Determination of Retinol, α-Tocopherol, and β-Carotene in Serum by Liquid Chromatography with Absorbance and Electrochemical Detection

William A. MacCrehan¹ and Emil Schönberger²

We describe a method for the determination of retinol, α-tocopherol, and β-carotene in serum, using a liquid-chromatographic separation with wavelength-programmed ultraviolet/visible absorbance and amperometric electrochemical detection with a glassy carbon electrode. After protein denaturation and addition of an internal standard, tocol, 250-μL samples are twice extracted with hexane. The reversed-phase, gradient-elution chromatographic separation provides baseline resolution of: the all-trans isomer of retinol from the cis isomers, α- from γ-tocopherol, and all-trans-β-carotene from α-carotene and from cis-β-carotene isomers. The linearity of response and the detection limits for the two detectors for the three analytes are measured. A comparison of the values obtained for serum extracts shows good agreement between the absorbance and electrochemical detectors.

Additional Keyphrases: micronutrients · chromatography, reversed-phase · cis-β-carotene

In a quality-assurance role, the National Bureau of Standards (NBS) is supporting clinical research funded by the National Cancer Institute into the cancer-chemopreventive properties of vitamin A (all-trans-retinol), vitamin E (α-tocopherol), and pro-vitamin A (all-trans-β-carotene).¹ The quality-assurance program is for laboratories involved in epidemiological studies, assaying these micronutrients in serum to evaluate the nutritional status of treated and control populations. For NBS analyses, we have developed a method to assay the three analytes by using liquid chromatography with ultraviolet/visible absorbance detection (LCUV) and oxidative electrochemical detection (LCEC), a new approach for the determination of all three micronutrients in serum.

In the past, all three analytes were determined by extraction followed by colorimetry (1–4). That approach is subject to nonspecific interference from other matrix constituents, such as carotenoids and xanthophylls as well as unresolved isomers of the analytes. With the advent of liquid-chromatographic separations, unrecognized interferences present in the earlier techniques became apparent, and those procedures are no longer considered reliable.

Many liquid-chromatographic methods have been proposed for the single (5–11) as well as the simultaneous multiple determination (12–21) of the three micronutrients in serum. Fixed-wavelength ultraviolet/visible absorbance detection can be used to measure the analytes at their expected serum concentrations, but this approach relies heavily on the degree of chromatographic resolution to provide bias-free results. Fluorescence detection (20,21) has been used to enhance both selectivity and sensitivity of assays for retinol and tocopherol. Oxidative electrochemical detection has also been used for the determination of tocopherol (9, 19, 22), but its use for detecting retinol and carotene has only been suggested (22). The electro-oxidation of retinol (23,24) and β-carotene (25) has been studied only in nonaqueous solvents. Recently, we have studied the oxidation of retinol at a glassy carbon electrode in methanol/water solvent such as those used in LC separations (26). The oxidation is a multiple-electron process that forms several products. The approach has been found suitable for the thin-layer amperometric detection of chromatographically separated all-trans as well as the cis isomers of retinol (26).

The objectives of this work were (a) to evaluate the extraction procedure used to recover the analytes from sera, (b) to develop a reversed-phase separation that would provide baseline resolution of the analytes of interest from other serum constituents and from their isomers, and (c) to investigate the utility of oxidative amperometric detection for the simultaneous determination of retinol, α-tocopherol, and β-carotene.

Materials and Methods*

Reagents/Standards

Standard solutions of the three analytes and the internal standard were prepared by dissolving crystals (all-trans-retinol, all-trans-β-carotene) or oils (α-tocopherol, tocol), in absolute ethanol; the concentrations were determined spectrophotometrically, corrected for impurities that absorb at

