that in most instances a complete blood count (CBC) was also included; in fact, 84% (104/124) of our subjects did have a simultaneous CBC and the rest had had sufficiently recent CBC evaluations to be considered normal.

Because our laboratory and Heinrich's are utilizing two different sources of antibody, there is potential for differences in reference ranges. This is clearly demonstrated by the fact that in the course of our evaluation, we ourselves observed higher values for the same specimens as determined by use of an assay from yet another manufacturer. We can say, however, that ferritin concentrations as measured by the Ventrex (Ventrex Laboratories, Inc., 217 Reed St., Portland ME 04103) method agree well with those determined by a reference laboratory that measures ferritin by an in-house assay with use of Ramco antibody (Ramco Laboratories, Inc., Houston, TX 77098). Forty-one paired results were evaluated, for samples with ferritin concentrations ranging between 1 and ~1600 μg/L. The mean result from the reference laboratory was 156.68 μg/L, that with the Ventrex method was 156.51 μg/L (the correlation coefficient, \( r^2 = 0.996 \)). The 55Fe absorption test is not used in our institution, nor have we studied the correlation between ferritin and bone-marrow iron stores. However, as Heinrich states, several such studies have been published (1–3), and even though the results do not always agree, the authors of these publications indicate that the clinical value of the assay, as compared with that of other indicators of iron status, is good. We did not state that serum ferritin measurement is used in our institution as the sole diagnostict determinant of iron-deficiency anemia, nor should it ever be so considered. We most certainly agree that other factors in iron metabolism must also be evaluated. However, if ferritin is included as one variable, it is imperative to evaluate the result in light of the age and sex of the patient. If we accept Heinrich's statement that 38% of his subjects had latent iron deficiency (we question the use of the term "prelatent") and were found to have serum ferritin concentrations of 5–35 μg/L, it would imply that most (27/37) premenopausal women, 10 of 29 menopausal women, and five of 58 men would suffer from latent iron deficiency anaemia, assuming equivalence in the results from the two different methods (4). We find it difficult to believe such statistics, but they do further show the necessity for evaluating ferritin concentration not only in relation to sex, but also to age.

We also agree that interpretation of ferritin alone is problematic in patients with probable iron deficiency in the presence of inflammatory disorders. However, it is still considered to be a valuable diagnostic aid in combination with other indicators of iron status and certainly rules out the necessity performing an invasive procedure such as a bone-marrow aspiration for iron-storage determination on most patients. This we consider to be of benefit. Finally, since our communication appeared in this journal (4), our clinical experience continues to confirm our original findings.

References


Use of Potassium Dichromate as a Surrogate Standard for \( \beta \)-Carotene Determination

To the Editor:

The absorbance of an alcohol/petroleum ether extract of serum is commonly measured in determining "carotenoids" in serum (1, 2). Use of potassium dichromate is recommended as a surrogate \( \beta \)-carotene standard, because \( \beta \)-carotene is unstable in petroleum ether. The absorbance of extracted carotenoids and dichromate can be compared in a spectrophotometer at 420 nm (1) or in a Klett–Summerson colorimeter with a "#44" light filter (3). Pett and Le Page (2) determined "colorimetrically" that a 200 mg/L solution of potassium dichromate was equivalent to 1.12 mg/L \( \beta \)-carotene. We have checked this equivalence, using a Model 124 spectrophotometer with 1-nm bandpass (Colesman Instruments Division, Perkin-Elmer Corp., Oak Brook, IL 60521).

A 2.10 mg/L solution of \( \beta \)-carotene (Aldrich Chemical Co., Milwaukee, WI 53201) was prepared in petroleum ether, 30–60 °C (Mallinkrodt, Inc., St. Louis, MO 63147). A 200 mg/L solution of potassium dichromate (Mallinkrodt) was prepared in water. Figure 1 shows the absorbance spectra of these solutions as determined against blanks of petroleum ether and water, respectively, and obtained with the Coleman 124 spectrophotometer in fused-silica cuvets with 10-mm lightpath. \( \beta \)-Carotene demonstrated a well-defined triple peak in the region 400–500 nm; the dichromate solution gave a poorly defined peak or plateau in the same region.

The absorbance of the 2.10 mg/L \( \beta \)-carotene solution is 0.370 A at 420 nm; that of dichromate standard at the same wavelength is 0.320 A. Thus, at 420 nm the 200 mg/L dichromate standard is equivalent to 1.76 mg of \( \beta \)-carotene per liter with the Coleman 124.

The absorbance of the 2.10 mg/L \( \beta \)-carotene solution is 0.528 A at 445 nm; that of the dichromate standard at the same wavelength is 0.282 A. Thus, at 445 nm the 200 mg/L dichromate standard is equivalent to only 1.12 mg of \( \beta \)-carotene per liter.

The absorbance of the 2.10 mg/L \( \beta \)-carotene standard is 0.522 A at 450 nm. The calculated absorptivity of a 10 g/L solution of \( \beta \)-carotene in the Coleman 124 at 450 nm is 2486, in good agreement with others (4).

