Improved Method for Measurement of Retinol and Didehydroretinol in the Modified Relative Dose Response Test to Detect Vitamin A Deficiency

To the Editor:

Vitamin A deficiency (VAD) is one of the most devastating dietary deficiencies worldwide. It causes significant increases in childhood and maternal morbidity and mortality among the poor of the nonindustrialized world. Several methods are available to assess VAD. The determination of retinol in blood is one of the most frequently used methods, but it has several disadvantages. Serum retinol is decreased only in severe VAD when liver stores are nearly exhausted. In addition, infection depresses the retinol concentration in blood, possibly leading to misclassification of individuals.

Because the majority of vitamin A in the body is stored in the liver, tests have been developed to measure vitamin A stores that tend to provide more reliable information about vitamin A status. The two most common tests of this nature are the relative dose response test (RDR) and the modified RDR test (MRDR). Of these, the most practical method for field collection is the MRDR. This test has the advantage of requiring only one blood sample (1). An equivalent of 5.3 μmol of didehydroretinol is given in the morning and a blood sample is taken 5 h later. Retinol and didehydroretinol concentrations in the blood are measured by HPLC, and the ratio of didehydroretinol to retinol is calculated. A percentage >6% is indicative of VAD.

The MRDR is an excellent test, but it has been underutilized, in part because of the poor chromatographic separation of didehydroretinol from retinol and the arduous sample preparation. The objectives of this study were therefore to (a) improve the chromatographic separation of retinol from didehydroretinol and (b) speed up the sample preparation process to allow higher sample throughput. The new procedure has been tested in a large HIV/vitamin A study in Zimbabwe using heel-prick samples in infants 6–12 months of age.

A 100-μL serum sample was denatured by mixing with 100 μL of ethanol containing butylated hydroxytoluene (5 g/L) and retinol acetate (0.5 μmol/L) as an internal standard. Hexane (400 μL) was then added, and the tubes were capped. The entire rack of tubes was vigorously mixed by hand for 1 min, using a vertical shaking motion. After centrifugation, 300 μL of the supernatant was evaporated to dryness, and the residue was dissolved in 100 μL of mobile phase (acetonitrile–water, 85:15 by volume). We injected 80 μL of this solution into the HPLC. The analytes were separated on a Thermo Hypersil Prism RP column [150 × 3 mm (i.d.); 3 μm bead size] at a flow rate of 1.5 mL/min, with detection at 350 nm. For economic reasons, the mobile phase was recycled for ~300 injections.

Typical serum chromatograms obtained with the new and traditional methods are shown in Fig. 1. In the traditional method, the separation is performed with a Waters Resolve C18 column [150 × 4.6 mm (i.d.)] and methanol–water (90:10 by volume) as mobile phase. When we switched to the Thermo Hypersil Prism RP column and acetonitrile–water as mobile phase, the peaks were sharper and well separated, and the run time was shortened. As a result, the CV and sensitivity of this measurement are improved. The day-to-day CV was 3.7% (n = 5 days) for a sample with high didehydroretinol 0.056 μmol/L and 5.9% (n = 5 days) for a sample with low didehydroretinol (0.03 μmol/L). Our previous CV values and literature values were typically 10% (2).

The new method has several advantages. Because only 100 μL of serum is used in this method, it is applicable to small samples, such as those from finger or heel pricks. Until now, all published methods specified at least 250 μL of serum. The detection limit for didehydroretinol in relation to retinol for a typical plasma sample with 0.7 μmol/L retinol is 1%, which is far below the cutoff value of 6%.

For large studies, the traditional method is time-consuming and delays information dissemination and intervention. To increase the throughput in these studies, some improvements have been made. The proposed protocol requires only one hexane extraction, rather than the three hexane extractions used in the traditional method. This reduces the workload substantially and has only a minor influence on the sensitivity of the measurement. The ratio between didehydroretinol and retinol is the critical value in the MRDR; we examined the residue after the first extraction and found no difference in the ratio. The second improvement is the use of one-piece disposable plastic autosampler vials with a conical sample compartment (Merck Eurolab). Because these hold up to 300 μL of solvent and have a wide opening on the top, hexane can be evaporated directly from these vials, saving one transfer step. With these modifications and a simple inexpensive mechanical evaporator unit with 68 lines (Evap-O-Rac System, Cole Parmer), it is possible to prepare 68 samples in one batch in ~4 h. Consequently, throughput can be increased from 20 samples a day to >60.

