Changes in Dried Blood Spot Hb A1c with Varied Postcollection Conditions

To the Editor:

Diabetes affects >5% of US adults (15% of those older than 60 years) and is on the rise in adolescents. Glycosylated hemoglobin (Hb A1c), a cumulative marker of blood glucose concentrations over the previous 2 months, has become a powerful clinical tool for diabetes management (1, 2) and is predictive of future complications of diabetes (3). Large-scale community-based studies often preclude the option of phlebotomy and would benefit from simplified sample handling. Epidemiologic studies have begun to use Hb A1c in dried blood spots as a biomarker (4). We studied Hb A1c from dried blood spots by validating the storage conditions appropriate for large-scale epidemiologic/outreach studies to test the hypothesis that measurements of Hb A1c in dried blood spots are valid under low-intensity storage conditions.

All participants were studied at the Brigham and Women’s Hospital, Boston, Massachusetts; all procedures were conducted in accordance with the Declaration of Helsinki.

Blood samples were drawn via standard phlebotomy procedures into EDTA-containing tubes for duplicate HPLC analyses of Hb A1c (Tosoh G7 automated HPLC assay, Brigham and Women’s Hospital Hematology Laboratory). Intra-run imprecisions (CVs) were 0.5% and 0.9% at Hb A1c proportions of the total hemoglobin of 0.041 and 0.1335, respectively. Intra-run CVs were 1.6% for control samples with Hb A1c values of 0.064 and 0.7% for control samples with Hb A1c values of 0.11. Blood for spotting was drawn into an identical EDTA tube, and a syringe was used to let each drop of blood fall onto randomly assigned blood-spot cards (3 spots/card) within 1 min. Inversion of the syringe minimized the settling of red blood cells. Samples were air-dried for at least 20 min and then placed into single-sample, air-tight bags that included a desiccant pouch. Trios of blood-spot cards were processed identically. After storing the samples at room temperature for 0, 2, or 4 weeks, we shipped the samples to Biosafe Laboratories for Hb A1c analysis (conditions 1–3) or placed them in freezer storage (−80 °C) for an additional 4 or 12 weeks (conditions 4–6, 7–9). Sample trios were shipped by US mail, presumably at room temperature, in batches that included multiple individuals/conditions per package. Hb A1c measurements of dried blood spots were performed blinded to storage conditions and protocol. We used Roche Hba1c Hemolyzing Reagent to elute from 3.00-mm punches of homogeneous parts of the blood spot and analyzed the eluate with the Roche Modular P system with Roche Hb A1c reagents, an immunoturbidimetric assay with results that show good correlation with the standard Hb A1c methodology for whole blood \( r^2 = 0.9708; \gamma = 0.85x + 0.81 \) (n = 115). The Roche assay exhibited intrarun CVs of 2.1% at Hb A1c values of 0.05 and 1.4% at Hb A1c values of 0.076. Interrun CVs were 4.1% and 3.5% for the same Hb A1c values. We applied mixed-effects ANOVA models to compare the effects of different storage conditions and times on Hb A1c values measured from dried blood spots vs values measured by the HPLC assay. We repeated the analysis vs values from dried blood spots that had undergone minimal storage. To identify conditions that degraded samples, we compared SD and bias measures for the trio (3 cards).

Twelve individuals [4 diabetic and 8 nondiabetic individuals; mean (SD) age, 46.8 (12) years; mean body mass index, 31 (9) kg/m\(^2\)] completed the protocol. The SD of trios was <0.0012 (i.e., proportion of glycosylated hemoglobin) across all storage conditions and times; differences between the times of storage at room temperature \( F(2,88) = 1.44; P = 0.242 \), of storage in freezers \( F(2,88) = 1.90; P = 0.156 \), or their interaction \( F(2,88) = 1.41; P = 0.236 \) were not statistically significant. The mean bias of the assays of dried blood spots compared with the gold standard HPLC assay was −0.00099 (i.e., proportion glycosylated hemoglobin) (range, 0.00044–0.00300). The bias was greater at higher Hb A1c values (Fig. 1). This bias is expected because of systematic differences in the immunoturbidimetric and HPLC assay methods described above. Bias did not vary with the Hb A1c value when the referent condition was dried blood spots with minimal storage handling (data not shown).

The main effect of the duration of room temperature storage was significant \( F(2,304) = 5.26; P = 0.006 \), but the main effect of the freezer duration was not \( F(2,304) = 0.76; P = 0.467 \). Interactions between room-temperature and freezer-storage durations were significant \( F(4,304) = 10.56; P < 0.0001 \) but not in a systematic manner, suggesting that the number of storage-condition transitions affects bias.

