Liquid-Chromatographic Assay for Retinol (Vitamin A) and Retinol Analogs in Therapeutic Trials

Sharon W. McClean, Mark E. Ruddel, Earl G. Gross, John J. DeGiovanna, and Gary L. Peck

A "high-performance" liquid-chromatographic separation of retinoids (retinol, isotretinoin, all-trans retinoic acid, retinal, etretinate, and retinyl acetate) in serum is described. The separation was used in developing a quantitative assay for retinol (vitamin A) and two therapeutic analogs, isotretinoin (13-cis-retinoic acid) and etretinate (Ro 10-9359). The procedure requires 1 mL of serum. Overall analytical recovery for retinol, isotretinoin, and etretinate from serum was 100% (SD 7%). The between-day coefficient of variation for specimens with concentrations ranging from 0.70 to 0.95 mg/L was <4%. Normal reference intervals for serum retinol in men and women are 0.61 to 1.33 and 0.44 to 1.19 mg/L, respectively.

Additional Keyphrases: reference interval • treatment of skin disorders • sex-related effect • isotretinoin • etretinate

Retinol (vitamin A) and some of its analogs (retinoids) are of current interest because of the association of decreased concentrations of retinol in blood with an increased incidence of cancer (1) and because of the beneficial effects that retinoids have on various dermatological disorders, both benign and malignant (2-5).

Measurement of retinol by "high-performance" liquid chromatography (HPLC) does not confound retinol with retinyl esters as does the Carr–Price procedure (6), nor does it suffer from the interferences typical of fluorometry (7). In addition, it offers another advantage: several retinoids can be measured in the same assay. De Ruyter and De Leenheer (8) developed an isocratic separation of retinol and retinyl esters. Frolik et al. (9) separated tretinoin (all-trans-retinoic acid) from its synthetic isomeric isotretinoin (13-cis-retinoic acid). Puglisi and DeSilva (10) developed several separation schemes, each capable of separating a pair of retinoids. We have developed a serum sample preparation scheme and HPLC isocratic separation for retinol, tretinoin, isotretinoin, and retinol, and for etretinate (Ro 10-9359) and two of its metabolites. Retinyl acetate is used as the internal standard.

Accuracy and precision studies were centered on isotretinoin and etretinate, along with retinol, because these two synthetic retinoids are currently being used in therapeutic trials. Isotretinoin is being and has been investigated for use in treatment of basal cell carcinomas and treatment-resistant acne. Etretinate is being studied for its effect in several keratinizing disorders, including psoriasis and Darier's disease (4).

Materials and Methods

Chemicals

Acetonitrile and acetic acid, both "HPLC" grade, were from Waters Associates, Milford, MA 01757, and J. T. Baker Chemical Co., Phillipsburg, NJ 08865, respectively. The water was glass-distilled. Butanol and K2HPO4 were "Baker Analyzed" grade (J. T. Baker). Crystalline retinol, retinyl acetate, retinal, and tretinoin were from Sigma Chemical Co., St. Louis, MO 63178. The two investigational drugs, isotretinoin (Ro 4-3780) and etretinate (Ro 10-9359), were from Hoffmann–La Roche, Inc., Nutley, NJ 07110. Structures for these two compounds have been published (9).

Sample Preparation for Liquid Chromatography

To 1 mL of serum in a 1.9-mL conical-tip snap-topped plastic centrifuge tube, add 25 μL of the internal standard, retinyl acetate, 40 mg/L in butanol/acetonitrile (equal volumes). Then add 400 μL of the extraction solvent, the butanol/acetonitrile mixture. After vortex-mixing for 60 s, add 300 μL of a dipotassium monohydrogen phosphate solution (1.2 mg/L) to the mixture. Vortex-mix for 30 s, then centrifuge the tubes at 8700 × g for 2 min. Inject 50 μL of the supernate directly onto the column. The sample is prepared under yellow lights.

For quantitation, use a single standard containing 0.5 mg of each analyte per liter of the extraction solvent. Determine unknown concentrations by comparison of the retinoid/retinyl acetate area ratios with those of the standards.

Liquid Chromatography

The liquid-chromatographic equipment (Waters Associates) included a Model 6000A Solvent Delivery System, a WISP autoinjector, a Model 450 Variable Wavelength Detector set at either 324 nm or 360 nm, and a Data Module recording integrator. Retinol absorbs maximally at 324 nm. The higher wavelength is preferred for etretinate and isotretinoin. The separation was effected at ambient temperature, on a 25 cm × 4.6 mm (i.d.) reversed-phase ODS-2 column from Whatman Inc., Clifton, NJ 07014. The mobile phase was acetic acid/water/acetonitrile (0.5/20.0/79.5 by vol), used at a flow rate of 3.0 mL/min.

