Nonaqueous Reversed-Phase Liquid Chromatography and Fluorimetry Compared for Determination of Retinol in Serum

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A new assay for retinol in human serum, based on nonaqueous reversed-phase liquid chromatography, is presented. Sample preparation includes addition of the internal standard, retinyl propionate, deproteinization of 100 μL of serum with acetonitrile, and extraction with hexane. The standard curve is linear up to 2 mg/L. The assay is characterized by excellent sensitivity (detection limit, 15 μg/L) and good within-run and between-run precision (CVs of 2.6 and 2.7%, respectively), and results compare favorably with those by fluorimetry. We assayed 135 samples from hospitalized patients by both methods. Although the two sets of values correlated well (r = 0.955) the fluorimetric method occasionally suffers from interferences. In practice, fluorimetry proves valuable as a routine method, while liquid chromatography meets the criteria of a potential reference method.

Additional Keyphrases: potential reference method · vitamin A

The superiority of liquid-chromatographic over fluorimetric and colorimetric vitamin A assays is generally recognized. Yet, fluorimetry, unlike colorimetry, continues to be a useful technique for retinol determination in blood, because of its simplicity, sensitivity, and speed. Most fluorimetric retinol assays, however, are subject to potential interference from phytofluene (1-4). To remove this interference, sample clean-up steps based on column chromatography have been designed (1-3), often at the expense of simplicity and speed. Some workers tried to avoid this complication by using a correction formula (2), while a few methods allegedly do not have such interference (5-7).

Recently, more selective retinol extraction methods have been developed (8,9), which are said to minimize the coextraction of endogenous substances, including carotenoids and retinyl esters (9). In addition to endogenous substances, exogenous compounds such as drugs may potentially interfere with fluorimetry as well. In such cases, use of a more specific chromatographic technique is imperative.

Several liquid-chromatographic methods for determination of retinol in serum have been reported (9-17). Straight phase systems (9-11) offer the advantage of compatibility with organic solvents, which permits direct injection of extracts without the need for an evaporation step. However, the major shortcoming of this type of method so far has been the lack of a suitable internal standard (9,11). Compounds that have been proposed in this regard include an aromatic retinoid (10) and retinol methoxime (18), both of which are not readily available and thus must be synthesized. As an alternative, reversed-phase systems have gained wide popularity for vitamin A determination (12-17), particularly because bonded-phase materials are more stable, reproducible, and convenient than silica. Retinyl acetate has been frequently used as an internal standard (14-17).

Recently, we showed that nonaqueous reversed-phase chromatography (NARP) is a powerful technique for separating carotenoids and vitamin A derivatives (19). This concept, originally described by Parris (20), has since then been occasionally used for the differentiation of fat-soluble vitamin standards (21) as well as for determination of retinyl palmitate in oil, margarine, and cereals (22, 23). The advantages of improved solubility of low-polarity samples in totally organic eluents are obvious and apply equally to retinol and retinyl esters. The poor solubility of the latter compounds in methanol–water and even pure methanol has hampered so far their quantification in biological materials by liquid chromatography (24-26). Yet, semi-aqueous eluents continue to form the basis of virtually all liquid-chromatographic systems for retinol and retinyl esters (14-17, 24-26).

This paper describes the use of NARP as a basis for a retinol assay in human serum. Sample preparation includes deproteinization with acetonitrile and extraction with hexane. We find this new method particularly useful as a reference method to verify results obtained with a "routine" fluorimetric assay, if required.

