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RAPID ANALYSIS OF THE MAJOR CLASSES OF RETINOIDS BY STEP GRADIENT REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING RETINAL (O-ETHYL) OXIME DERIVATIVES

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SUMMARY

A rapid step-gradient reversed-phase high-performance liquid chromatography (HPLC) method is presented for analysis of the major classes of retinoids in tissues. Retinal was converted into a new derivative, retinal (O-ethyl) oxime, since the standard derivative, retinaloxime, co-elutes with retinol on reversed-phase HPLC. The most abundant naturally occurring retinyl esters, retinyl palmitate and retinyl stearate, were eluted within 12 min to complete the separation. Retinoids were extracted in the presence of an antioxidant, butylated hydroxytoluene, and a lipid carrier, cholesterol. Recoveries of 98–100% were obtained from tissue samples by internal addition for the retinoids tested (retinol, retinal and retinyl palmitate), and the absolute recovery of endogenous retinal from rat eyecups was confirmed by spectrophotometric measurements of rhodopsin. Extraction was carried out in an air atmosphere and under subdued incandescent light rather than requiring inert atmosphere and safe-light conditions used in most methods. *Cis–trans* isomers were not separated under the reversed-phase HPLC conditions employed. Quantitation was carried out using retinyl acetate as internal standard and the day to day precision was better than 3.5%. A sensitivity of about 1 ng is obtained for all retinoids using absorbance monitoring at 325 nm and a C_{18} 5 μm column with 12% reversed-phase loading. The tocopherols can also be separated and detected simultaneously with similar sensitivity by this method using a fluorescence detector in series [G. J. Handelman, L. J. Machlin, K. Fitch, J. J. Weiter and E. A. Dratz, *J. Nutr.*, 115 (1985) 807].

INTRODUCTION

Studies on vitamin A metabolism have been greatly simplified during the past ten years by high-performance liquid chromatographic (HPLC) techniques. Vitamin

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A and its major metabolites (retinal, retinolic acid and retinyl esters) occur widely in biological systems and have many important roles that are under active study[1,2]. There are several published methods for separation of vitamin A compounds on straight-phase HPLC systems[3,4]. When they can be employed, reversed-phase HPLC systems are usually preferable to straight phase because of their superior retention time stability. In addition, the solvents used in reverse phase are less volatile and less toxic than typical solvents used in straight-phase HPLC. However, two of the reasons that reversed-phase HPLC separations of the major classes of vitamin A compounds has not previously been satisfactory are:

(i) Retinal is usually covalently bound in biological systems as an aldimine (Schiff base)[5,6]. To extract quantitatively the retinal, hydroxylamine is usually used to chemically cleave the Schiff base linkages to form retinaloxime derivatives[7]. However, it has not been possible to separate the retinaloxime from vitamin A (retinol) by reversed-phase HPLC despite numerous attempts (C. C. Farnsworth and E. A. Dratz, unpublished results). In the present work, it was found that by using O-ethylhydroxylamine instead of hydroxylamine to cleave the retinal Schiff base and forming the retinal (O-ethyl) oxime derivative, the desired separation was obtained. The recovery of retinal as the O-ethyloxime was greater than 99%.

(ii) The major storage forms of vitamin A, the retinyl esters, show long retention times, broad peaks and low sensitivity with current reversed-phase HPLC separation methods[8–13]. Recently, a paper was published where a relative rapid analysis of different retinyl esters with a good sensitivity (10 ng) was accomplished by gradient elution reversed-phase HPLC, but this method does not include the retinal compounds[14]. We have developed a reversed-phase HPLC step gradient to elute the retinyl esters within 12 min, which provided rapid analysis of all the major classes of vitamin A compounds with a 1 ng detection limit.

Optimum sensitivity is obtained for each compound by avoiding separation into geometric isomers. With the addition of a fluorescence detector in series all the tocopherols can also be analyzed in the same chromatographic run with similar sensitivity[15].

