Measurement of Creatine in Urine by Creatinase, Sarcoinine Oxidase, and Peroxidase Reevaluated

To the Editor:

In the original creatinine kit method, creatininas (EC 3.5.2.10) is used as the starting reagent to convert creatinine to creatine at preincubating the sample with all other reagents. After conversion of creatinine to creatine, this latter compound is converted to sarcosine and urea by creatinases (EC 3.5.3.3). Sarcoinine is converted to glycine, formaldehyde, and peroxide by sarcosine oxidase (EC 1.5.3.1). The Trinder reaction (1)—the oxidative coupling of 4-amino-phenazone with a phenol (2,4,6-tribromo-3-hydroxybenzoic acid) by peroxidase (EC 1.11.1.7)—is used as the color indicator reaction. By omitting creatininas (supplied as separate tablets), this kit method has been proposed for measuring creatine in urine (2-5) or in serum (6). Here, we evaluate the use of the proposed creatine assay in urine (2-5).

Throughout the evaluation procedure, we used fresh urine samples to avoid bacterial contamination. We did not obtain satisfactory regression data when we compared the proposed creatine assay (y) with another enzymatic method (z) (7): y = 0.577x + 7.79 (r = 0.80; Sx = 26.1; n = 28; range of x = 70-598 μmol/L). Because analytical recoveries of creatine added to 10 different urine samples (measured range of y = 21-155 μmol/L) were 58.8% ± 15.4% (mean ± SD), we suspected interferences in the proposed assay. These 10 different urine samples were obtained from healthy laboratory workers (n = 4) and from patients (n = 6) on no special diet or receiving no medication.

Indicator reactions involving peroxidase are known to be very sensitive to the presence of reducing substances (3, 9). For this reason ascorbate oxidase (EC 1.10.3.3) is included in the reagent system to prevent interference by endogenous ascorbic acid. However, a recent study emphasized that ascorbic acid still interferes in the enzymatic creatinine kit method (8). Other substances (e.g., dopamine) in serum and urine may also interfere in the assay.

We investigated the possible use of a pretreatment procedure for urine samples involving Ba(OH)₂ and ZnSO₄. This procedure has earlier been used to eliminate reducing substances in a serum glucose assay (10). We determined the optimum amounts (range studied: 0-28 mmol/L) of Ba(OH)₂ and ZnSO₄ that allowed maximum recovery. Adding 0.25 mL of 150 mmol/L Ba(OH)₂ and 0.25 mL of 150 mmol/L ZnSO₄ to 1.0 mL of sample, centrifuging the pretreated samples (15 min, 3000 x g), and analyzing the clear supernate by the proposed creatine assay yielded significantly improved recoveries (93.8% ± 17.2%, mean ± SD; P < 0.001). The high variation in recovery (SD 17.2%) is probably caused by differences in the concentrations of the interfering substances.

Because we were interested in the cause of the effect of the pretreatment procedure on the performance of the proposed creatine assay, we studied its effect on ascorbic acid and dopamine in the assay. To demonstrate that the pretreatment procedure eliminated the interference from these compounds, we omitted ascorbate oxidase from the reagent. Ascorbate oxidase is not provided separately in the creatinine kit, so we alternatively used the procedure described by Suzuki and Yoshida (11), whereby we could omit the addition of ascorbate oxidase (reagents were supplied free of charge by Soper Biochem, Nieuwegein, The Netherlands).

We prepared a creatine solution (100 μmol/L) containing ascorbic acid (100 μmol/L) and dopamine (5 μmol/L), respectively. Without sample pretreatment we obtained for these ascorbic acid- and dopamine-containing creatine solutions recoveries of 33% and 80%, respectively. After sample pretreatment recoveries were 92% and 98%, respectively.

We conclude that the pretreatment procedure increases the recovery of added creatine in the proposed creatine assay, apparently by eliminating interfering substances such as ascorbic acid and dopamine. These results suggest that systematically pre-treating urine samples with Ba(OH)₂ and ZnSO₄ is advisable. Moreover, we have demonstrated that interferences present in the earlier described creatine assays in urine (2-5) give rise to unsatisfactory regression data and low recoveries; eliminating these interferences gives far better recoveries.

In the original creatinine kit method the interference by endogenous creatine and sarcosine is prevented by preincubating the samples with all reagents except the starting reagent. In the proposed assay for creatine measurement in urine (2-5), the possible interference by endogenous sarcosine—although hypersarcoisemia and hypersarcosinuria are rare (12)—was not studied. Oversteegen et al. (6) introduced a sample blank to account for possible interferences (which were not specified by the authors) by adding p-chloromercuribenzoic acid (PCMB) to the reagent (3.6 mg/10 mL of reagent) to inactivate the enzymes. After addition of this amount of PCMB to the reagent, we measured (by appropriate enzyme assays: 13-15) an almost complete inhibition of creatinase activity, a 50% decrease in sarcosine oxidase activity, and no decrease in peroxidase activity. These effects on creatinase and sarcosine oxidase activities by PCMB have been described earlier (13, 14). Using that suggested procedure to incorporate a sample blank, we were unable to correct adequately for the presence of endogenous sarcosine, which may be present—although rarely—at high concentrations (16-18).