In conclusion, the improved
MRDR-HPLC method is more reliable and faster. With these modifications, the MRDR may find wider application for the detection of VAD.

References

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Effects of Blood Collection Methods on Gelatin Zymography of Matrix Metalloproteinases

To the Editor:
Matrix metalloproteinases (MMPs), zinc-dependent proteinases belonging to the family of matrixins, are involved in the processes of normal development and growth as well as in several pathologic states (1). MMP-2 (gelatinase A; EC 3.4.24.24) and MMP-9 (gelatinase B; EC 3.4.24.35) play crucial roles in the proteolytic cascade leading to extracellular matrix degradation (2). To evaluate the usefulness of MMPs as tumor markers, several authors have measured MMP-2 and MMP-9 in the blood of cancer patients, using serum or plasma samples, and reached contradictory conclusions (3, 4). The preanalytical steps of blood sampling and processing influence the concentrations of MMPs and their inhibitors (5, 6), but no data are available concerning the effect of blood specimen collection methods on the appearance of the full spectrum of MMP isoforms on gelatin zymograms.

We simultaneously collected blood samples from 20 healthy volunteers (median age, 36 years; range, 23–55 years) into plastic tubes with no additive, plastic tubes with a silica gel-coated surface as coagulation accelerator, lithium heparin-coated plastic tubes, and potassium EDTA-coated plastic tubes (Monovette Systems; Sarstedt). The tubes were kept at room temperature and centrifuged immediately after venipuncture (1600 g for 15 min at 4 °C), and the supernatants were stored at −80 °C until analysis. Human gelatinases were prepared as described previously (7). Gelatin zymography was performed under nonreducing conditions on 7.5% polyacrylamide mini slab gels (Bio-Rad), copolymerized with 1.5 g/L 90 Bloom gelatin (Sigma) (8). Aliquots containing 50 μg of total protein (Bio-Rad) were used for each zymographic test. To assay gelatin lysis, scaled aliquots of proteins were run in triplicate and submitted to computer-assisted densitometric scans using Image Pro-Plus software (Cybernetics); the semiquantitative results were expressed as a percentage vs control or calibrator.

The gelatin zymography technique allows detection of all MMPs in circulating blood, including the largest isoform (225 kDa; Fig. 1). The four bands showing gelatinase proteolytic activity were fibroblast-derived pro-MMP-2 (72 kDa) and neutrophil-derived pro-MMP-9 (92, 130, and 225 kDa) (7, 8).

As shown in Fig. 1A, pro-MMP-2 is commonly present in both plasma and sera from healthy humans, although in most samples other major proteolytic activities were seen at M, 92, 130, and 225 kDa. All gelatino-

Fig. 1. Gelatin zymograms of plasma and serum MMPs.
Samples (50 μg of total protein) were analyzed on 7.5% gels containing 1.5 g/L 90 Bloom gelatin. The calibrators (lane Marker) were capillary whole-blood gelatinases; molecular masses (kDa) are indicated. (A), lanes 1 and 2, gelatinases from plasma samples collected into potassium EDTA-coated and lithium heparin-coated plastic tubes, respectively; lanes 3 and 4, MMPs from serum collected in tubes with clot activator and with no additive, respectively. (B), blood MMP zymograms incubated in the presence of 2 g/L potassium EDTA (lanes 1 and 2), 30 μL/L lithium heparin (lanes 3 and 4), or the incubation buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 5 mmol/L CaCl2, 1 mmol/L ZnCl2, 0.2 mL/L Brij-35, 2.5 mL/L Triton X-100, and 0.2 g/L NaN3) without additives (lanes 5 and 6).