Materials and Methods

Chemicals. Analytical-grade acetonitrile was purchased from the Aldrich Co., Beerse, Belgium. Other "chemically pure" solvents came from Hoechst, Frankfurt, F.R.G., and were redistilled before use. Hexane was refluxed over metallic sodium before distillation. Retinol was from Fluka, Buchs, Switzerland; retinyl propionate from AEC, Commeny, France. Retinyl palmitate, retinyl acetate, lycopene, and retinol were from Sigma Chemical Co., St. Louis, MO 63178. All other test substances, including α-retinyl acetate, 13-cis-retinyl acetate, 3-dehydroretinol, phytoene, and retinyl stearate, were gifts from F. Hoffmann-La Roche, Basle, Switzerland. Phytofluene was extracted from tomatoes by homogenization in acetonitrile: dichloromethane: methanol (70:15:15 by vol), in a Potter–Elvehjem homogenizer. An aliquot of the homogenate was injected into the liquid-chromatographic system. Detection was at 330 nm. The major chromatographic peak displayed the highly typical ultraviolet spectrum of phytofluene and had a capacity ratio \( k' = (t_c - t_0)/t_0 \), where \( t_c \) and \( t_0 \) are the retention times of the compound of interest and of an unretarded peak, respectively, of 18.1. This value agreed well with the theoretical value predicted from a comparison with reference standards of the two close analogs, lycopene \( (k' = 8.9) \) and phytoene \( (k' = 22.0) \). The capacity ratios and the number of conjugated double bonds in the molecules are linearly related.

Chromatography instrumentation. We used a constant-flow pump (Varian 5020; Varian Instrument Group, Palo Alto, CA) with a Rheodyne injection valve (70 μL), a Model 1030 variable-wavelength detector (Varian), a Hewlett-Packard 3390A integrator, a Model 3380A chromatography workstation (Varian), and a Model 5720A gas chromatograph (Varian) for the determination of fatty acids. The column used was a 25 cm × 4.6 mm i.d. Nucleosil 100 C18 (19 mm) packed with 5 μm Nucleosil 100 C18 (20 μm) (Macherey-Nagel, Düren, Germany). The mobile phase was a mixture of hexane:isopropanol:water (85:10:5, v/v), which was filtered using a 0.22 μm filter (Millipore, Bedford, Mass.) and degassed by ultrasonic treatment before use. The flow rate was 1 mL/min at 30°C. We determined the peak at 280 nm, with an exposure time of 1.0 s at 1.0 A. units. The separation was completed in 40 min.

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Alto, CA 94303), a Valco N60 sample valve with a 50-µL loop (Valco Instrument Co., Houston, TX 77055), and a Varian 5000 variable-wavelength detector (Varian). Ultraviolet spectra of chromatographic peaks were recorded on-line with an HP 1040A detector equipped with an HP 85 computing system, a HP 82901M flexible-disc drive, and a HP 7470A graphics plotter (all from Hewlett-Packard Co., Palo Alto, CA 94304). The 25 × 0.46 cm column, prepacked with 7-µm Zorbax ODS, was purchased from Du Pont, Wilmington, DE 19898. For elution we used a mixture of acetonitrile: dichloromethane:methanol (70:15:15 by vol, flow rate 1–2 mL/min, ambient temperature).

**Fluorimetry instrumentation.** For fluorimetric measurements we used a Model 1000 filter fluorimeter (Perkin-Elmer Corp., Norwalk, CT 06856). A 336-nm filter was used to select the excitation wavelength; the emission wavelength was set at 450 nm.

**Standard solutions.** Standard solutions of retinol and retinyl propionate were prepared in ethanol. Stored at −20 °C and protected from light, they are stable for at least six weeks. On chromatography, no significant extra peaks indicative of degradation products have been observed. We determined the concentration of the retinol solution spectrophotometrically, using a ε₃₂₅ value of 46 000 mol⁻¹ cm⁻¹. The absorbance of the 4.5 mg/L retinyl propionate solution averaged 0.860 (at 326 nm).

**Patient materials.** We assayed 135 sera from unselected hospitalized patients who had undergone gastrointestinal vascular surgery. Blood was sampled both before and after the operation. Some patients received preoperative therapy, including cardiotonic, diuretic, and vasodilating agents, or were on a restricted diet. We made no attempts to categorize samples according to these different groups.

**Procedures**

**Fluorimetry.** In a brown screw-capped conical-tip tube, combine 100 µL of serum, 200 µL of acetonitrile and vortex-mix vigorously for 30 s. Add 3 mL of n-hexane and mix on a rotary mixer for 5 min. Centrifuge, isolate the organic layer, and immediately measure its fluorescence at λₑₓ 336 nm, λₑ𝑚 450 nm. For standardization, add known amounts of retinol (10 to 80 ng) to 100 µL of a 50 g/L albumin solution and proceed as above.