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³ Nonstandard abbreviations: NBS, National Bureau of Standards; LCUV, liquid chromatography with ultraviolet/visible absorbance detection; LCEC, liquid chromatography with electrochemical detection; BHT, 2,6-di-t-butyl-4-methylphenol.
⁴ Certain commercial equipment, instruments, or materials are identified in this paper in order to specify adequately the experimental procedures. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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the wavelength of measurement (see below). The tocopherol internal standard was added in equal concentrations to standard mixtures and the serum samples before extraction. All-trans-retinol (type IV), retinyl acetate (type I), retinyl palmitate (type IV), all-trans-β-carotene (type IV), and all-trans-a-carotene (type V) were obtained from Sigma Chemical Co., St. Louis, MO. dl-Tocool was a gift from Hoffmann-La Roche, Basel, Switzerland. d-α-Tocopherol ("99% minimum" grade), d-γ-tocopherol, and d-α-tocopherol acetate came from Eastman Kodak, Rochester, NY. 2,6-Di-t-butyl-4-methylenophenol (BHT) was obtained from Calbiochem, La Jolla, CA. Lutein, zeaxanthin, cryptoxanthin, and nonaprene were a gift (G. R. Beecher, USDA, Beltsville, MD). All were stored at −4 °C upon receipt and thawed just before preparation of ethanol solutions.

The concentrations of the three analyte standards were determined with a double-beam grating spectrophotometer vs an absolute ethanol reference. The absorbivity values (L/cm) in ethanol that we chose for the calibration are: all-trans-retinol ε_{524 nm} = 1850 (27), α-tocopherol ε_{292 nm} = 75.5 (13), and all-trans-β-carotene ε_{450 nm} = 2620 (28). The solutions were diluted such that the absorbance of the solution was always between 0.1 and 0.9 A. Once prepared, the purity of the individual standard solutions was evaluated by the "slow-gradient" LC/UV separation described below. If the total impurity at λ_{max} was between 1% and 5%, as determined by the total area under all chromatographic peaks (other than that for the desired analyte), then the absorbance of the solution was corrected for the impurities; otherwise, the solution was discarded and fresh material was taken for measurement. From the individual solutions, we prepared dilute standards in ethanol, adding BHT (20 mg/L) as a preservative (5). The approximate final concentrations in our working standards for the analyte mixture (in mg/L) were: all-trans-retinol 0.5, tocol 7, α-tocopherol 7, and all-trans-β-carotene 0.2. Aliquots of this mixture were stored in glass vials at −70 °C and were stable for at least one month. Before use, we warmed the solutions to about 40 °C, agitated them ultrasonically, then cooled them to room temperature.

LC Apparatus

For the simultaneous determination of the three analytes, we used a two-pump, computer-controlled solvent-delivery system. Each pump had two reciprocating pistons with pressure-feedback compensation. The automated sample injector was used with Teflon-septa-sealed vials. To separate the analytes and their isomers, we used a 0.46 × 25 cm Vydac (The Separations Group, Hesperia, CA) octadecylsilyl-modified silica (C18) column (201TP) packed with 5-μm particles. This column has a polymeric bonded phase with large (30-nm) pores, and can separate all of the retinol isomers in 30 min (26). For absorbance detection we used a wavelength-programmable detector (Model 783; Kratos Analytical, Ramsey, NJ) with a noise specification of 1 × 10⁻⁵ A (1-s time constant, 236 nm), and a deuterium lamp. The electrochemical detector was a thin-layer amperometric detector cell (Model TL-6A; Bioanalytical Systems, West Lafayette, IN) modified by replacing the original working electrode block with a custom Teflon holder and a 1.2 mm-diameter glassy carbon (Fluorocarbon Co., Anaheim, CA) rod. The filling solution for the silver/silver chloride reference electrode contained methanol/water (70/30 by vol before mixing) and 0.1 mol of KCl per liter. All potentials are specified with respect to this reference electrode. We used this reference solution to help address the problems in the commercial electrode of the widely disparate internal (3 mol/L aqueous KCl solution) and external (methanol/water mobile phase) electrolytes. A 2-s time constant was used for both detectors.