We determined that simplified sample handling (without refrigeration or freezing) could be a valid means for obtaining reliable Hb A1c estimates. Storage at room temperature for up to a month or freezer storage for up to 3 months after collection yielded stable Hb
AC1 values. Across a range of storage conditions, we observed bias of approximately \(-0.001\) (proportion glycosylated hemoglobin) relative to results obtained with the gold standard HPLC method. We conclude, given the limitations of a small sample size and design that did not examine all permutations of storage conditions, that storage at room temperature is adequate; however, we encourage investigators to process and assay their samples as rapidly as practicable. The data obtained in this validation of methods and conditions appropriate for large-scale community and outreach studies extends the usefulness of Hb AC1 as a marker.

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Fig. 1. Effects of storage time at room temperature, duration of freezer storage, and their interaction on bias in Hb AC1 values measured from dried blood spots, compared with the gold standard HPLC method.

Individual data points are the mean of a trio (3 spots/card) of measurements of dried blood spots collected at the same time and processed identically. Data are presented as the change in absolute Hb AC1 values (i.e., proportion glycosylated hemoglobin) and compared with the mean of duplicate measurements by the HPLC method. Horizontal dashed line represents the mean bias for that condition.
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References

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S-Nitrosothiols in Blood:
Does Photosensitivity Explain a 4-Order-of-Magnitude Concentration Range?

To the Editor:

S-nitrosothiols such as S-nitroso glutathione (GSNO) are nitric oxide derivatives with potent biological actions. There is disagreement regarding the blood concentrations of nitrosothiols, which are reported to range between nondetectable and approximately 10 000 nmol/L (1). Problems and pitfalls in the analysis of nitrosothiols have been attributed to their wide concentration range in biological samples (1). Many of these analytical problems arise from the chemical nature of S-nitrosothiols and are difficult to control, but other problems may be avoidable (1).

Wu and coworkers recently reported that the photoinstability of S-nitrosothiols during sampling of whole blood may be a likely source of error and variation in S-nitrosothiol measurement (2). In general, we think that the findings of this study (2) are incorrect and suffer from analytical and methodologic shortcomings, as are discussed below.

1. Selectivity
Wu and coworkers assumed that their electrochemical method was selective for S-nitrosothiols and that any signal obtained was attributable to S-nitrosothiols (2). They reported that the ammonium/ammonia system may interfere with S-nitrosothiol analysis by this method (3) but did not report on a quantitative basis the potential contributions by NH3, NH2CH2, NH(CH2)2, and other volatile, labile, and readily oxidizable substances present in porcine blood. For instance, carbamino compounds are abundant in blood and spontaneously decompose to NH3 and CO2. Additionally, the authors did not report experiments that demonstrated the selectivity of this method for S-nitrosothiols, e.g., the use of HgCl2 (1).

2. Insufficient Experiments on Photoinstability of S-Nitrosothiols
Wu and coworkers (2) provided no solid and convincing evidence for the photoinstability of endogenous S-nitrosothiols, although they may have been constrained by the “Brief Communication” format. The following are additional experiments they should have included: (a) unequivocal demonstration of the high photoinstability of endogenous and externally added S-nitrosothiols, which would have excluded other possibilities for S-nitrosothiol degradation; and (b) measurements in plasma and erythrocytes. S-nitrosothiols are indeed sensitive to light; however, other published studies (1, 4) have not reported endogenous S-nitrosothiols to be as profoundly sensitive to light as proposed by Wu et al. (2). Our own experiments show that externally added GSNO to be equally stable in rat blood, whether “exposed” to daylight or protected from it (Fig. 1). This experiment also demonstrates, in contrast with what Wu and coworkers have stated (2), that N-ethylmaleimide does not induce NO liberation from GSNO.

3. Incorrect Calculation
In our opinion, Wu et al. did not correctly calculate the sensor signals from “covered” and “exposed” blood (2). If one refers to Fig. 1 in their report, it is easily observed that the initially constant current changes markedly with addition of blood to the chamber in which the electrode is placed and drops to levels clearly below the starting level. The greatest difficulty in assessing such current signals quantitatively is how to set the correct reference level. The baseline current level before blood addition can
not be used as the reference value for the signal resulting from blood addition.

