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DETERMINATION OF VITAMIN A AND VITAMIN A ACETATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method with fluorescence detection has been devised for the analysis of vitamin A (retinol) and its acetate. The method employs a C_{18} reversed-phase column and methanol as an eluent. The detection of these two compounds is monitored with fluorescence excitation at 348 nm and emision at 470 nm. Detector noise established the lower limit of quantitation at approximatly 0.5 ng. Plasma samples were employed to evaluate the accuracy, reproducibility, and applicability of the method. Less than 1 ng of vitamin A in plasma (as low as 1 μl) can be quantitated by this procedure.

INTRODUCTION

Vitamin A is known to be essential for normal vision, bone growth, reproduction, and epithelial cell formation. The possible role of vitamin A and its analogues (retinoids) in the prevention and therapy of cancer has received considerable recent research interest[1–4]. Currently, vitamin A status is assessed by measuring retinol levels in blood plasma or tissue by either fluorometric methods[5–7], colorimetric methods[8,9], or by high-performance liquid chromatography (HPLC) with UV detection[10–15]. Colorimetric analysis and fluorometric methods are subject to interference from such endogenous substances as carotenes, phytofluene, and exogenous contaminants. Furthermore, these two methods are more time consuming and require considerable analytical skill and larger sample sizes to yield useful results[16]. These analytical problems can largely be eliminated by employing HPLC. The HPLC method with UV detection, however, shows a relatively high background, as many compounds in plasma and other biological materials are UV-absorbing, so that quantitative measurement in the low nanogram range is usually unsatisfactory.

We have developed a rapid and sensitive HPLC method using fluorescence detection for the analysis of vitamin A (retinol) and its acetate. The method requires only a simple extraction without further need for sample clean-up. Less than 1 ng retinol in plasma (as low as 1 μl) can thus be quantitated.

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EXPERIMENTAL

Reagents

HPLC-grade hexane, methanol, and 95% ethanol were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). β-Carotene and trifluoroacetic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Highly purified *trans*-retinol and retinol acetate were provided by Hoffmann-La Roche (Nutley, NJ, U.S.A.). All chemicals and reagents were used without further purification.

Preparation of standards

A retinol stock solution containing 1 mg/ml was prepared in methanol and stored in actinic glassware at −20°C. The stock solution proved to be stable for at least 10 weeks and no significant decrease in retinol or appearance of impurities was noted[14,15]. Retinol working standards containing 0.0312–1.0 μg/ml were prepared before use and kept for up to 4 weeks at −20°C and protected from natural light. The relative fluorescence intensity, expressed as peak area, was used to quantitate the results.

A retinol acetate stock solution containing 1 mg/ml in methanol was diluted to 0.5 μg/ml in ethanol for use as a deproteinizing solution and as an internal standard. Internal working standards were prepared weekly and protected from light.

Because carotenes are relatively unstable[9], standard solutions were prepared less than 4 h prior to use. