To the Editor:

In their letter, Leventis et al. compare methods for 25-hydroxyvitamin D [25(OH)D] measurements. In light of recent reports, and given the complexities of accurately measuring the physiologically relevant species among metabolites of vitamin D, we consider that the title and the conclusions of their letter may be potentially misleading. One of the methods they studied is based on antibody recognition and isotopic detection (DiaSorin), whereas the other is based on vitamin D-binding protein recognition and chemiluminescence detection (Nichols Institute Diagnostics). These important differences may be responsible for the different results. We have communicated to Nichols Advantage assay users in customer bulletins issued on June 15, 2004, and December 6, 2004, that our customers of a study showing that, after treatment of patients with 25(OH)D2, the assay gave measured increases in 25(OH)D concentrations (1). In their letter, Leventis et al. also indicate that in the posttreatment samples, both of the assays that they studied detected an increase in 25(OH)D concentration compared with baseline. Representations of the Vitamin D External Quality Assessment Scheme, in a Technical Brief published in this journal (2), indicated that the Nichols Advantage assay does detect 25(OH)D2 although with lower absolute values.

We do not dispute that different vitamin D assays may yield different results. One possibility is that the natural vitamin D-binding protein in the Nichols Advantage assay quantifies the physiologically relevant metabolite(s) of vitamin D2 better than does an antibody-based assay. This has led us to start investigating the identities of metabolites generated by vitamin D2 using HPLC and mass spectrometric methods. A recent report by Heaney et al. (3) is noteworthy in that it reflects substantially more rapid metabolism or clearance of the vitamin D2 metabolites. Further research is necessary to uncover the identities of vitamin D metabolites and their clinical significance. Until this is done, conclusions such as those drawn in the letter of Leventis et al. should be considered as preliminary and premature.

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References


Detrimental Effect of Formaldehyde on Plasma RNA Detection

To the Editor:

Recent studies have shown that detection of fetal DNA or RNA in maternal plasma is useful for prenatal investigation of certain fetal genetic traits (1, 2) or pregnancy-associated complications (3, 4). Fetal DNA has been shown to amount to 3.4%–6.2% of the total DNA in maternal plasma (5). Thus, the reliability of circulating fetal nucleic acids analysis is dependent on the ability to sensitively and specifically detect and distinguish such fetal molecules from a background of maternal nucleic acids. Hence, methods that enable enrichment of the proportion of fetal nucleic acids in maternal plasma would, in theory, facilitate robust analysis of circulating fetal nucleic acids. Dhallan et al. (6) recently explored the use of formaldehyde for the enrichment of circulating fetal DNA. The authors reported marked increases in the proportion of fetal DNA in maternal blood samples preserved with formaldehyde. Although controversies exist regarding the effect of formaldehyde on fetal DNA enrichment (7, 8), we nonetheless wanted to investigate whether form-
aldehyde could be used for the enrichment of circulating fetal RNA.

We collected EDTA-blood samples (12 mL) from each of 6 pregnant women (gestational age, 17–19 weeks) with informed consent. We immediately treated 6 mL of each blood sample with 150 μL of 10% neutral-buffered solution containing formaldehyde and left the remaining 6 mL untreated; we then subdivided the treated and untreated blood portions into 3 aliquots. One aliquot each from the treated and untreated groups was processed by centrifugation to obtain plasma (9), either immediately or after 6 or 24 h of storage at 4 °C. RNA was extracted from the plasma by a combined Trizol LS and column-based method as described previously (9) and was further subjected to real-time quantitative reverse transcription-PCR analysis.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (9) (Fig. 1A) and a placenta-specific mRNA, human placental lactogen (hPL) mRNA (10) (data not shown), were detected in the maternal plasma aliquots without formaldehyde treatment but in none of the paired aliquots treated with formaldehyde.