**Liquid chromatography.** In a brown screw-capped conical-tip tube, combine 100 µL of serum, 15 µL of retinyl propionate solution, and 200 µL of acetonitrile and vortex-mix vigorously for 30 s. Add 3 mL of n-hexane and mix on a rotary mixer for 5 min. After centrifugation, isolate the organic layer and evaporate it at room temperature under reduced pressure. Reconstitute the residue with 80 µL of the above-described chromatographic solvent and inject a 50-µL aliquot onto the liquid-chromatography column. For calibration, analyze 100-µL aliquots of a serum pool supplemented with 10 to 80 ng of retinol.

A standard curve, obtained by plotting peak-height ratios (retinol/retinyl propionate) vs the standard concentrations, intercepts the y-axis because of endogenous retinol in the serum pool. Construct a second standard curve, parallel to the former one, by subtracting this intercept. Unknown concentrations are determined by comparing peak-height ratios with the new curve.

**Results and Discussion**

NARP is ideally suited for separating vitamin A derivatives. Because of the unusually high retentivity of Zorbax ODS for retinol, strong mobile phases containing dichloromethane can be used, which afford excellent sample solubility (19, 21). Figure 1 illustrates the separation of six vitamin A analogs. In this particular experiment, a slightly more polar mobile phase, without dichloromethane, was used to optimize resolution. Remarkably, even all-trans retinyl acetate and its geometric isomer, 13-cis-retinyl acetate, could be readily separated on the reversed-phase column, although the latter co-eluted with α-retinyl acetate. Table 1 lists capacity ratios of retinol and related derivatives in this system, as well as under standard conditions. Stronger mobile phases are advantageous to ensure fast elution of higher retinyl esters. Even under those strong elution conditions retinol remains sufficiently retained to be quantified if required. As can be inferred from Table 1, phytoflueone—a common interference in most of the current fluorimetric retinol assays (3–4)—does not interfere in the present method (k' = 18.1, compared with 0.9 for retinol).

Figure 2 shows a representative chromatogram of a serum extract containing the internal standard. Total chromatographic analysis time is only 5 min. The standard curve is linear to at least 2 mg/L. A comparison of the slopes of the calibration curves runs over during seven days yielded a CV

| Table 1. Capacity Ratios (k') of Retinol and Related Compounds |
|-----------------|-----------------|-----------------|
|                  | Eluent 1*       | Eluent 2*       | Eluent 3*       |
| 3-Dehydroretinol | 0.66            | 1.23            | —               |
| Retinol          | 0.90            | 1.83            | 0.64            |
| Retinal          | 1.06            | 1.98            | 0.75            |
| α-Retinyl acetate| 1.15            | 2.27            | —               |
| 13-cis-Retinyl acetate | 1.22   | 2.32            | —               |
| All-trans-retinyl acetate | 1.27 | 2.45            | 0.91            |
| Retinyl propionate | 1.52         | 3.02            | 1.07            |
| Retinyl palmitate | 17.70       | —               | 5.64            |
| Phytoflueone     | 18.10           | —               | —               |
| β-Carotene       | 18.10           | —               | —               |
| Retinyl stearate | 26.90           | —               | 7.55            |

* Eluent 1: acetonitrile/methanol/dichloromethane, 70:15:15 by vol.  
* Eluent 2: acetonitrile/methanol, 90:10 by vol.  
of 3.8%. The yield of retinol extracted from serum with acetonitrile/hexane reportedly is nearly 100% (9). We found an analytical recovery of 90.6(SD 2.6)% (x = 0.68 mg/L, n = 9) for retinyl propionate, a figure which essentially supports the result obtained by Wu et al. (8) for retinyl acetate under similar conditions (91.4 ± 2.9%). Table 2 summarizes precision and sensitivity data for both the liquid-chromatographic and the fluorimetric method.