Separation Conditions

Distilled-in-glass solvents (B & J Brand; American Scientific Products, McGaw Park, IL) were used in preparing the mobile phase. The electrolyte we used was a combination of Suprapur-grade ammonium hydroxide and acetic acid (Merck, Darmstadt, F.R.G.), which provided a buffer of pH 3.5. This electrolyte was included in all mobile-phase mixtures at a final concentration of 0.02 mol/L. The electrolyte not only was necessary to conduct electrical current for the amperometric detector, but also enhanced the reversed-phase separation of the analytes from their isomers. For simultaneous determination of the three analytes, we used the following "slow-gradient" solvent program: initial solvent, water/methanol/n-butanol (15/75/10, by vol); final solvent, water/methanol/n-butanol (2/88/10 by vol). The initial solvent composition was used for 3 min, then was changed linearly to the final solvent composition over a 15-min period, and was held at the final composition for 17 min before being returned to the initial solvent composition. The flow rate was constant at 1.5 mL/min. The "fast-isocratic" mobile phase was water/methanol/n-butanol (2/87.4/10 by vol), with an initial flow rate of 1.5 mL/min; the flow rate was increased to 3.0 mL/min after 10 min, held constant for 5 min, then returned to 1.5 mL/min.

Sample Preparation

We used three steps to extract the micronutrients from serum for LC analysis: protein denaturation, organic solvent extraction, and solvent exchange. Frozen sera, stored in glass ampules at −70 °C, were thawed thoroughly to room temperature, and vortex-mixed for 5 s before sample aliquots were removed. We placed 250 μL of serum in a 13 mL centrifuge tube, added 25 μL of an ethanolic solution of the internal standard, tocol, and vortex-mixed briefly. We then denatured the proteins with 250 μL of ethanol containing BHT, 15 mg/L. After vortex-mixing the sample for 1 min with 1.5 mL of hexane extractant, we centrifuged (~2000 × g) it for about 2 min, then removed 1.0 mL of the upper layer. We added a fresh 0.5 mL of hexane to the remaining lower layer, vortex-mixed for 1 min, then centrifuged 2 min. We collected 300 μL of the second extract and combined it with the first extract. To remove the hexane, we placed the sample in a container with a two-holed polyethylene lid and blew dry nitrogen gas over it, to avoid oxidation by atmospheric oxygen. Finally we dissolved the residue in 250 μL of ethanol containing BHT, agitating the sample ultrasonically for 1 min. Occasionally, the redissolved extract was cloudy, and an additional centrifugation was necessary before LC analysis. We placed the redissolved extract in a glass sample vial with an air-tight Teflon-lined septum, and injected 25 μL into the chromatograph.

Results and Discussion

Extraction of the Analytes from Serum

Although retinol, tocopherol, and carotene are highly insoluble in water, they are soluble in serum because they are transported by lipoproteins. The proteins involved may be 80% to 90% lipid enclosed in a hydrophilic protein sheath.
Specific retinol-binding-proteins are known to selectively transport retinol in vivo. The presence of lipoprotein encapsulation imposes several constraints on the available approaches to the determination of these micronutrients in serum. First, it makes impossible meaningful recovery experiments for these analytes in the serum matrix based on a simple addition of the free analyte dissolved in solution. As noted by Bieri et al. (7), the procedure of addition of analyte solution to the serum prior to extraction fails to adequately simulate the naturally encapsulated analytes because of the slow kinetics of uptake by the lipoproteins. The slow kinetics of the analyte transfer to and from the lipoprotein encapsulation is demonstrated by the failure of hexane to directly extract the analytes without the addition of protein denaturant. In such an experiment we found that tocopherol is only partially extracted, with little recovery of retinol and carotene. Although this standard addition approach has been used to determine extraction efficiencies in many studies (12, 14–18, 20–22), it may provide unrealistic "recovery" results for these analytes.