By setting the baseline reference levels to values close to the nadir of the signals observed after blood addition, our calculation of the traces shown in Fig. 1 of the report by Wu et al. (2) indicates that the differences between “exposed” and “covered” blood are much less than the authors claim (2). The prolonged and marked changes in signal caused by addition of the blood render accurate quantitative analysis impossible. Moreover, the signal for the GSNO calibrator (3 μmol/L) added to “exposed” blood is clearly lower than the signal produced by the GSNO calibrator (3 μmol/L) added to “covered” blood. Interestingly, the ratios of the signals from putative endogenous S-nitrosothiols to those of the calibrator GSNO in “exposed” and “covered” blood differ insignificantly.

4. COMPARISON WITH DATA FROM THE LITERATURE

It is very strange that about 75% of the S-nitrosothiols in blood disappeared in the study of Wu et al. (2) within only about 15 s of blood sampling. This finding suggests that not protecting blood from light during sampling (the common practice of most scientists in this area, including those who have reported micromole-per-liter concentrations of S-nitrosothiols) would yield extremely low S-nitrosothiol concentrations; however, this is reportedly not the case (1).

Other analytical and preanalytical factors are more likely to be responsible for the great discrepancies in reported S-nitrosothiol concentrations in blood (1).

5. PHYSIOLOGICAL CONSIDERATIONS AND CONSEQUENCES

In our opinion, GSNO concentrations in the blood of the order of 3 μmol/L, as the authors claimed to have detected (2), would be not compatible with health in mammals and humans, given the potent biological activity of GSNO (1).

Fig. 1. Time course of the plasma concentration of GSNO added to rat blood unexposed and exposed to daylight.

In the dark, N-ethylmaleimide was added (to alkylate sulfhydryl groups) to rat blood (hematocrit, 35%) to a final concentration of 40 mmol/L. GSNO was then added to a final concentration of 4 μmol/L with respect to the whole-blood volume. The blood sample was divided into 2 equal aliquots, of which one aliquot was incubated in the daylight and the other in the dark. After incubation for the indicated times, blood aliquots were taken, and GSNO concentrations were measured with a modified Griess assay [Giustarini et al. (5)] after conversion to nitrite by HgCl₂. GSNO concentration was calculated from the difference of the spectra recorded in the absence and presence of HgCl₂. The experiments were carried out at room temperature (22–23 °C).

References

In Reply

We appreciate the thoughtful comments by Rossi and Tsikas regarding our previous report (1); however, we remain confident in our assessment of the photoinstability of S-nitrosothiols (RSNOs) in blood as monitored by our sensor-based method. Rossi and Tsikas’ specific concerns are addressed point by point below.

SELECTIVITY
Although not detailed in our report (1), the selectivity of the electrochemical RSNO sensor has been examined carefully. Remarkable RSNO selectivity is observed because of the high chemical specificity of organoselenium-catalyzed RSNO decomposition (2). Indeed, the sensor exhibits essentially no response toward nitrite, nitrate, N-nitrosamine, ascorbate, acetaminophen, or uric acid, whereas 1 mmol/L S-nitrosoglutathione yields an approximately 300-pA response (see Fig. 1). Iron-nitrosyls, including hemoglobin-nitrosyls, cannot permeate the outer dialysis membrane of the sensor and thus never contact the organoselenium catalyst that generates the NO [see sensor design in (3)]. Hydrogen peroxide has also been studied but does not yield a response below 100 μmol/L. Most importantly, a NO sensor devoid of the immobilized organoselenium species is always placed in the same diluted blood sample, and the signal from that sensor is used to correct for any interference signal that may occur for the RSNO sensor because of unknown components in the blood. Furthermore, at pH 7.4, amine species, including those referred to by Rossi and Tsikas, exist mostly in protonated forms and would not have any appreciable permeability through the Teflon AF–treated gas-permeable membrane of either the RSNO or NO sensors. In addition, with our sensor-based method, we have found that extracorporeal circulation of blood from a living animal (rabbit) through tubing possessing immobilized Cu(II) sites (a known catalyst for RSNO decomposition) on the inner wall greatly reduces the detectable concentrations of RSNOs in the blood, suggesting that the species being monitored are indeed RSNOs.