EXPERIMENTAL

Retinoids were analyzed by reversed-phase HPLC using a 25 × 0.46 cm C_{18} 5 μm (12% loading) Econosphere column (Alltech) and a direct connect 5 × 0.46 cm pre-column. The HPLC system consisted of two Altex 110 pumps, an Altex mixer and an Altex 210 injection valve with a 20-μl loop. An Altex programmer was used to control solvent changes. Detection was carried out by ultraviolet absorption at 325 nm (Hitachi Model 100-10 spectrophotometer) with an Altex 18-μl flow cell. A Spectra-Physics minigrator was used to establish the retention times. Chromatograms were recorded on a Hewlett-Packard 7132 chart recorder and quantitation was done by peak height measurements of authentic standards and unknowns relative to an internal standard (retinyl acetate) as described below.

An HPLC step gradient solvent system was developed with methanol and isopropanol (Fisher HPLC grade) at a flow-rate of 1.5 ml/min during the analysis. An initial 3.5 min isocratic elution was employed with solvent A (0.5% ammonium acetate in 100% methanol). This was followed by a short linear step to 50% of solvent

B (100% isopropanol), carried out over 1.5 min to avoid sudden pressure changes and large refractive index changes. This latter solvent mixture was run for 8.5 min to achieve the elution of the long chain retinyl esters. During this period the pressure in the HPLC system increased by 500–1000 p.s.i. After the separation a 1.5 min period was used to return linearly to solvent A. Column re-equilibration with solvent A was carried out for 5 min at a flow rate of 2 ml/min. The pressure in the HPLC system decreased during this re-equilibrating step. The analysis and re-equilibration program requires 20 min. The solvent programmer and integrator were started with a switch on the injection valve.

All-*trans* retinoic acid, retinol, retinal, retinyl acetate, and retinyl palmitate standards were obtained from Sigma (St. Louis, MO, U.S.A.). These standards were tested for purity by running each compound separately on HPLC. Standards were stored as concentrated solutions in hexane at −20°C, and were stable under these conditions. Fresh dilutions were made daily into methanol for internal addition experiments, and absorption spectra were measured for each methanol dilution for quantitation.

Extractions of biological tissues were carried out under subdued incandescent light except when calibrations using spectrophotometric assays of rhodopsin were performed under dim red light. Bovine retina was used as a convenient source of retinal for retinal recovery and for between day reproducibility experiments. Single whole bovine retinas were homogenized in 5 ml buffer containing 10 m*M* HEPES, 20 m*M* sodium chloride, 0.1 m*M* EDTA, pH 7.0 (retina buffer). Homogenates were centrifuged for 2 min at 120 *g*. The supernatant was collected, aliquots were transferred to vials and stored at −80°C. Crude bovine retinal pigment epithelium homogenates were used for retinyl palmitate recovery experiments. Retinal pigment epithelium suspensions, produced by scraping bovine eyecups in retina buffer, were homogenized and stored as described above.

Recoveries were calculated by using the method of internal addition. Extractions were carried out in 7-ml glass vials (Kimble, No. 60910-L) with PTFE lined caps (Thomas No. 2390-H22). For recovery calculations three separate samples were

TABLE I

RETINAL RECOVERY EXPERIMENT

The values shown are for duplicate analysis.

Sample number	*Analysed material*	*Peak height ratio* $\frac{\textit{retinal (O-ethyl) oxime}}{\textit{retinyl acetate}}$
1	Retina	0.480 0.472
2	Retina + retinal*	1.054 1.061
3	Retinal	0.596 0.588

* The predicted peak height ratio for retina with added retinal is 1.068, and the average measured value is 1.058, which indicates a recovery of greater than 99%.

analysed in duplicate, as shown for a retinal recovery experiment in Table I. Experimental samples No. 1 and No. 2 contained 100-μl aliquots of tissue homogenate (obtained from bovine retina in this experiment), followed by 100 μl of 0.1 *M* PIPES buffer pH 6.5 containing 0.1 *M* O-ethylhydroxylamine hydrochloride (Fluka) [or alternatively O-methylhydroxylamine (Aldrich) or hydroxylamine in some experiments]. Aliquots of 100 μl methanol containing butylated hydroxytoluene (BHT) (50 μg/ml) and cholesterol (50 μg/ml) were transferred to each vial followed by a second 100-μl aliquot of methanol, either pure for sample No. 1 or spiked with all-*trans* retinal (1 μg/ml) for sample No. 2. Sample No. 3 (reference) contained 100 μl retina buffer without tissue. Aliquots of 100 μl O-ethylhydroxylamine solution, 100 μl methanol containing BHT and cholesterol, and 100 μl methanol spiked with all-*trans* retinal (1 μg/ml) were added to this sample. Positive displacement SMI pipets were used for accurate internal additions using methanol solutions. The mixtures were vortexed for 60 s and incubated at room temperature for 5 min in the dark.