Validation of the Assay

Within-run precision was estimated by assaying a serum pool prepared from patients who were being treated with either isotretinoin or etretinate. Each determination was the result of a separate extraction as well as a separate injection onto the column. Between-run precision was determined by assaying once a day for 20 days a pooled specimen of serum supplemented with the respective analyte.

Analytical-recovery studies were done in which four additions were made to a serum pool. These additions resulted in concentrations ranging from 0.05 to 1.0 mg/L for each of the three compounds: retinol, etretinate, and isotretinoin.

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Stability Studies

To assess stability of retinol, isotretinoin, and etretinate in serum and in the extraction solvent, we exposed the solutions for various intervals to fluorescent light at 22 °C before analysis. Other portions of the same solutions were kept in the dark at 22 °C for the same comparison.

We investigated the effect of two anticoagulants on retinoid measurements. Blood was drawn from individuals into two evacuated tubes, one without and one with an anticoagulant, either ethylenediaminetetraacetate (EDTA) or potassium oxalate/sodium fluoride. In the case of EDTA, we used both 20-mL and 7-mL tubes and the specimens were drawn to produce half-filled as well as filled tubes. We also added EDTA to screw-topped glass tubes into which we emptied blood from the syringe used for the collection.

Peak Identification

Retention times of unknowns were compared with those of standards for identification. Identifications were additionally confirmed spectrophotometrically from absorbance ratios. The absorbance for a given peak was measured at each of three wavelengths (340, 360, 380 nm) and ratios were calculated from pairs of measurements. Three such ratios were calculated for each peak.

Reference Range for Retinol

Serum was collected from 172 ostensibly healthy adults, to establish a reference interval for retinol. These specimens were assayed by this HPLC method, with the detector set at 324 nm. Parametric statistics were used to calculate the mean and reference range.

Results and Discussion

All of the retinoids that we examined are well separated. The first chromatogram (Figure 1a) is of a standard mixture of retinoids dissolved in an equimolar mixture of acetonitrile and butanol; elution occurs in order of decreasing polarity. Figure 1b shows a chromatogram of a serum sample from a patient who was not receiving any therapy with retinoid. Only retinol and the internal standard, retinyl acetate, appear. Figures 1c and d depict chromatograms of serum extracts from patients who were receiving isotretinoin and etretinate, respectively. Isotretinoin, retinol, and etretinate peaks in serum were identified by comparison with elution times and multiwavelength absorbance ratios of standards. For the ratio determinations, solvent flow was stopped when the recorder indicated an eluting compound was in the flow cell; measurements were then made at three wavelengths. The standards and their like-eluting unknowns all had absorbance ratios that agreed within 5%. A large peak just following the solvent front in Figure 2c is not seen in Figure 2d. We believe this may be 4-oxo-13-cis-retinoic acid, the major metabolite of isotretinoin (11). The metabolite, more polar than isotretinoin, would be expected to elute near the solvent front, as does this unknown compound. In addition, the unknown compound appears to reach higher concentrations than isotretinoin with chronic administration of isotretinoin, which is consistent with what others have found (11). However, authentic 4-oxo-13-cis-retinoic acid was not available to use in confirming this. Sera from patients receiving etretinate show two extra peaks, marked peak 1 and peak 2 in Figure 1d, eluting at 3 and 4 min. From elution times and multiwavelength absorbance ratios, we were able to identify the peak that elutes at about 4 min as the ethyl ester of etretinate (Ro 10-1670).

Addition of dipotassium phosphate to the extraction mixture enhanced the separation of the organic and aqueous phases in several ways. With the salt, the interface between the phases was more defined, the organic phase was optically clearer, partitioning was faster, and the phase volume ratios were consistent. Extraction of 1 mL of serum with 400 µL of solvent produced a 2.5-fold concentration of the sample. Butanol was combined with acetonitrile in the extraction solvent to improve the reproducibility of analytical recovery. Without butanol we found that recovery decreased with increasing triglyceride concentration of the specimen. When butanol was used, recovery was independent of the triglyceride concentration.