INSUFFICIENT EXPERIMENTS
We demonstrated that the reproducibility of sensor measurements of RSNOs for blood samples drawn sequentially from the same animal is within 10%. Given this reproducibility and the repeatable 75% decrease in the RSNO sensor signals, with the only difference being light protection, it is reasonable to infer that light exposure is the primary cause. Most importantly, we explicitly stated that the blood was drawn through a 12-in length of...
narrow-bore butterfly tubing, in which the effect of light exposure may be considerably greater than in larger-size containers (e.g., syringes), because of mixing and a high ratio of surface area to volume within the tubing. Mammalian blood has an appreciable absorbance at the wavelength of RSNO photodecomposition (560–580 nm) (4). Therefore, measurements of photodecomposition of blood RSNOs in tubes/containers of larger volumes that light cannot penetrate will not yield the same results. For the stability data that Rossi and Tsikas presented in their Letter, they indicated neither the blood volume nor the ratio of the surface area to the volume of the blood container. In addition, they did not indicate whether the blood was mixed; therefore, the ambient light might have penetrated only into a very thin layer of blood adjacent to the vessel wall. Furthermore, the sensor method responds to a variety of RSNO species in blood (including, to some degree, macromolecular RSNOs via a transnitrosation reaction with added thiol reducing agent) (2, 3), not just S-nitrosoglutathione (the only RSNO species that Rossi and Tsikas tested). These species may well have different photodissociation rates.

INCORRECT CALCULATIONS
In amperometry, current signals are additive and an analyte concentration of zero does not necessarily mean zero current. In a solution containing no analyte of interest, a background current is always present because of the oxidation/reduction of water or trace impurities in the inner electrolyte of the gas sensor. For our RSNO sensor, the immobilized organoselenium catalyst decomposes the small molecular RSNOs in blood to yield NO gas. The NO diffuses through a membrane and is immediately oxidized on the Pt-black electrode to yield anodic current proportional to the NO (and, in turn, the RSNO) concentration. It is appropriate to calculate the RSNO concentration from the difference between the baseline and steady-state signals. The “dip” that sometimes occurs immediately after blood addition reflects a transient fluctuation in the current due to mixing and/or slight temperature changes (the sensor measurement cell is thermostated at 34 °C, and the blood cools toward room temperature in the syringe before the sample can be added to the measurement cell).

LITERATURE DATA
Rossi and Tsikas acknowledge the wide discrepancies in literature-reported RSNO concentrations, and hence our sensor-measured values at the higher end of this range do not automatically disqualify our findings. We agree that there may be other factors contributing to these discrepancies, and that consideration was precisely the goal of reporting our initial observations regarding the effect of shielding the sampling tubing from light (1). Without conducting experiments with an identical blood-collection method or with the variable RSNO species found in blood, their data do not necessarily refute our findings. We suggest that they circulate blood samples spiked with S-nitrosoglutathione and other RSNOs through very narrow-bore tubing (e.g., 1 mm i.d.) and then report whether RSNO concentrations decrease.

PHYSIOLOGICAL CONSIDERATIONS/ CONSEQUENCES
We concur that RSNOs are known to exert physiological functions like those of NO, such as vasodilation; however, we have repeatedly found that infusion of RSNOs (e.g., S-nitroso-N-acetylpenicillamine) into rabbits under anesthesia for 4 h to yield micromole-per-liter concentrations in whole blood (as measured with the sensor) has no major consequences (e.g., a drop in blood pressure of only 15 mmHg and no significant effect on platelet function).

We recognize that our findings may be controversial. The methodologies that have thus far been used for measuring RSNOs have many flaws. Blood contains a host of proteins with active sites (including sites with Cu(II) and selenium) that can spontaneously catalyze RSNO decomposition [e.g., glutathione peroxidase (5)]; however, we believe that the rate of this decomposition is normally slow, as evidenced by Rossi and Tsikas’ results, but may be accelerated by light of the appropriate wavelength. Such decomposition can occur only when photons can penetrate into the bulk of the blood phase. We are currently conducting studies to further verify this photocatalytic effect.

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Letters to the Editor

Interference from Rose Bengal with Total Bilirubin Measurement

To the Editor:

We report an unusual cause for a false-positive interference in the total bilirubin method on the Beckman Coulter DxC 800 analyzer and for the hemolytic index (HI) measured on the Roche Modular D. A total bilirubin result of 53 μmol/L was obtained for a patient who had an earlier result of 7 μmol/L on the DxC 800 analyzer. The direct bilirubin concentration remained at <4 μmol/L, and no other results changed in subsequent multibiochemical profiles. The sample had a red/pink tinge. There was no clinical explanation for the increased bilirubin value. A sample obtained the next day had a total bilirubin result of 7 μmol/L. The patient was a participant in a trial for the treatment of severe melanoma lesions with PV-10 [100 mmol/L rose bengal (4,5,6,7-tetrachloro-2’,4’,5’,7’-tetrachlorofluorescein disodium; MW, 1017.65 Da) in 9 g/L NaCl (i.e., 10% rose bengal disodium in 9 g/L NaCl); Provectus Pharmaceuticals]. PV-10 causes tumor necrosis, possibly owing to the release of cathepsins (1). The trial protocol is to inject PV-10 directly into the lesion and to collect a blood sample within an hour. The sample in question was collected 20 min after the injection. Most likely, the PV-10 entered the bloodstream rapidly because it had been injected deeply into healthy tissue or the lesion was well vascularized.