After the incubation, 1.0 ml chilled hexane containing 50 ng/ml retinyl acetate as internal standard was added. Samples were vortexed for 1 min in a special clamped holder and centrifuged for 1 min at 1000 *g*. The supernatant was collected and the aqueous lower layer was extracted twice with hexane without retinyl acetate. The pooled hexane fractions were filtered through a 0.2-μm PTFE filter in a Swinex adapter (Millipore), evaporated under nitrogen and dissolved in 100 μl of methanol for injection into the HPLC system. After HPLC separation the peak-height ratios of retinal (O-ethyl) oxime *versus* retinyl acetate were calculated for each experimental and reference sample, as shown in Table I, to determine the recoveries.

Pigmented agouti rats (ACI), which had been maintained under dim cyclic light conditions, were dark adapted overnight. Eyes were removed under dim red light after injection of a lethal dose of nembutal. Rat eye cups, including the retina, were cut into small pieces and homogenized in retina buffer. Extraction and manipulation of the rat eye exctracts was carried out under dim red light. Portions of the rat eye homogenate were extracted and analyzed by HPLC as described above and other portions were extracted with 6% Ammonyx LO (Onyx Chemical Co.), centrifuged for 5 min at 6000 *g* and assayed for rhodopsin as light sensitive absorbance at 500 nm[16].

RESULTS

The separation of the main classes of retinoids by the step gradient reversed-phase HPLC method presented is shown in Fig. 1. Retinoic acid, retinol, retinyl acetate, retinal (O-ethyl) oxime, and retinyl palmitate are well separated within 12 min. Retinoic acid elutes 25 s after the void volume of the column. The trace below the chromatogram in Fig. 1 is a blank solvent injection and shows a broad feature between 6 and 8 min, caused by the refractive index change due to the step gradient of isopropanol. The use of a step gradient minimizes retention times and yields sharp peaks for the late eluting retinyl esters, which reside at the front of the column during the separation of the other retinoids in methanol. The sharpness of the late eluting retinyl ester peaks enhances their detection sensitivity. The magnitude of the refractive index anomaly in the baseline is sensitive to flow cell design but can be minimized by proper alignment.

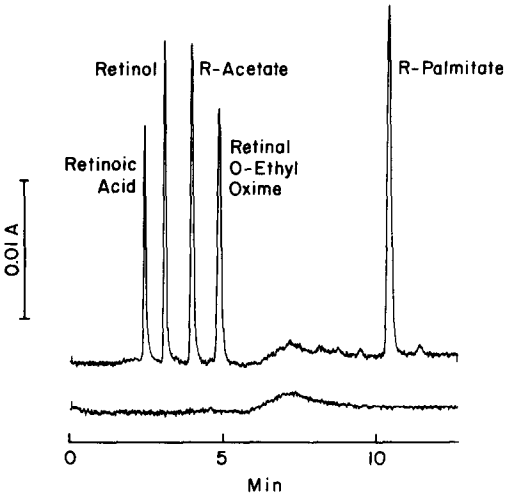

Fig. 1. Step gradient HPLC separation of the main classes of retinoids on a C_{18} 5 μm reversed-phase column. Samples were eluted at a flow-rate of 1.5 ml/min with solvent A (0.5% ammonium acetate in 100% methanol) for 3.5 min. The eluent was changed with a 1.5 min short linear gradient to 50% solvent B (isopropanol). The lower curve is the detector response to a blank injection and the same solvent program. The broad feature in the baseline between 6 and 8 min in both the chromatogram and the blank injection is caused by the refractive index gradient between solvent A and B. R-Acetate and R-Palmitate are retinyl esters.