To overcome the serum lipoprotein-binding of the analytes for solvent extraction, one must first denature the proteins, then use a water-immiscible organic solvent to extract the unencapsulated analytes. The final aspect of the lipoprotein-binding of the serum micronutrients that must be resolved before complete recovery is possible is the requirement of effective competition of the extraction solvent with the serum lipids for solvation of the analytes. Hexane has been most frequently used for this purpose, with a large ratio of the volume of organic extractant to serum providing suitable competition to overcome lipid-binding. However, even if the distribution ratio of the analytes into the hexane from the precipitated serum were infinite, the recovery would still be <100%, owing to the volume of hexane (about 20% in our method) that does not separate into the upper layer, presumably because it is bound in lipid micelles in the aqueous lower layer. The unrecovered volume of extractant graphically demonstrates the importance of the serum lipids in the procedure and points to the advisability of the addition of a suitable internal standard to correct for the volume of extractant not recovered.

We chose to use tocol (29) as internal standard to correct for the variations in extractant volume. Tocol is structurally very similar to α-tocopherol but lacks the three methyl groups on the phenolic ring. It is eluted in an uncrowded portion of the chromatogram and responded to both absorbance and electrochemical detectors. We also examined the use of three alternative internal standards: two esters of the vitamins—retinyl acetate (12, 18, 20) and α-tocopherol acetate (7, 12)—and a β-carotene analog, nonaprene (30, 31). Retinyl acetate was unstable and eluted with two shoulders on the LCUV chromatogram. α-Tocopherol acetate was not suitable as internal standard for LCEC, because the esterification of the electro-active phenolic hydroxy group of the tocopherol ring increased the applied potential required to +1100 mV, where detector selectivity and sensitivity are poor. Nonaprene, a promising internal standard for carotenoid analysis (30, 31), is an analog of β-carotene having an additional isoprene unit; however, in our method, nonaprene co-eluted with serum components that were retained longer than β-carotene, making it unsuitable as an internal standard.

We evaluated the effect of different protein denaturation solvents on analyte recovery. Acetone, acetonitrile, methanol, and perchloric acid (100 mL/L), in equivolume ratio to the serum sample, yielded less recovery of all three analytes than ethanol did.

Because of the loss of extractant volume, we examined the utility of a second extraction of the serum denatured with fresh solvent. We used a pooled serum sample and measured the analytical recovery of the added internal standard, tocol. After a single extraction, recovery was 81.0 (SD 15.2)% (n = 9). Combining a second extraction with the first recovered 96.8 (SD 3.0)% of the added tocol (n = 72). The recovery of the three analytes was also improved by including the second extraction. Although this additional extraction adds to the complexity of the procedure, it is necessary for assay precision.

We evaluated alkaline hydrolysis of the serum prior to the extraction, as outlined by Thompson et al. (8), for the determination of micronutrients in serum, comparing the results with those obtained in our method. Results were identical, within statistical error, for β-carotene. For all-trans-retinol, however, the alkaline extraction method provided higher results than our method did, because of the additional free retinol recovered by hydrolysis of serum retinol esters. No α-tocopherol was recovered from the alkaline hydrolysate because of conversion to the hydrophilic phenolato anion.

We also tested a standardization approach based on the addition of increments of an ethanol solution of the three analytes to bovine serum albumin, followed by extraction (15, 16). The rationale for this approach is that this albumin is commercially available, contains none of the three micronutrients, and provides a matrix similar to human serum. Although matrix-matching for standardization often has merit, we found serious bias with this particular method: only about 70% of the added all-trans-retinol and α-tocopherol and about 40% of β-carotene were recovered from the bovine serum albumin matrix. Because of the low recovery of the standards in this matrix, the final determined results for the three micronutrients in test human serum were quite high. Thus, this method is not recommended (32).

Separation of the Analytes

Some methods for the determination of all three micronutrients involve an isocratic separation (15, 16, 18). However, the advantage of rapid repetitive analyses is offset by the significant compromises that must be made in the selectivity of the chromatographic separation for all-trans-retinol and α-tocopherol. Because the reversed-phase retention of these two analytes is much less than for β-carotene, baseline resolution of the micronutrients from their isomers and other serum components in a short isocratic chromatographic procedure is not possible with current column technology.