A scan of the affected sample with a Beckman Coulter DU 640 spectrophotometer (460–610 nm) revealed a peak absorbance at 562 nm. A diluted aliquot (1 part in 10 000 with normal saline) of the PV-10 solution showed a color and intensity similar to the colored sample and had a peak absorbance at 549 nm. We prepared 3 different PV-10 dilutions with normal saline and a dilution of 1 part PV-10 in 10 000 with a sample of pooled patient plasma with a bilirubin concentration of 10 μmol/L. We analyzed these dilutions for total bilirubin and serum indices with the Beckman Coulter DxC 800 and Roche Modular D analyzers (Table 1).

Table 1. Results of total bilirubin measurement and hemolytic and icteric indices for samples containing PV-10 (rose bengal).a

<table>
<thead>
<tr>
<th>System/assay</th>
<th>PV-10 (1/1000 with saline)</th>
<th>PV-10 (1/2000 with saline)</th>
<th>PV-10 (1/10 000 with saline)</th>
<th>Pooled plasma</th>
<th>PV-10 (1/10 000 with pooled plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beckman Coulter DxC 800</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin, μmol/L (reagent)</td>
<td>208</td>
<td>109.5</td>
<td>23.8</td>
<td>9.8</td>
<td>36.2</td>
</tr>
<tr>
<td>HI</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Icteric index</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Roche Modular D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin, μmol/L (reagent)</td>
<td>−9.7</td>
<td>−3.9</td>
<td>−0.5</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>HI, mmol/L</td>
<td>1.35</td>
<td>0.825</td>
<td>0.155</td>
<td>0.037</td>
<td>0.645</td>
</tr>
<tr>
<td>Icteric index, μmol/L</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

a The Beckman Coulter analyzer measures the HI and icteric index semiquantitatively: an icteric index of 1 is equivalent to 0–25.7 μmol/L total bilirubin, and an HI of 1 is equivalent to 0–0.03 mmol/L free hemoglobin. PV-10 dilutions were prepared by diluting 1 volume PV-10 solution to the indicated total volume.
concentration increased in proportion to the PV-10 concentration on the DxC 800 and in proportion to HI values on the Roche Modular D. From the saline dilutions, we calculated the PV-10 concentration in the affected patient blood to be approximately equal to a dilution of 1 part in 5000, or approximately 20 μmol/L.

To determine the mechanism of the interference, we manually added the bilirubin reagents and the pooled plasma spiked with PV-10 in the same ratios as per the manufacturers’ methods. Absorbances were measured on the Beckman Coulter DU 640 spectrophotometer. The 2 bilirubin methods measure primary/secondary absorbances at 520 nm/580 nm with the Beckman Coulter method and 546 nm/600 nm with the Roche method. Blanking is performed after the addition of reagent A and reagent B and without sample in the Beckman Coulter method, and after reagent 1 and sample are added in the Roche method. Both assays use diazo methods, with azobilirubin being the final product. In both methods, the PV-10 is either chemically altered or compounds are formed with the first reagent in both methods; the compound(s) has a peak absorbance at 562 nm. Blanking with the Beckman Coulter method would not correct for nonspecific chromogens from samples with PV-10, hence the interference. In contrast, blanking with the Roche method is able to correct for nonspecific chromogens from the sample; in fact, the negative values in Table 1 suggest that it may overcorrect. With the Beckman Coulter serum indices method, the sample is added to saline, and absorbances are measured at 570 nm and 600 nm for the HI. The presence of PV-10 causes a substantial absorbance increase at 570 nm.

On the Beckman Coulter analyzers, the icteric index and the direct bilirubin concentration (in which sample blanking is performed with reagent A) remain unchanged in the presence of PV-10. Discordant Beckman Coulter total bilirubin results with no change in the icteric index or the direct bilirubin value and/or an increased HI from the Roche analyzer in the absence of changes in other analytes (e.g., K⁺, lactate dehydrogenase, and so on) may indicate the presence of PV-10. The manufacturer of PV-10 has been informed of these findings. The PV-10 interference illustrates how different method-blanking procedures can help eliminate interferences.