Table II shows a summary of the retention times for all the retinoids studied in this HPLC system. Table II shows that the retinaloxime co-elutes with retinol and that the separation between retinal (O-ethyl) oxime and retinyl acetate (internal standard) is better than the separation between retinal (O-methyl) oxime and retinyl acetate. The structures of retinal and the different retinaloxime derivatives that were studied are shown in Fig. 2. Note that neither the *syn–anti* isomers of the oximes nor the *cis–trans* isomers of the polyenes were separated.

A standard curve for retinal (O-ethyl) oxime is shown in Fig. 3. Linearity has only been shown over the 1–60 ng range, because this method was developed to detect small amounts of retinal in tissues, close to the detection limit. However, the

TABLE II

RETENTION TIMES OF THE RETINOIDS STUDIED IN THE STEP GRADIENT REVERSED-PHASE HPLC SYSTEM DESCRIBED IN THIS PAPER

Compound	*Retention time (s)*
Retinoid-acid	145
Retinol	182
Retinaloxime	182
Retinal	204
Retinyl acetate	233
Retinal (O-methyl) oxime	260
Retinal (O-ethyl) oxime	283
Retinyl palmitate	630
Retinyl stearate	690

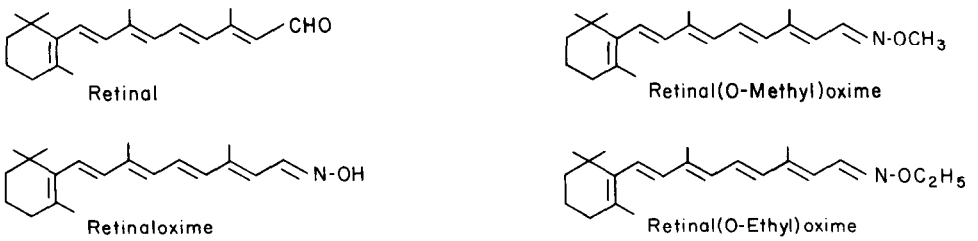

Fig. 2. Structures of all-*trans* retinal (retinal), retinaloxime and the retinal (O-alkyl) oxime derivatives used in this study.

lack of any detectable curvature and experience with HPLC suggests that the system will probably be linear to much higher levels.

Fig. 4 shows the analysis of the retinoids from an aliquot (one third) of a dark adapted rat eyecup extract. The more slowly eluting retinyl ester, peak 5, has been shown to be retinyl stearate by Bridges *et al.*[17]. Retinyl palmitate and retinyl stearate are by far the most abundant naturally occurring forms of retinyl esters. The separation of retinyl esters with other fatty acids was not investigated, however, the excellent separation of retinyl palmitate and retinyl stearate suggests that this method might be used to separate most of the common naturally occurring retinyl esters.

Table I shows the results of a retinal recovery experiment using internal addition of retinal as described in Experimental. The predicted ratio of retinal (O-ethyl) oxime *versus* retinyl acetate differs by less than 1% from the measured ratio and this shows a recovery of greater than 99%. Added retinal tends to bind to free amino groups in tissue by Schiff base linkages and its extractability is expected to mimic the extractability of the endogenous retinal, which is largely bound to protein by the same chemical linkage. The high recoveries with internal addition suggests that retinal covalently bound in tissue is completely converted to the retinal (O-ethyl) oxime

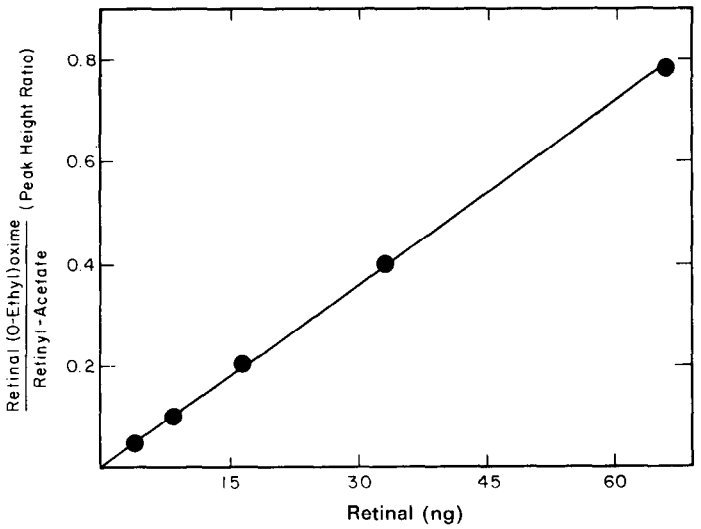

Fig. 3. Standard curve for retinal (O-ethyl) oxime over the 1–60 ng range.