Recovery studies comparing sera supplemented with standards in an organic matrix resulted in average recoveries of 106% (SD 3%), 96% (SD 10%), and 100% (SD 5%) for isotretinoin, retinol, and etretinate, respectively. Overall, the average recovery was 101% (SD 7%). Retinoids are poorly soluble in aqueous solutions. Consequently, protein-based standards must be made from highly concentrated organic standards and referenced to them. In our hands the long-term stability of the organic standards was greater than that of

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**Table 1. Reproducibility of the Assay**

<table>
<thead>
<tr>
<th></th>
<th>Within-run</th>
<th>Between-run</th>
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<tr>
<td></td>
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<tr>
<td>Isotretinoin</td>
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</tr>
<tr>
<td>Retinol</td>
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<tr>
<td>Etretinate</td>
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<td>0.96</td>
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<td></td>
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<tr>
<td></td>
<td>6</td>
<td>1.97</td>
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**Fig. 1.** Elution profile of retinoids in (A) a 1:1 acetonitrile/butanol mixture supplemented with retinoids, (B) serum from a normal individual, (C) serum from individual receiving therapy with isotretinoin, and (D) serum from individual receiving therapy with etretinate. Retinyl acetate is the internal standard. Chromatograms were obtained with the detector set at 360 nm.
Table 2. Stability of Retinoids to Fluorescent Light at 22 °C

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Retinol</th>
<th>Isotretinoin</th>
<th>Etretinate</th>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Organic</td>
<td>100</td>
<td>102</td>
<td>100</td>
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<table>
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<tr>
<th>Percentage of zero hour concn left after exposure for times (h)</th>
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<th>2</th>
<th>5</th>
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<tbody>
<tr>
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<td>100</td>
<td>101</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Isotretinoin</td>
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<td>Etretinate</td>
<td>100</td>
<td>89</td>
<td>77</td>
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</table>

Table 2. Stability of Retinoids to Fluorescent Light at 22 °C

*The organic matrix was 9/1 (by vol) mixture of 1:1 acetonitrile:butanol and methanol. D Degradation products of etretinate interfere with etretinate peak. This value may overestimate the amount of etretinate remaining.

protein-based standards. For these reasons, with the good recoveries we obtained, we elected to use a standard in extraction solvent rather than a protein-based standard. Only a single standard was used with each run, because with the 80-μL injection volume the peak ratios (retinoid/retinyl acetate) were linearly related to concentration up to 6 mg/L. With the detection wavelength at 360 nm and the range on 0.04 absorbance unit full scale we obtained a response factor (area/concentration) of 0.13, 0.052, and 0.040 A mg⁻¹L for retinol, isotretinoin, and etretinate, respectively. At 360 nm the limit of detection for each of these was 0.04, 0.02, and 0.03 mg/L. At 324 nm the limit of detection for retinol was 0.02 mg/L. Its response factor was 0.047 A mg⁻¹L at 324 nm.

Table 1 shows the within-run and among-run reproducibility of the assay. These data were obtained with the detector at 360 nm. One could expect that the precision for low concentrations of retinol would improve at 324 nm, where it has a 2.7-fold higher absorptivity.

The stability of retinol, isotretinoin, and etretinate in the presence of light is summarized in Table 2. Retinol showed no significant change even after 23 h of exposure when in serum. In the extraction solvent over half of the retinol was lost by 23 h. Etretinate and isotretinoin underwent photodecomposition in both the serum and extraction solvent, degradation in the extraction solvent being greater than that in the serum. Serum seems to stabilize the retinoids against photodecomposition, perhaps owing to protein binding or to the absorption of light by other molecules in the serum.

When either oxalate/fluoride or EDTA was used, the apparent concentration of retinoids in the plasma specimen was consistently lower than in serum (Table 3). The decreases shown for EDTA were obtained with filled tubes. The effect was doubled by filling the evacuated tube only half full. Decreases of the same order of magnitude as shown in Table 3 were also seen when either was added to serum. Thus the effect does not seem to be related to the coagulation process. We did not see extra peaks forming in the pattern for oxalate/fluoride plasma as we did in the case of EDTA-treated plasma. With the addition of EDTA a peak appeared that was not seen in the serum and that eluted at a time close to that for all-trans-retinoic acid. De Ruyter et al. (12) report finding endogenous tretinoin (all-trans-retinoic acid) in EDTA-treated plasma samples. The amount of this substance that we see in the EDTA plasma is directly proportional to the concentration of EDTA in the plasma. Thus we felt it to be a degradation product. Although we occasionally see a small peak in serum specimens where tretinoin elutes, it is present in quantities too low for our system to quantitate.

We found that the reference intervals for retinol in serum are sex related. The distributions were close to gaussian; the means and medians were identical within each group. The means and ranges were:

<table>
<thead>
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<th>Sex</th>
<th>Mean (Range ±2 SD)</th>
<th>mg/L</th>
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<tbody>
<tr>
<td>Men (n = 96)</td>
<td>0.97 (0.61–1.33)</td>
<td></td>
</tr>
<tr>
<td>Women (n = 150)</td>
<td>0.82 (0.44–1.19)</td>
<td></td>
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</table>

Our assay differs from that of Frolik et al. (9) in two ways. The isomers, tretinoin and isotretinoin, are more quickly separated, making assay of less-polar retinoids more practical. In addition, lyophilization of the sample is not required as part of our sample-preparation procedure. The method of Puglisi and DeSilva (10) was designed for optimum separation of pairs of retinoids. It involves different sample-preparation schemes and different column conditions, depending on the retinoids of interest. Our method was developed to allow separation of retinoids with a wider range of polarities.

