \(CV_A\) of \(<15\%\) at NP concentrations within the reference interval is recommended. If an eventual goal is to rely on monitoring of marker trends over time, then an optimal imprecision of \(<10\%\) is advocated.

It is interesting that those who produce allegedly evidence-based guidelines, recommendations, and scientific statements always seem to end up with “round numbers” such as 15%, 10%, and 5% as goals for \(CV_A\) (3). Using readily available data on \(CV_A\) and \(CV_B\), it is easy to work out whether the recommendations are
cogent, particularly the latter, because the model of Cotlove et al. (2) is actually concerned with the clinical setting of monitoring individuals.

Consider that CV is 40%, as suggested by Apple et al. (1) and recently studied in detail (4). A significant change in serial results occurs only if the reference change value (RCV), sometimes called the critical difference, is exceeded. RCVs are easily calculated as \(2^{1/2} \times Z \times (CVA^2 + CVI^2)^{1/2} (3)\), where \(Z\) is the number of standard deviations appropriate to the probability selected. If CV was 15%, then the RCV for \(P < 0.05\) would be 118%, and if CV was 10%, the RCV would be 114%. Thus, the statement made by Apple et al. (1) that an optimal CV of 10% would replicate assays or replicate samples, Bruins et al. (6) that an optimal CV of 10% would be advocated for monitoring individuals is not evidence based. If CV is much larger than CV, then it is simply not worthwhile reducing CV to less than one-half of CV even in this clinical use of results.

Whether the RCV can be reduced to make NPs more useful for monitoring individuals over time is a question not addressed by Apple et al. (1) but is discussed briefly by Bruins et al. (4). In particular, do replicate assays or replicate samples, as advocated for cholesterol and high-sensitivity C-reactive protein (5), help in this regard to improve the utility of a test in monitoring individuals? This again can be calculated easily because:

\[
\text{RCV} = 2^{1/2} \times Z \\
\times \left[ \left( \frac{CVA^2}{nA} \right) + \left( \frac{CVI^2}{nS} \right) \right]^{1/2}
\]

where \(nA\) is the number of replicate assays, and \(nS\) is the number of patient samples (5). If each sample was analyzed twice, a good and simple way to reduce CV, if CV was 15%, RCV would become 115%, and if CV was 10%, RCV would become 113%. Thus, reducing CV even further through duplicate analyses has very little effect on RCV because CV is much larger than CV. Lowering CV from 15% to 10% and lower is simply not worthwhile, and the recommendations (1) seem less than objective. In contrast, if duplicate samples were taken, the RCV would be 89% if CV was 15%, and RCV would be 83% if CV was 10%. If CV is larger than CV, then taking multiple samples is the strategy to adopt to reduce RCV.

It is also easy to calculate the number of samples required to obtain an estimate within a certain percentage of the true individual homeostatic set point of the individual from the formula based on a simple standard error of the mean estimate (3):

\[
n = \frac{Z \times (CVA^2 + CVI^2)^{1/2}}{D}
\]

where \(D\) is the percentage deviation allowed from the true homeostatic set point. Thus, if CV was 15%, 70 samples (clearly an untenable number) would be needed to estimate the homeostatic set point of an individual within 10% at \(P < 0.05\). Reducing CV to 10% does not make much difference: 65 samples would be required.

NPs are very like high-sensitivity C-reactive protein when the relative magnitudes of CV and CV are considered objectively, and it has been suggested that professional guidelines on the latter are less than objective (5). It is not difficult (6) to calculate the effects of CV on RCV, on the results of analyzing a sample more than once and/or taking more than 1 sample, and on the number of samples needed to obtain an estimate of an individual’s homeostatic set point within a stated closeness at a predetermined probability.