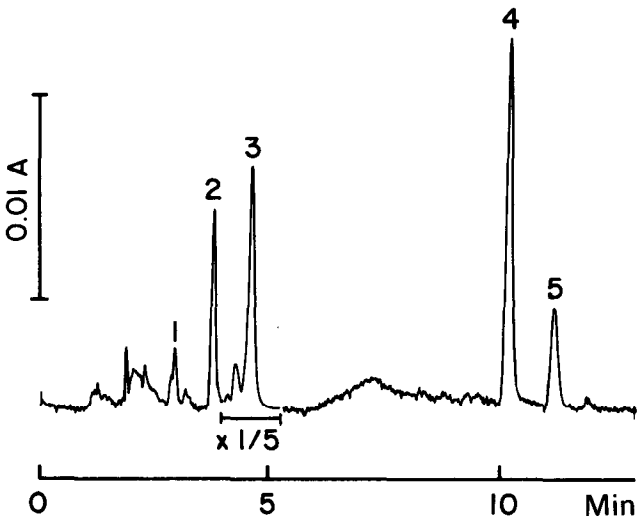

Fig. 4. Extract of a dark adapted rat eyecup analyzed under the same chromatographic conditions described in Fig. 1. The material analyzed corresponds to 35% of the eyecup. Peaks: 1 = retinol; 2 = retinyl acetate; 3 = 11-*cis*-retinal (O-ethyl) oxime; 4 = retinyl palmitate; 5 = retinyl stearate.

derivative. Similar experiments have been performed with bovine retinal pigment epithelium, which is a rich source of retinyl palmitate, to show a similar high recovery of retinyl palmitate.

Absolute recoveries of retinal were measured by comparison of the amount of rhodopsin in dark adapted rat eyecup homogenates (analysed by direct spectrophotometric assay), with the amount of retinal (O-ethyl) oxime extracted from these homogenates (analysed by HPLC). Rhodopsin was extracted in dim red light with aqueous detergent and each rhodopsin contains one mole of retinal. Using duplicate spectrophotometric measurements according to Raubach *et al.*[16], 1.60 nmoles rhodopsin per rat eye was found. When analysis were performed by HPLC the standard solution of retinal used for calibration was the pure all-*trans* isomer, whereas the retinal extracted from the dark adapted eye is recovered as the 11-*cis* isomer. The ratio of the extinction coefficients of all-*trans* and 11-*cis* retinaloximes are the same in alcohol and hexane at the absorption maxima[18]. The ratio of extinction coefficients of all-*trans* and 11-*cis* retinaloximes at 325 nm (1.44), has also been taken to be the same in methanol, as was reported in hexane[19]. Using this extinction coefficient ratio, 450 ng retinal per eye was measured by HPLC in duplicate samples, which equals 1.58 nmoles rhodopsin. Therefore, the absolute recovery of retinal from the rat retina was found to be 99%.

The between day reproducibility of the method was established by preparing pooled retina and retinal pigment epithelium (RPE) homogenates, as described above. These tissue homogenates were stored in numerous aliquots at −80°C. Aliquots of the same pool were thawed and analysed on different days. By daily spectrophotometric calibration of retinal standard solutions in methanol, the values obtained for the amount of retinal in aliquots from the same pool of retina homogenate, showed that the day to day precision of the method was better than 3.5%[15]. Similar

calibrations were performed to measure the amount of retinyl palmitate from a pool of RPE homogenate, for which the same day to day precision was obtained.