So far, we have encountered no interference whatsoever with our assay. No endogenous compound co-elutes with retinol, as evidenced by the "purity" of the peak: ultraviolet spectra, recorded on-line at four different points of the peak, matched perfectly (λmax 324 nm). We have been unable to demonstrate the presence of phytofluenae in serum from human volunteers by our method. This apparently should be attributed to the selectivity of the acetonitrile/hexane extraction, as suggested by other authors (8, 9). In addition, because of the long retention time for phytofluenae in our system, the low amounts of this compound in serum extracts—if it is present at all—will remain undetected. Reportedly (27), the concentration of phytofluenae in blood is an order of magnitude lower than that of retinol.

Major advantages of our system for retinol determination are the exceptional efficiency and stability of the column when used in the NAP mode. Even after injection of hundreds of serum extracts, the column maintained theoretical plate numbers of as high as 10,000 to 15,000 and continued to yield perfectly symmetrical peaks. These favorable properties may be in part associated with the good solubility of low-polarity samples (hexane extracts) in the nonaqueous eluent. So far, there has been no need to regenerate or replace the column. To make the method suitable for quantification of retinyl esters in serum, a less-selective extraction procedure such as the Bligh-Dyer approach (24) should be used.

We assayed 135 samples from hospitalized patients by both the liquid-chromatographic and the fluorimetric method. As demonstrated in Figure 3, the two approaches correlated well (r = 0.955). Mean values obtained by chromatography and fluorimetry were 0.57 (range, 0.03–1.81) mg/L and 0.57 (range, 0.04–2.13) mg/L, respectively. This good agreement between results by the two techniques and the lack of a positive or negative bias lead us to assume that in the present fluorimetric assay there are no major interferences, e.g., from phytofluenae. This good correlation is even more pronounced in the lower range of retinol concentrations. However, some samples from hospitalized patients occasionally displayed excessively high fluorescence—i.e., beyond the instrument's range—presumably caused by the presence of drugs. Such off-scale readings did not permit calculation of corresponding retinol concentrations, so these data are not included in Figure 3. Re-analysis of these samples by chromatography invariably resulted in normal values.

The superiority of the present liquid-chromatographic assay over the fluorimetric approach in terms of sensitivity, precision, and specificity suggests that it meets the criteria of a reference method for retinol determination. Yet the simplicity, low cost, and high sample throughput of the fluorimetric method have led us to adopt it as a routine assay in our laboratories, where it has been in use for over a year. From an economic standpoint, most clinical laboratories cannot afford to use liquid chromatography routinely unless a sufficiently large and steady supply of samples is guaranteed, or no suitable alternative technique is available. However, its use becomes imperative when the presence of interferences in the samples precludes fluorimetry. In addition, periodic verification by chromatography of results obtained by fluorimetry remains useful.

### Table 2. Precision and Sensitivity Data

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<th>Within-run</th>
<th>Between-run</th>
<th>Detection limit, μg/L</th>
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<tr>
<td></td>
<td>CV, %</td>
<td>x, mg/L</td>
<td>n</td>
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<td>Liq. chrom.</td>
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<td>0.80</td>
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<tr>
<td>Fluorimetry</td>
<td>4.8</td>
<td>0.70</td>
<td>11</td>
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*Defined as the retinol concentration (in a 50 g/L albumin solution) yielding a peak height corresponding to four times the signal/noise ratio of the detector.

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Fig. 2. Chromatogram of a human serum extract

Eluant, acetonitrile/dichloromethane/methanol, 70/15/15 by vol, flow rate, 1 mL/min; detection at 330 nm, 0.02 A full scale. Peak identification: 1, retinol; 2, retinyl propionate (internal standard).

Fig. 3. Retinol concentrations in serum as determined with the liquid-chromatographic method (x) and the fluorimetric method (y).

Regression analysis: y = 0.995x + 0.22, r = 0.955, n = 135. Points exceeding 1.5 mg/L are not depicted. Samples exhibiting abnormal interferences in the fluorimetric assay are not included. Four duplicate points are present.
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