Two types of analytical compromises are illustrated when a "rapid isocratic" separation is used (Figure 1): (a) peak area integration of the three analytes is made less precise by the presence of partly co-eluting "shoulders," and (b) the accuracy is lowered by exactly co-eluting components, which are revealed only in long-gradient separations. Despite the long elution and recycle time required for gradient separations, we could not tolerate the attendant losses in the precision and accuracy resulting from the use of this fast-isocratic separation.

Thus, we used slow-gradient separation (Figure 2), which provides significantly better resolution of the three micronutrients from their isomers than shown in previously published reversed-phase analyses (6, 7). We chose this wide-pore, polymeric, octadecyl stationary phase in conjunction
Fig. 1. Fast-isocratic separation of the micronutrients in a serum extract
Peaks: 1, retinol; 2, tocot; 3, 7-tocopherol; 4, a-tocopherol; 5, a-carotene; 6, all-trans-b-carotene; 7, cis-b-carotene

Fig. 2. Slow-gradient-elution separation of micronutrients in a serum extract
Peaks: 1, all-trans-retinol; 2, tocot; 3, 7-tocopherol; 4, a-tocopherol; 5, lutein; 6, zeaxanthin; 7, cryptoxanthin; 8, a-carotene; 9, all-trans-b-carotene; 10, cis-b-carotene
with a methanol/water/n-butanol mobile phase, based on our previous experience with separation of all-trans and the mono-cis isomers of retinol (26). We found, as have others (6, 7, 33), that bonded-phase separations are enhanced by the addition of a third solvent possessing polar and nonpolar ends (such as n-butanol). The addition of a supporting electrolyte was also beneficial to the separation of these micronutrients. With this gradient separation, all-trans-retinol is separated at the baseline from its 13-cis isomer, \( \alpha \)-tocopherol is separated from \( \gamma \)-tocopherol, and all-trans-\( \beta \)-carotene is separated, also at the baseline, from the cis isomers and \( \alpha \)-carotene. Recently, the 15,15’-cis and 9-cis isomers of \( \beta \)-carotene have been separated from the all-trans and \( \alpha \)-isomer in determinations in foods (34).

Detection of the Analytes in Serum

We evaluated the use of LCEC for the determination of the three micronutrients because of the sensitivity and selectivity offered by the technique. LCEC has previously been used for tocopherol determination (9, 19). However, a recent tocopherol method (9) noted an unexpected pronounced pH effect for the hydrodynamic current-voltage curve of the tocopherol isomers, the signal being zero for pH values < 4. We examined the pH dependence of the \( \alpha \)-tocopherol electrochemical signal by rotating disk voltammetry over the pH range of 1.6 to 10 with a glassy carbon disk. A single oxidation wave \( (E'_{1/2} = +0.55 \text{ V}) \) was found for all pH values < 8. Above pH 8, the wave splits, a second wave appearing at lower potential \( (E'_{1/2} = +0.35 \text{ V}) \); the sum of the wave heights remained constant. At low pH, the oxidation of the protonated form of the phenolic moiety predominated, whereas the second (easier) oxidation wave appeared at the higher pH because of the oxidation of the phenolate anion \( (pK_a \approx 10) \). The pH dependence for the electro-oxidation of phenols has been described (35): at pH values below \( pK_a \), the OH moiety is oxidized to a radical cation in the first oxidation step; at pH values near or above \( pK_a \), the oxidation potential decreases because the deprotonated phenolate anion may be more easily oxidized to a free radical. No change in the protonation level of tocopherol is expected in the pH range of 2 to 7, and thus no change in the electro-oxidation signal would be expected. We have concluded that the electro-oxidation of tocopherol shows no pH dependence from pH 2 to 7, and thus we use an ammonium acetate buffer (pH 3.5).