DISCUSSION

This method was developed for nutritional and metabolic studies. Vitamin A compounds are central in vision and it has been established that retinal, retinal Schiff bases, retinol, and retinyl esters occur in the visual cycle although many of the detailed steps are still controversial[5,20–24]. Retinoids also play important roles in the immune- and reproductive system, and are essential for maintenance of epithelial cells[1,2]. If the distribution of the geometric isomers of vitamin A compounds is desired, the analysis must currently be performed with straight-phase HPLC. If, however, the total amount of each vitamin A compound is desired, reversed-phase HPLC systems are more convenient. With reversed-phase HPLC systems currently available, only retinoic acid, the first eluting minor component, has so far been separated into the various geometrical *cis* and *trans* isomers[25,26], whereas the isomeric compounds of the less polar retinoids are not spread into several peaks. Therefore, the reversed-phase determinations have more sensitivity for analysis of small tissue samples and the complexity of the data to be worked up is much less than if separate isomers are measured. Another reason to prefer reversed-phase HPLC techniques is the superior day to day stability of retention times of these systems, which is useful for firm identification of compounds of interest. In nearly a year of retinoid analysis on a conventional C_{18} 5-μm column we have obtained retention times with maximum variations of less than 5 s.

Figs. 1 and 4 show that the step gradient reversed-phase HPLC technique we have developed rapidly analyses the major classes of vitamin A compounds. It has been shown[7] that for a quantitative analysis of retinal derivatives, a chemical cleavage of the retinal Schiff base linkages is required, and this is usually done by forming retinaloxime derivatives. However, the retinaloxime co-elutes with retinol on reversed-phase HPLC [Farnsworth and Dratz, unpublished data]. A new derivative was found, retinal (O-ethyl) oxime, which separates well from all the other vitamin A compounds.

During the conversion of retinal Schiff bases or free retinal to oximes each geometrical isomer of retinal generates an additional pair of stereoisomers: a *syn* and *anti* oxime[19]. To make sure that both stereo-isomers co-elute on our reversed-phase system, the all-*trans* retinal (O-ethyl) oxime fraction was collected, re-extracted in hexane, injected on a 25 × 0.46 cm straight-phase 10 μm silica column and eluted with hexane–dioxane (95:5) at a flow-rate of 2 ml/min. On this straight-phase system it was possible to separate and collect two different fractions that had a product yield of 83:17, for the *syn* and *anti* isomers of both retinal (O-alkyl) oximes.

A limitation of the method presented is caused by differences in molar extinction coefficients for *cis–trans* isomers of retinoids[18]. The mixed *syn–anti* isomers of retinal oximes present no limitations since it was shown by straight-phase HPLC above that 83% of the retinal (O-ethyl) oxime is the *syn* isomer, and an effective extinction coefficient can be calculated. The bulk of the retinoids exist in the all-*trans* form in most tissues, except for the eye, where the 11-*cis* isomer predominates. The 11-*cis* isomer has a 30–40% lower peak extinction coefficient than the all-*trans* iso-

mer. Depending on the tissue analysed, correction factors can be used to compensate for differences in molar extinction coefficients between *cis* and *trans* isomers. However, to greatly diminish the influence of differences between the molar extinction coefficients of *cis–trans* isomers, detection can be performed at the isosbestic point, which is 290 nm for retinoids in alcoholic solutions[27], and 302 nm in hexanes[28]. Detection at the isosbestic point leads to a 3–4 times lower sensitivity due to the lower extinction coefficient. If highest accuracy for total retinoids is desired it is also possible to collect fractions, extract them into hexane, and analyse them by straight-phase HPLC.

Compared to current reversed-phase HPLC methods for retinyl esters we have improved both the speed and the sensitivity of the analysis (to a detection limit of 1 ng, with a signal noise ratio of 5:1). Most methods suffer from long retention times[8–13] which produces broad peaks and a loss of sensitivity. Recently, Furr *et al.*[14] published a reversed-phase HPLC separation for retinyl esters using a linear solvent gradient of (A) methanol–water (80:20) and (B) methanol–tetrahydrofuran (50:50). They separated the different retinyl esters relative rapid between 15–20 min, however amounts less than 10 ng where not quantitable in their system. Tetrahydrofuran was avoided in our work since it has a propensity for peroxide formation and peroxides can start free-radical reactions that may destroy retinoids.