The dichromate standard can be used in two ways on the Coleman 124, either (a) measure absorbance at 420 nm, and assign an equivalence of 1.76 mg/L \( \beta \)-carotene, or (b) measure absorbance at 445 nm, and keep the equivalence of 1.12 mg/L assigned by Pett and Le Page. The latter is the better choice. At 420 nm, the slopes of the spectra of \( \beta \)-carotene and dichromate are opposite in sign. At 445 nm the spectra of \( \beta \)-carotene has symmetrical peak, while that of dichromate is approaching a plateau.

The difference in spectra between \( \beta \)-carotene and dichromate may be a
compensating error or negligible when a wide-bandpass spectrophotometer or a photoelectric or white-light colorimeter is used. The equivalence of the surrogates the standard should be determined on the instrument used.

The "stufenphotometer" was news in the days of Pett and Le Page; their unit of measurement was millimeter depth of solution, not absorbance. The limitations imposed by the equipment available should be kept in mind when interpreting data from the older literature.

References


Rudolph G. Mueller
Gordon E. Lang
St. Mary's Hospital
P.O. Box 503
Milwaukee, WI 53201

Metabolite Interference in Homogeneous Enzyme Immunoassay of Phenytoin

To the Editor:

Phenytoin concentrations obtained by homogeneous enzyme immunoassay (EMIT) of sera from uremic patients may be higher than concentrations obtained chromatographically (1-4). The major metabolic pathway for phenytoin (diphenylhydantoin) in humans is oxidation to 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), then conjugation to HPPH-glucuronide (5), which can attain concentrations of 10 to 100 mg/L in sera of uremic patients, although the HPPH concentrations usually are <1 mg/L (3, 4, 6).

If antibodies against phenytoin recognize the glucuronide metabolite, this could account for the reported discrepancy. This hypothesis seemed unlikely, from previous experience with antibody specificity (7), but HPPH-glucuronide was at that time unavailable for direct testing. We have now tested the hypothesis.

Pallante and Fenselau have synthesized authentic HPPH-glucuronide and characterized it by mass spectrometry, using their immobilized UDP-glucuronosyl transferase (EC 2.4.1.17) method (8). To our knowledge, this material had not been previously isolated.

The synthetic HPPH-glucuronide migrated as a single spot with Rf 0.37 on a Merck silica gel 60 F-250 thin-layer chromatographic plate developed in butanol/methanol/benzene/water (20/12.5/10, by vol). This spot was visible under ultraviolet light (254 nm), and it developed a blue color characteristic of glucuronic acid and its conjugates when sprayed with naphthoresorcinol reagent (9). Under the same conditions, HPPH displayed an Rf of 0.91 and glucuronic acid an Rf of 0.21.

Stock solutions of HPPH-glucuronide were quantitated by two methods: (a) a modified naphthoresorcinol test, with which an adduct between glucuronic acid and 1,3-dihydroxynaphthalene can be measured colorimetrically (10); and (b) ultraviolet absorbometry of the HPPH liberated by acid hydrolysis of the glucuronide (11). Samples with HPPH-glucuronide concentrations ranging from 5 to 50 mg/L were prepared in EMIT anti-epileptic drug calibrators (lot J02), which contained 0 to 20 mg of phenytoin per liter. The calibrator solutions are made from pooled, drug-free, freon-treated human serum that has been lyophilized and reconstituted with distilled water. The samples were analyzed according to the recommended (12) protocol with both lot J01 and lot K01 EMIT reagents. The results are presented in Figure 1.

The HPPH response is comparable for both lots, but the HPPH-glucuronide response is significantly lower for lot K01. The increase in apparent phenytoin caused by HPPH-glucuronide depends not only on the HPPH-glucuronide concentration but also on the phenytoin concentration. At low phenytoin concentrations the relative HPPH-glucuronide interference is greater than at high phenytoin concentrations.

To assess how much metabolites may affect the values for apparent phenytoin we prepared three samples (A,B,C) in pooled, drug-free, normal human serum to simulate ratios of phenytoin, HPPH, and HPPH-glucuronide that might be present in sera of uremic patients. The results of testing these samples with EMIT reagents lots J01 and K01 were as follows:

<table>
<thead>
<tr>
<th>Conc, mg/L</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>10</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>HPPH</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>HPPH-glucuronide</td>
<td>20</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

Assayed phenytoin concn, mg/L

Lot J01  12.0  5.0  28.0
Lot K01  11.0  5.1  20.0

Evidently, high HPPH-glucuronide concentrations increased the apparent phenytoin concentration with reagent lot J01 to a greater degree than with reagent lot K01. We have, as yet, been unable to obtain appropriate clinical samples to confirm this difference for uremic patients being treated with phenytoin.

Three factors influence the apparent increase in phenytoin observed in uremic patient sera: (a) interpatient variability in phenytoin disposition, (b) lot-to-lot variation of the EMIT reagent response to HPPH and HPPH-glucuronide, and (c) the concentration of phenytoin and its metabolites in a particular sample. Therefore Syva currently recommends that phenytoin determinations performed by the EMIT method on uremic patient sera be interpreted...