Some details of the success of the PV-10 trial have been published (1), and full details undoubtedly will be published in the near future. In light of the present findings, the trial protocol for the timing of postinjection sample collection should be reviewed. Plans are under way for additional trials of PV-10 for treating other cancers. Therefore, these interferences may become more common with the assays outlined above and potentially with others on different analytical systems.

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Some Notes on Visinin-Like Protein 1 and Alzheimer Disease

To the Editor:

We wish to comment and add some potentially useful information on certain aspects of the report of Lee and collaborators, which was recently published in Clinical Chemistry (1). In an earlier study, the authors had proposed visinin-like protein 1 [VILIP-11 or VSNL1; VSNL1 gene (visinin-like 1)] as a
Letters to the Editor

potential biomarker for stroke because they had detected this intracellular calcium-binding protein in cerebrospinal fluid (CSF) in a rat model of stroke and in the plasma of patients after stroke (2). The group extended these findings to Alzheimer disease (AD) in a second publication (1). VILIP-1 concentrations were significantly altered in the CSF of AD patients; hence, the authors concluded that VILIP-1 might also be a useful novel biomarker for AD-related brain injury. Lee et al. put forward the hypothesis that measures of VILIP-1 might reflect neuronal injury with subsequent release of the intracellular protein VILIP-1 into the CSF. The authors stated that both Aβ and τ reflect different pathologic features of AD, whereas VILIP-1 may reflect the end result of the disease, namely neuronal cell death. We would like to add important information concerning the hypothesized relationship between the calcium sensor protein VILIP-1 and AD (3–5). Owing to the limited space, we cannot discuss here the findings on the production of VILIP-1 in rat brain (1); instead, we would like to emphasize that VILIP-1 immunoreactivity is also abundantly present in the nondemented human brain with a regional and cellular distribution of the protein similar to that in rat brain (3). With a knowledge of the topochemistry of VILIP-1 in human brain, we have addressed 3 questions about VILIP-1 in the brains of AD patients: (a) Is the cellular localization of the protein altered in AD? (b) Is the protein content altered in AD brains? and (c) is there any anatomical association of the neuropathologic hallmarks of the disease (plaques, tangles) with VILIP-1? We found that the number of VILIP-1–immunoreactive neurons was significantly reduced in the temporal cortex of AD patients, whereas the total number of neurons was unchanged (4). These data point to a disease-related loss of VILIP-1–producing neurons. Moreover, western blot analyses of brain tissue extracts of AD patients revealed that VILIP-1 is less concentrated in AD brains (5). Importantly, other groups have confirmed the reported changes in VILIP-1 protein production in AD brains by means of gene microarray analysis (6). Both the reduced number of VILIP-1–immunoreactive cortical neurons and the decreased tissue content in AD fit with the CSF data presented by Lee and colleagues. Furthermore, extracellularly located VILIP-1 was detected in close association with typical pathologic hallmarks of AD, such as dystrophic nerve cell processes, amorphous and neuritic plaques, and extracellular tangles, pointing to an involvement of this calcium sensor protein in the pathophysiology of changed calcium homeostasis in AD (4, 5). Moreover, these data provide reason to suppose that VILIP-1 may not only be a CSF marker of cell injury in AD (1) but may also be causally related to AD. Because VILIP-1 is associated with fibrillar tangles in AD brains, we tested whether VILIP-1 has an influence on τ hyperphosphorylation. VILIP-1 production enhanced hyperphosphorylation of τ protein and enhanced calcium-mediated cell death in transfected neuronal cell lines. These findings suggest that this calcium sensor protein may indeed influence τ phosphorylation and have a role in calcium-mediated neurotoxicity in AD. The observed reduction in VILIP-1–producing cells in AD thus may indicate selective vulnerability (5). The close association of VILIP-1 with τ pathology is of special interest because it is in good agreement with the findings of Lee et al. (1) that VILIP-1 values correlate highly with phosphorylated τ in patients, but not with Aβ values.

We hope that our added information on VILIP-1 will enable the readers of Clinical Chemistry to put these highly interesting data on the potential biomarker VILIP-1 in perspective with respect to a possible pathophysiological relationship between VILIP-1 and AD.