Fig. 4 illustrates the value of this method as a micro determination technique for analysis of retinoids in small amounts of tissue. The extract of one third of a rat eye cup yields large easily measured peaks with a conventional variable wavelength detector. For example, 3 ng of retinyl stearate (peak 5), a minor component, is detected with a signal to noise ratio of about 10:1. Detectors are commercially available with a 10 times higher signal to noise ratio.

Isopropanol was a convenient second solvent component of our reversed-phase HPLC separation system. It is miscible with methanol, non-toxic, stable and does not appear to cause any solvent compatibility problems. The only problem with isopropanol is caused by its hygroscopic character. Small traces of water in this solvent cause large increases in retention times for the long-chain retinyl esters. This can prevented by dividing a freshly opened bottle into several smaller reservoir bottles. Each reservoir bottle is opened once to insert a sintered HPLC solvent filter and pump tubing after which it is tightly sealed. With such a procedure problems with variable retention times of retinyl palmitate and retinyl stearate were eliminated.

The inclusion of dry 0.5% ammonium acetate in solvent A (methanol) is required to elute retinoic acid as a sharp, well resolved peak[29]. If a retinoic acid analysis is not desired, the ammonium acetate can be omitted and the other retinoid compounds will elute within a few seconds of the same retention times.

It is important to use antioxidants during extraction. The polyene character of the retinoid compounds makes them susceptible to a variety of oxidizing conditions[3,29,30]. For example, vortexing a sample in air during extraction can lead to significant losses of retinoids[4] if antioxidants are omitted from the extraction solvents. BHT has been reported to be a stabilizer of fat soluble vitamins during extraction[31], and was found to be an effective protective agent against retinoid degradation that is especially important for recovery during evaporation of hexane under a stream of nitrogen.

Cholesterol was added as a lipid carrier, which was crucial for obtaining good

recoveries of retinyl esters. The retinyl esters dissolve poorly in methanol, particularly when they are in a dry film on the surface of a glass vial. However, when the retinyl esters are contained in an oily film of cholesterol they dissolve quantitatively in methanol. The recovery of retinyl palmitate and retinyl stearate was improved from about 50% to nearly 100% when cholesterol was added as a carrier. In addition, it was important for good quantitative reproducibility in the analysis of all the retinoids that the internal standard, retinyl acetate, was added in hexane rather than in methanol, since retinyl acetate has poor stability in aqueous alcoholic solutions[32]. The retinyl acetate and other retinoids are stable for months at −20°C in the hexane extract. Retinoid containing extracts should be held in hexane and not in methanol if they cannot be analysed immediately. Once the extracts are dried down and dissolved in methanol they should be injected into the HPLC with minimum delay.

Most methods for retinoid extraction and quantitation are carried out under an inert atmosphere and under yellow safe-lights[4,7]. However, by incorporating BHT for antioxidant protection and cholesterol as a host lipid carrier it was possible to obtain nearly 100% recovery of the retinoids while working under subdued incandescent light and an air atmosphere. Nitrogen is only needed for evaporation of the hexane. These improvements contribute to the ease, convenience and reproducibility of our method. The use of subdued incandescent lighting appears to cause minor *cis–trans* isomerization of retinoid extracts, when oximes were formed promptly before extraction. If isomerization was substantial it should not have been possible to obtain the high reproducibility and precision within day, or between days since timing of various steps was not standardized. Isomerization would change the ratio of *cis–trans* isomers, and the peak height ratios would change, because differences in molar extinction coefficients. Brown *et al.*[33] reported that retinol requires the presence of iodine to isomerise efficiently in visible light and Hubbard[28] reported that the oxime derivatives isomerise at a much slower rate than free retinal.

The column with a 12% loading of the C_{18} reversed-phase coating is superior to more commonly used columns with a 16% loading. The advantage of lighter loading is that similar retention times are obtained at a slower flow-rate. For example, retinol elutes from our column at 183 s at a flow-rate of 1.5 ml/min while on a similar column with 16% loading it requires a flow-rate of 2 ml/min to elute with the same retention time. A slower flow-rate is preferable because columns develop more plates at a slower flow-rate, and a decreased amount of solvent is used in the HPLC system.

We often perform high sensitivity measurements of all the major tocopherols simultaneously with these retinoid assays by simply connecting a fluorescence detector in series and lengthening the initial methanol elution period from 3.5 to 10 min[15].

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