We believe that an assay for vitamin A that also measures therapeutic retinoids will be useful. For instance, this assay is now being used to investigate the serum concentrations associated with therapeutic and toxic responses and to study factors affecting the absorption and the mode of transport of retinoids in the blood.

References


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Danazol and Its Principal Metabolites Interfere with Binding of Testosterone, Cortisol, and Thyroxin by Plasma Proteins

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Danazol and its three principal metabolites (2-hydroxy-methyl ether-thiostereone, 2-hydroxy methyl-1,2-dehydroethisterone, and ethisterone) competitively displace cortisol and testosterone from plasma proteins. This effect is in addition to the reported inhibition of the production of testosterone-binding globulin and thyroxin-binding globulin. We saw no competitive inhibition of thyroxin binding. Concentrations of total testosterone, total cortisol, and total thyroxin were low, whereas percentages of free testosterone, free cortisol, and free thyroxin were abnormally high in women being treated with danazol. Values for testosterone, cortisol, and thyroxin in danazol-treated patients should therefore be appropriately corrected before interpretation. Protein-binding assays for testosterone or cortisol that involve testosterone- or cortisol-binding globulin may be invalid in danazol-treated subjects because of the competitive binding of danazol and its metabolites to these proteins.

Additional Keyphrases: variation, source of hormones protein-binding assays

The drug danazol (17α-pregna-2,4-dien-20-yne[2,3-d]-isoxazol-17-o) is structurally related to both testosterone and ethisterone (17α-ethinyll testosterone) and is commonly used for treating endometriosis and benign breast disease. These conditions produce no abnormalities in endocrine function and no effect on steroid or thyroxin binding. The present study was begun when samples of plasma obtained from such danazol-treated patients showed no specific testosterone binding. Danazol affects thyroid-function tests (1), displaces progesterone from guinea pig plasma proteins (2), binds to transcortin (2), and decreases the actual concentration of both thyroxin-binding globulin and testosterone-binding globulin to one-third or less of normal, but does not change the concentration of cortisol-binding globulin (3). Danazol treatment interferes with radioimmunoassay of testosterone in some systems (4).

Danazol is rapidly metabolized in vivo, and some 60 end products have been observed in monkey urine (5), the principal urinary and fecal end products being 2-hydroxy methyl-ethisterone (2-HME), 2-hydroxy methyl-1,2-dehydroethisterone (Δ1-2HME), and ethisterone (5). The concentration of unmetabolized danazol in human plasma is about 260 μg/L at 2 h after a 400-mg oral dose (2), and the concentration of 2-HME is about five- to 10-fold that of danazol itself (2). Thus, danazol or its metabolites (or both) could affect the binding of thyroxin, cortisol, and testosterone in plasma both by competitive displacement and by decreasing the actual concentration of the binding protein.

Accordingly, we studied the effects of danazol and three of its metabolites on the plasma binding of testosterone, cortisol, and thyroxin in vitro and in vivo. We determined the binding of testosterone, cortisol, and thyroxin in plasma from women taking danazol.

Materials and Methods

We sampled plasma from eight volunteer patients who were being treated with danazol (400 mg, twice daily) for endometriosis. These samples were stored at −20 °C until analysis in duplicate.

A pooled specimen of plasma was produced by combining samples from women younger than 40 years who were not receiving danazol. Stock solutions of danazol (0.1 to 50 mg/L), ethisterone (0.1 to 100 mg/L), 2-HME (0.1 to 1000 mg/L), Δ1-2HME (0.1 to 1000 mg/L), and DL-norgestrel (0.01 to 0.5 mg/L) were prepared in this pooled plasma. The patients' samples and the dilutions of danazol and its metabolites were then evaluated for binding of a testosterone by the use of a diethylaminoethyl (DEAE)-cellulose column (6), (b) cortisol by charcoal absorption (7), and (c) thyroxin by Sephadex gel filtration (8), determined as described in these references except that tritiated cortisol was substituted for tritiated progesterone for determination of cortisol binding (7).

The displacement produced by each steroid was compared with that produced by testosterone, cortisol, or thyroxin as previously reported for determination of cross reactions with an antibody (9). Briefly, this consisted of construction of logit/log regression lines for both the standard and the

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