Because formaldehyde has previously been shown to interact with Trizol and affect its ability to extract RNA from tissue (11), we investigated whether this was the cause of our observed data. Thus, instead of adding formaldehyde directly to the whole-blood samples, we mixed formaldehyde with Trizol LS and used that solution to extract RNA from plasma samples that had not been treated previously with formaldehyde. RNA was readily detectable from plasma samples extracted with Trizol LS solutions containing up to 2 mL/L formaldehyde, which was equivalent to the final concentration of formaldehyde in the blood samples evaluated in the earlier part of the study, but not when the formaldehyde concentration in the solution was 20 mL/L (Fig. 1B). These data suggest that formaldehyde does affect the efficacy of RNA extraction by Trizol LS, but not at the concentration used to preserve the maternal blood samples. We then used a non-Trizol–based extraction method, the QIAamp viral RNA Mini Reagent Kit (Qiagen), to extract plasma RNA from blood samples to which formaldehyde had or had not been added. RNA was not amplifiable from any of the plasma extracts obtained from the formaldehyde-treated blood (Fig. 1C).

Although the underlying mecha-
nism is unclear, based on these results, we conclude that formaldehyde has a detrimental effect on plasma RNA detection. Irrespective of the extraction protocol used, it appears that no amplifiable RNA in plasma can be obtained from formaldehyde-treated whole-blood samples.

References

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DOI: 10.1373/clinchem.2004.045807

Paraprotein Interference in an Assay of Conjugated Bilirubin

To the Editor:
Artificially increased total bilirubin and artifactually low HDL have been described recently in a patient with a monoclonal IgM paraprotein (1). Similar interferences have already been described for serum samples containing paraproteins when tested for bilirubin [with a reagent from the same manufacturer (2)], phosphate (3–7), creatinine (8), calcium (9), urea nitrogen (10), iron (11), C-reactive protein, and antistreptolysin-O (12). Here we describe interference in the measurement of conjugated bilirubin by a different analyzer in sera from 3 patients (A, B, and C) with a monoclonal IgG–κ paraprotein. Conjugated bilirubin was initially measured with the Olympus AU2700 automated analyzer using the Olympus conjugated bilirubin assay. For patient A (40-year-old man), the reported conjugated bilirubin was 37.5 mg/L, total bilirubin was 2.0 mg/L (reference interval, 0.0–11.0 mg/L), and total protein was 156 g/L. For patient B (64-year-old man), the reported conjugated bilirubin was 12.0 mg/L, total bilirubin was 3.3 mg/L, and total protein was 89 g/L with a monoclonal IgG–κ component of 25.9 g/L. For patient C (42-year-old woman), the reported conjugated bilirubin was 10.9 mg/L, total bilirubin was 2.0 mg/L, and total protein was 136 g/L with a monoclonal IgG–κ component at a concentration of 97.2 g/L. The concentration of the paraproteins was determined by serum protein electrophoresis with densitometry and total protein measurement. No spurious creatinine, calcium, inorganic phosphate, urea nitrogen, or iron measurement using Olympus assays on the Olympus AU2700 analyzer were detected in the 3 samples.

The Olympus serum total bilirubin assay is an end-point chromogenic assay (13). The reagent contains an “accelerator” (caffeine) to solubilize unconjugated bilirubin, together with a diazonium salt (2,5 dichlorophenyl-diazonium-tetrafluoroborate), in the presence of surfactant to avoid protein precipitation, in a weakly acid medium (pH 5). The color (pink) intensity of the azobilirubin produced is proportional to the total bilirubin concentration. For determination of the conjugated fraction, the solubilizing agent and the surfactant are lacking in the reagent, and the medium is strongly acidic (pH 1) to eliminate conjugated isomers of bilirubin from measurement. At this low pH, proteins typically precipitate. To avoid that precipitation, this reagent contains a “protein stabilizing agent”. Visually, the patient samples were nonicteric and showed no evidence of hemolysis or lipemia. Clinically, the patients were not jaundiced, and there was no supporting evidence for hemolysis or liver disease. On a different analyzer that uses the dry-chemistry methodology (Vitros 950; Ortho-Clinical Diagnostics), the results for conjugated bilirubin were 0.6, 1.0, and 0.4 mg/L in patients A, B, and C, respectively.

We also compared results between intact sera and the serum ultrafiltrates (Ultrafree CL; nominal molecular mass cutoff, 30 kDa; Millipore) from the same 3 patients. Serum from a patient without myeloma and a comparable concentration of conjugated bilirubin was included as a control. The conjugated bilirubin concentrations in the serum ultrafiltrates were 0.9, 0.6, and 0.3 mg/L for patients A, B, and C, respectively. No