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Comparison of a New Procalcitonin Assay from Roche with the Established Method on the Brahms Kryptor

To the Editor:

Procalcitonin (PCT) is a 13-kDa peptide and a precursor of calcitonin. In a healthy population, PCT concentrations are negligible (1). In systemic bacterial and fungal infections, plasma concentrations are raised, whereas concentrations remain fairly low in infections of viral or nonspecific cause (2). Recent studies have demonstrated the potential of PCT as a parameter to guide antibiotic therapy in different groups of patients, i.e., patients with chronic obstructive pulmonary disease experiencing respiratory tract infections (3, 4). The most frequently used medical decision points at which the use of antibiotic therapy is considered are 0.25 μg/L and 0.50 μg/L, depending on the patient population (3, 4).

The first PCT assays were based on manual immunochemistry methods (Brahms PCT LIA). These assays have been replaced by fully automated immunochemistry methods (Brahms Kryptor, Brahms LIAISON, Olympus SphereLight 180). Recently, the PCT assay has been modified for use on a consolidated routine immunochemistry analyzer family, the Roche Elecsys, cobas, and the Roche Modular E170 systems. We evaluated the analytical performance of this new assay by following the EP10 protocol, a document from the Clinical and Laboratory Standards Institute to test precision, linearity, recovery, carryover, and drift. Samples were prepared at different concentrations, from 0.24–2.85 μg/L. Three aliquots of each concentration were assayed on 5 different days, in a specific assay order. The within-run CV ranged from 3.0% for the lowest concentration to 1.3% for the highest concentration. The between-day CV ranged from 6.3% for the lowest concentration to 2.8% for the highest. These levels of imprecision were comparable with those reported for the PCT assay on the Brahms Kryptor (5). The mean recovery was 99%. There was no evidence of nonlinearity or sample carryover. The limit of quantification, i.e., the lowest concentration of analyte that can be quantified with a between-run imprecision of <20%, met the manufacturer’s specification of 0.06 μg/L. In addition, we compared the new PCT assay from Roche on the Modular 170 with the widely accepted PCT assay from Brahms on the Kryptor (5). For analytical comparison, we used 229 samples of patient serum obtained from 195 different patients who were admitted to our hospital for lower respiratory tract infections (81, exacerbation of chronic obstructive pulmonary disease; 114, pneumonia). The patients participated in an ongoing study in our hospital on the etiology of exacerbations of chronic obstructive pulmonary disease, a study approved by the local ethics committee. Samples were also collected from 34 patients after antibiotic treatment. The majority of the serum samples were obtained within 24 h of admission. Samples not immediately analyzed were stored at −80 °C until analysis. PCT concentrations ranged from 0.02 μg/L (limit of detection, i.e., the lowest concentration of analyte that can be reliably measured as being qualitatively present in the sample) to 57 μg/L. PCT concentrations were <0.10 μg/L in 126 samples, ≥0.10 μg/L and <0.25 μg/L in 34 samples, ≥0.25 μg/L and <0.50 μg/L in 19 samples, and ≥0.5 μg/L in 50 samples. Nearly all of these patients, including the 126 patients with PCT concentrations <0.10 μg/L, were treated with antibiotics, reflecting the potential benefit of PCT-guided antibiotic therapy for preventing antibiotic overuse. Confirmation of this possible benefit awaits further study. Methods were compared by orthogonal Deming analysis (y = 0.95x − 0.09 μg/L, where x is the PCT assay from Brahms on the Kryptor and y is the PCT assay from Roche on the Modular instrument; Syx = 1.02; r = 0.99) and by medical decision points. No outliers were detected (i.e., distance from the regression line exceeding 10 times the Syx value). The concordance between the 2 assays was 99% and 98% at the cutoff values of 0.25 μg/L and 0.50 μg/L, respectively. Fig. 1 shows the comparative data for the clinically important interval of 0–1.0 μg/L. The predicted medical decision points and 95% CIs for the Roche assay were 0.24 (0.23–0.25) μg/L and 0.49 (0.48–0.51) μg/L, respectively, as calculated by Deming regression analysis.

In conclusion, the new PCT assay on the Roche Modular shows a
good correlation and concordance with the PCT assay on the Brahms Kryptor in a highly relevant patient group. Thus, the PCT assay from Roche allows easy and accurate measurement of serum PCT on an automated clinical immunochemistry analyzer that is fully integrated in standard hospital care.

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Fig. 1. Procalcitonin concentrations as measured with the Roche Modular 170 assay and the Brahms Kryptor assay.

Procalcitonin concentrations were measured within the clinically important range of 0–1.0 μg/L (y = 1.03x − 0.02 μg/L; Sxy = 0.05; r = 0.96). Diagonal dashed line, y = x; solid line, Deming regression line; horizontal and vertical dashed lines, medical decision points.

