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 A\textsubscript{a}), Hb A\textsubscript{2}, 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 A\textsubscript{co}, A\textsubscript{2}, 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 that 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 in untreated dogs and into the same type tubes with no additive for CK.

The blood concentration of cTnI was measured by a modified Access® troponin assay (Beckman-Coulter) performed manually on 96-well plates according to the manufacturer’s recommended experimental conditions. cTnT was determined by an ELISA containing a biotinylated monoclonal cTnT-specific antibody from the third-generation cTnT STAT immunoassay (Roche Diagnostics) and a pair-matched monoclonal antibody against human cTnT (clone 1A11) obtained from Hytest. The biotinylated antibody against cTnT in the cTnT-containing immuno-complex was quantified by peroxidase-labeled streptavidin and 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), 55.2 (11.2) μg/L] 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 (0.6) μg/L] in the male dog was ~16-fold higher than that in the female dog [12.4 (0.5) 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
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 similar to 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).

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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 intradividual 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\(_I\) 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\(_I\) it is easy to work out whether the recommendations are
circulating RNA (cirRNA) were found in blood plasma of 33% of healthy men and 35% of healthy women. Notably, the cirRNA concentration in plasma was higher in men than in women (Table 1; also see Table 1 of the online Data Supplement).

We did not find any correlation between the ages of the patients and the concentrations of free or cell-surface–bound cirNAs. The total mean concentration of cirDNA in blood was higher in healthy men (1030 ng/mL of blood) than in healthy women (430 ng/mL; Mann–Whitney U-test, P <0.01). The cirRNA concentration was also significantly higher in men than in women (770 vs 100 ng/mL of blood; Mann–Whitney U-test, P <0.01).

We have found that the main portion of cirNAs in healthy donors is tightly or weakly bound to the cell surface, apparently because of interaction with cell-surface NA-binding proteins (16) or phospholipids of the cellular membrane through bivalent ions (11). In the blood of breast cancer patients, cirNAs circulate only in blood plasma and are not detected at the cell surface (6). This finding indicates that the cirRNA concentration in plasma is determined not only by the intensity of cell death (12) or by active secretion from cells (13) but also by the interaction of cirNAs with cell surface and plasma biopolymers. Increased concentrations of proteases accompanying tumor invasion in cancer patients (14, 15) can cause damage to the cell-surface NA-binding proteins and detachment of the cell-surface–bound cirNAs. Proteolytic enzymes are also responsible for endometrium turnover in the uterus (16), and background protease activity combined with known high nuclease activity in blood plasma (17) can affect the concentrations and distribution of cirNAs in the blood of healthy female donors and sick female patients.

The results of the present study demonstrate that although the concentration of cell-free cirDNA in the plasma of healthy donors does not depend on the sex of the donors, concentrations of cell-surface–bound cirNA differ in male and female donors. The sex of a patient should be taken into consideration when the concentration of cell-surface–bound NA is determined for diagnostic purposes. As far as cell-surface–bound nucleic acids are concerned, they represent the main part of cirNAs and, along with cirNAs from plasma fraction, provide a valuable source of material for PCR diagnostics (18).

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