The above does assume that the variation in NP concentrations can be described as random variation around a homeostatic set point, and this widely used model (3) has been implicitly and explicitly used by Apple et al. (1). However, it may be that this model is not totally appropriate for NPs. It could be argued that any change in BNP concentration in an individual should be considered as potentially and clinically relevant, even when smaller than the RCV calculated from the CV derived using the homeostatic model, because such a change would reflect an alteration in activation of the neuroendocrine system as a result of specific pathophysiologic mechanisms. In this alternative model, any change in NP concentrations in an individual should be interpreted by taking into account clinical history and examination, including the response to specific treatments, as well as laboratory findings. Perhaps clinical criteria should be used to evaluate the pathophysiologic relevance of a “significant” variation in NP concentrations (which can be mathematically defined as \(2^{1/2} \times Z \times CV\) in an individual patient. If this alternative model is considered appropriate, then the recommendation that it may be desirable to minimize CV (1) has considerable merit, but not just to CV of 10% as recommended. The lower the CV, the higher Z will be for any specific change, and the more significant will be the observed changes in NP. This consideration is not discussed by Apple et al. (1).

I again strongly suggest that those who produce allegedly evidence-based guidelines, recommendations, and scientific statements be urged to do all the calculations outlined here and think on their ramifications on clinical utility before disseminating their work.

References

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Analysis of Mass Spectrometry Profiles of the Serum Proteome

To the Editor:

Regarding the editorial “Analysis of Mass Spectrometry Profiles of the Serum Proteome” (1), we found many points to praise, but we must respectfully disagree with the conclusion that the software is “extremely conservative” about calling a peak and that the baseline correction introduces a substantial bias. Although we do agree with the assertion made by Dr. Coombes that data reduction algorithms should reflect the physical function of ion generation and detection elements reflecting a priori understanding of artifacts and routine processes involved in the creation of mass spec signals, it is difficult to directly compare baseline correction and peak detection algorithms because there are many parameters that can vary. The default parameters that we set for our baseline and peak detection settings were selected based on the physics of the mass spectrometer and data from both simulated and biological models. It should be noted that the setting of several user-adjustable parameters in our algorithm can have a dramatic effect on the number of peaks; one such parameter is the starting mass to begin estimating the noise (2). Increasing the value of this parameter to a mass above the matrix region where the detector is saturated will improve the accuracy of the noise estimate. It should also be noted that improvements over time in instrumentation and algorithms have dramatically reduced or eliminated this as an issue. The detection system of the current instrumentation has been improved by incorporating an innovative form of matrix attenuation, and the noise algorithm has been improved in CiphergenExpress, the data analysis software that is included with current instrumentation.

These results notwithstanding, we continue to evaluate improvements to peak detection and baseline algorithms and the incorporation of new methods as they become widely accepted because we recognize that downstream data processing and pattern recognition methods for biomarker discovery can be strongly influenced by baseline estimation and peak detection. We further agree with Dr. Coombes that the most successful implementation of clinical proteomics requires appropriate clinical study design, judicious use of sample preparation techniques, and rigorous independent validation, all of which hold true whichever data preprocessing and analytical approaches are used.

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Dr. Coombes replies:

To the Editor:

I am glad to see that Drs. Fung and Gavin find so much to agree with in my editorial (1). I would, however, like to briefly address their concerns. First, my claim that the Ciphergen software is “extremely conservative” about calling peaks is based on an analysis performed with 24 replicate spectra, which will be published elsewhere (2). We have made the raw spectra available at http://bioinformatics.mdanderson.org/Supplements/Datasets/KuererQC. I urge interested readers to download the data and test the conclusions for themselves. Second, my statement that their baseline correction introduces “substantial bias” should be clarified. Every baseline algorithm I have investigated (including the one we use in our own analyses) introduces substantial bias in peak heights in some portion of the spectrum. In my opinion, the difficulty arises because we do not have a good theoretical understanding of the processes that shape the baseline and must resort to ad hoc algorithms to remove it. One advance in the report by Malyarenko et al. (3) is an explanation of how the physics of the ion detector contributes to the baseline; this is an important first step toward better baseline correction.

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