We conclude that the proposed enzymatic creatine method (2-5) involving creatinase, sarcosine oxidase, and peroxidase does not reliably quantify creatine in urine.

References
the laboratory, samples may not be analyzed until many days after collection. Thus, in all of the xerophthalmia surveys conducted and published to date, whole blood was collected from the participants and centrifuged and the resulting serum samples were stored in liquid nitrogen or refrigerated before being transported to the analytical laboratory. This recommended practice (1) is expensive and presents a real problem, especially in rural areas in the Third World. Here we present a cheap and convenient method of resolving this particular problem.

It is well documented that free vitamin A (retinol) in solution is unstable in the absence of antioxidants or in the presence of light (2). In serum, vitamin A exists and circulates in the form of a large-M₆ protein complex (~80 000 Da) consisting of retinol, retinol-binding protein (RBP), transthyretin (TTR; formerly prealbumin), and thyroxine, in a stoichiometry of 1:1:1:2 (3). Driskell et al. (4) showed that vitamin A in this complex is stable in serum and that only when the vitamin A is released from the complex, by the addition of ethanol during the first part of the assay procedure, does it become subject to the same instability (photo- and oxidative) as the free vitamin in solution. These latter two problems may be overcome by performing this stage of the assay in subdued light and using suitable antioxidants. Because of the reported stability of the circulating complex in serum, we decided to investigate the possibility that the complex might be preserved in the solid state, i.e., in a dry spot formed by absorbing an aliquot of serum onto filter paper. Accordingly the following series of parallel experiments were designed and performed.

We used 2-mL samples of whole blood collected from 66 children involved in a survey being conducted to investigate xerophthalmia and vitamin A deficiency in Ndola (Zambia). The blood samples were then centrifuged at 1600 × g for 5 min to prepare the corresponding serum samples. Immediately after their preparation, we analyzed 200-µL aliquots of each of the specimens of serum for vitamin A in the Ndola laboratory by HPLC (5). A further 200-µL aliquot of each serum sample was then individually applied to small pieces (4 × 4 cm) of ethanol-washed, dry filter paper (Whatman no. 54) for storage, transport, and subsequent HPLC analysis in a UK laboratory. The total time elapsed between the collection of the blood samples in Zambia and their final assay in the UK was 5 months. The absorbed vitamin A complex was extracted from the filter paper and the vitamin A was liberated and transferred to the HPLC mobile phase by the following procedure, which was performed in subdued light. The entire area of the filter paper containing the dried sample was cut into small pieces (~5 × 5 mm) with a clean pair of scissors, and the pieces were then put into a labeled screw-capped Pyrex test tube (10 × 1.5 cm) to which we then added 3 mL of phosphate buffer (pH 7.4). The contents of the tube were intermittently mixed by shaking every 5 min for 15 min, and then 3 mL of an antioxidant ethanol ascorbic acid (10 g/L) was added, followed by 100 µL of a retinyl acetate internal standard ethanol solution (2 mg/L) required as the internal standard for the HPLC assay. The addition of these ethanolic solutions dissolves the vitamin A from the complex (4) so that, at this stage, free retinol is now dissolved in the buffer/ethanol mixture. After thoroughly shaking the mixture for 1 min, we then transferred the free vitamin A in the aqueous ethanol buffer to n-hexane by extracting it twice with 2 mL of n-hexane containing butylated hydroxytoluene (BHT), 0.1 g/L, as an antioxidant. The test tube and the contents were centrifuged at 1600 × g for 5 min, after which the top (n-hexane) layer was then removed with a glass Pasteur pipette. The transferred n-hexane solution was placed into a screw-capped glass test tube (6 × 1.5 cm) and then evaporated under a stream of dry nitrogen. For each residue, containing the vitamin A/retinyl acetate internal standard, was redissolved in 200 µL of the HPLC mobile phase and assayed (5).

The results of these experiments are presented in Figure 1, a scatter plot of the vitamin A concentration results obtained from analysis of the 66 dried blood spots on the filter papers collected in Zambia and analyzed in the UK 5 months later vs the corresponding results obtained from the analysis of the fresh sera immediately after collection in Zambia. The line shown in Figure 1 is that calculated for the best straight line (least squares) between the two sets of analytical results. The good correlation between the two sets of data was confirmed by applying the paired Student’s t-test, which gave a probability factor of 0.75. Further, the computed equation of the scatter plot least-squares straight line (r = 0.98) is [Vit A]paper = [Vit A]serum − 5.9, which shows that the assayed vitamin A concentrations in the stored

Cornelis Beyer
Ina H. Alting
Egbert T. Backer
Dept. of Clin. Chem.
Het Diaconessenhuis
Houtlaan 55
2300 RD Leiden
The Netherlands

Stability of Vitamin A Circulating Complex in Spots of Dried Serum Samples Absorbed onto Filter Paper

To the Editor:

The assay of vitamin A is important in evaluating populations at risk of xerophthalmia and cancer or for assessing the nutritional status of population groups. Unfortunately, the present methods of analysis for vitamin A in blood involve HPLC equipment; consequently, because most population surveys are done well away from...