Only one preliminary investigation of the use of LCEC for retinol and \( \beta \)-carotene has been reported (22). Bulk voltammetric studies of the electro-oxidation of \( \beta \)-carotene (23, 25) and retinol (23, 24) in nonaqueous solvents have been made. Recently, we investigated the electro-oxidation reaction and the use of LCEC for all-trans-retinol and its cis isomers (26). The electro-oxidation of retinol is a multi-step process, involving the initial formation of radical cations from the double bonds, followed by reaction with the protic solvents, with no involvement of the alcohol moiety. We found little or no selectivity for the electro-oxidation of any one specific double bond, in the five-bond conjugated system. A charge of 4.7 electrons per retinol molecule occurred rapidly in coulometric experiments. A similar sequence of electro-oxidation reactions might be expected for \( \beta \)-carotene, except the number of electrons transferred may be twice that for retinol (24).

As a basis for LCEC determination of the micronutrients, we constructed a hydrodynamic voltammogram (Figure 3). The current response, measured as peak area, was normalized by dividing the response at a given potential by the response at +1000 mV. The optimum potential for the simultaneous determination of all three analytes is +900 mV, and is limited by the requirement to detect retinol. For detecting \( \alpha \)-tocopherol and \( \beta \)-carotene, an applied potential of only +0.75 V would be necessary and would provide additional detector selectivity (see below).

For absorbance detection, we used an instrument that allowed rapid (<100 ms) wavelength programming to the absorbance maxima for each analyte. The wavelength changes and subsequent auto-zero adjustments were timed to occur at least 3 min before the elution of each micronutrient (Figure 2), thereby providing the data system an adequate baseline for measurement.

Calibration curves for the three analytes were linear through the physiological concentration range for both the absorbance and electrochemical detectors (Table 1). However, when we used mobile phases containing a large initial proportion of water, the \( \beta \)-carotene calibration curve displayed some slight curvature in both detectors. Table 2 shows the effect of the initial proportion of water on the \( \beta \)-carotene signal. The signal was also dependent on the material used in the column inlet frit. We speculate that the mechanism of loss of the \( \beta \)-carotene signal may involve precipitation of the injected sample onto the oxide surface of the steel frit, with slow redissolution of \( \beta \)-carotene into mobile phases that contain more water. This speculation is supported by the "tailing" of the \( \beta \)-carotene peak in solvents in which signal loss occurred.

We compared the detection limits of the absorbance and electrochemical detectors for the three analytes (Table 3), i.e., a signal three times the peak-to-peak baseline noise levels. The detection limits for the electrochemical detector were somewhat superior to absorbance detection for all of the analytes, especially for \( \alpha \)-tocopherol.

The relative selectivity of the two detectors for the determination of the three analytes in serum may be judged by

![Fig. 3. Hydrodynamic voltammograms for all-trans-retinol (□), tocol (+), \( \alpha \)-tocopherol (ocrine), and all-trans-\( \beta \)-carotene (Δ) at a glassy carbon electrode](image-url)
Table 1. Linear Regression Calibration Curves for the Micronutrients by Each Detector

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Conc range, mg/L</th>
<th>LCUV SE</th>
<th>Intercept</th>
<th>LCEC SE</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans-retinol</td>
<td>0–1.3</td>
<td>0.00841</td>
<td>-0.018</td>
<td>0.0196</td>
<td>-0.011</td>
</tr>
<tr>
<td>a-Tocopherol</td>
<td>0–16</td>
<td>0.0150</td>
<td>0.0032</td>
<td>0.0109</td>
<td>0.0024</td>
</tr>
<tr>
<td>All-trans-β-carotene</td>
<td>0–1.0</td>
<td>0.0264</td>
<td>-0.10</td>
<td>0.0356</td>
<td>-0.084</td>
</tr>
</tbody>
</table>

n = 14 each.

Table 2. Variation of Absorbance Signal for β-Carotene with Mobile Phase Composition and Frit Type

<table>
<thead>
<tr>
<th>Initial proportion of water in mobile phase, mL/L</th>
<th>Stainless steel frit</th>
<th>Teflon frit</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50 100</td>
<td>76 000</td>
</tr>
<tr>
<td>65</td>
<td>45 500</td>
<td>73 400</td>
</tr>
<tr>
<td>110</td>
<td>39 800</td>
<td>70 100</td>
</tr>
<tr>
<td>155</td>
<td>34 600</td>
<td>69 000</td>
</tr>
</tbody>
</table>

Table 3. Detection Limits for the Analytes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Absorbance</th>
<th>Electrochemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans-retinol</td>
<td>6.0 (150)*</td>
<td>4.1 (103)</td>
</tr>
<tr>
<td>a-Tocopherol</td>
<td>96 (2400)</td>
<td>0.65 (16)</td>
</tr>
<tr>
<td>All-trans-β-carotene</td>
<td>29 (730)</td>
<td>2.1 (53)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate pg of analyte in 25-µL injection volume.