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Familial Dysalbuminemic Hyperthyroxinemia: A Persistent Diagnostic Challenge

To the Editor:

Familial dysalbuminemic hyperthyroxinemia (FDH)1 is a well-characterized condition associated with increased circulating total thyroxine (T4) concentrations and normal physiological thyroid function. It is caused by mutations in the ALB (albumin) gene that increase the affinity of albumin for T4 by approximately 60-fold. When measured by a technique that minimally disturbs the equilibria between T4 and its serum binding

1 Nonstandard abbreviations: FDH, familial dysalbuminemic hyperthyroxinemia; T4, thyroxine; SyD, symmetric dialysis; FT4, free T4.
proteins, such as equilibrium or symmetric dialysis (SyD) performed in a near-physiological medium, the free T₄ (FT₄) value is characteristically within the reference interval. Assays that rely on the competition of a T₄ analog with unbound T₄ in the sample can give spuriously high results in FDH patients, because albumin binding of the T₄ analog is enhanced by the FDH mutation. “Two step” methods, in which the T₄ analog never comes into contact with serum albumin owing to a wash step immediately after capture, avoid this problem. Such assay methods are expected to give FT₄ results within the reference interval in FDH patients, but this expectation has been questioned (1, 2).

Thyroid-function tests, including 1- and 2-step methodologies, were examined in 4 affected individuals from different families who had their FDH diagnoses proved genetically by DNA sequencing of exon 7 of the ALB gene. Each individual carried the well-recognized R218H albumin mutation associated with this disorder (R242H, if the signal peptide is included). SyD was chosen as an alternative method, because this technique is unlikely to be affected by abnormalities in serum T₄-binding proteins. The assay was performed as previously described (3) but with a buffer of a more physiological composition (4). FT₄ assays were performed as described by the manufacturers with reagents and equipment provided by Abbott Diagnostics (ARCHITECT®), Beckman Coulter (Access®), PerkinElmer (DELFIA®), Roche (Elecsys E170), Siemens (Immulite® 2500 and ADVIA Centaur®), and Tosoh Bioscience (AIA®-1800). The Siemens, Roche, and Tosoh assays are 1-step analog methods, and the others are 2-step assays. Total T₄ was measured with the PerkinElmer DELFIA method.

Fig. 1 summarizes the FT₄ results for the different assays. The reference intervals used were those provided by the kit manufacturer, which were not necessarily those used by the respective laboratories. The values for thyroid-stimulating hormone were within the reference interval for all assays in all patients. For all methods, except for SyD, at least one of the patient results fell above the recommended reference interval for FT₄.

In FDH patients, the serum FT₄ results obtained with any of these assays can be misleading. The Siemens Centaur 1-step assay performed considerably better than some of the 2-step methods. This finding is consistent with a report on the predecessor of the Centaur that stated that it yielded the same results as equilibrium dialysis with FDH samples (5). If the limited size of the data set is taken into account, the Centaur, DELFIA, and Abbott methods compare reasonably well with SyD, with the differences between the methods possibly representing relatively small assay biases or the use of incorrect reference intervals. The results for 4 methods in routine use, however, show large deviation from those of the SyD method. Theoretically, both the 1-step and 2-step designs are based on valid principles; however, application of the 1-step approach is hampered by the fact that no T₄ analog has been found that fulfills the essential criteria of binding to the anti-T₄ antibody but not to T₄-binding proteins in the serum. Manufacturers have been trying to reduce this problem both by adding extraneous albumin and by adding inhibitors of T₄ binding to albumin (2). Such additions are not required for 2-step methods, although some manufacturers still use them. Addition of inhibitors of T₄ binding to albumin will lead to spuriously high values for FDH samples, because a much larger proportion of T₄ is bound to albumin in FDH samples than in typical samples; therefore, a higher proportion of the T₄ will be released by the action of the inhibitor during incubation. When SyD is performed in a buffer from one of the 2-step assays that yields high results for FDH samples, similarly high results are obtained (data not shown). Because serum samples from FDH patients still have the potential to generate false-positive FT₄ results in both 1- and 2-step immunoassays, it is essential that this benign condition be identified in situations in which measurements of serum samples produce high FT₄ results with thyroid-stimulating hormone values within the reference interval. A diagnosis
of FDH can be excluded by means of biochemical methods and by albumin genotyping. Because all mutations that have been associated with FDH to date involve residue 218 (242 with the signal peptide) in the albumin molecule, molecular genetic testing is comparatively simple and returns an unambiguous result.

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