Fig. 4. Comparison of absorbance and electrochemical detection of carotenoids

Lowest trace: absorbance detection at 450 nm; middle trace: amperometric detection at +900 mV; upper trace: absorbance detection at 325 nm. Peaks: 1, α-carotene; 2, all-trans-β-carotene; 3, cis-β-carotene; 4, retinol palmitate.

the ability to provide baseline-resolved chromatographic signals (cf. Figures 1 and 2). The selectivity of the absorbance detector was slightly better than the electrochemical detector for all-trans-retinol; however, with the gradient-elution solvent program, baseline resolution was possible with both detectors. The electrochemical detector provides superior selectivity for tocol and α-tocopherol, as evidenced by small chromatographic shoulders found on the tocol and α-tocopherol peaks in some absorbance chromatograms but not observed with the electrochemical detector. We were able to alleviate most of this occasional absorbance detection interference by using cursor-controlled manual integration. The selectivity of the absorbance detector was superior to that of the electrochemical detector for β-carotene (Figure 4): interfering serum components, including retinol palmitate and possibly other retinol esters, were detected electrochemically (middle trace) but not by absorbance measurements (lowest trace). The interfering retinol esters could be detected by their absorbance at the retinol optimum wavelength, 325 nm (upper trace). Although the retinol esters interfere in the LCEC at the applied potential of +900 mV, no interference with the β-carotene signal is observed if a lower detector potential of +750 mV is used. Perhaps it would be feasible to program a change in potential from +900 to +750 mV after the elution of retinol and tocol; this would allow the detection of α-tocopherol and β-carotene with maximum signal and better selectivity. Again, we currently rely on cursor-controlled manual integration to

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minimize this interference with the β-carotene signal in the electrochemical detector.

Determination of the Analytes

We evaluated the method by assaying (all in duplicate) a series of serum samples and one serum pool obtained from 27 Japanese Hiroshima-bomb survivors who have subsequently developed cancer. The procedure was calibrated with a single external standard, at the beginning and at the end of the day, to correct for small amounts of drift in the electrochemical detector response. Correlation plots for the micronutrient values determined with the absorbance and electrochemical detectors are presented in Figure 5. Linear least-squares analysis (n = 72) indicates an excellent correlation between the determined values by the two detectors for all-trans-retinol, with a slope of 1.01 and standard error of 0.016, and for α-tocopherol, with a slope of 1.02 and a standard error of 0.025. However, despite excellent correlation between the detectors, apparently some small bias is indicated for the determination of β-carotene (slope 1.08, standard error 0.02). Overcompensation in the manual integration of the retinol ester interference with the β-carotene peak may have produced a negative bias for the electrochemical detector results.

Figure 5 also demonstrates the relative lack of interference in the micronutrient determinations from unknown serum components or ingested drugs. Such interferences are not likely to provide response factors similar to those of the analytes in both detectors and thus would appear in the plots as points far removed from the line of identity.

The results for the analyses of the pooled serum are presented in Table 4. The agreement between the two detectors is good, and the uncertainty (CV) was acceptable (3% to 4%) for all three analytes.

Although the absorbance and electrochemical detectors measure the three micronutrients by completely separate principles with different attendant interferences, the results for both still depend on the same extraction and separation technique. Absolute validation of our method would require agreement with the analysis of a serum Standard Reference Material, for which the values of the three micronutrients would have been certified by two totally independent techniques. Current work at NBS is directed toward the development of such a Standard Reference Material.

References


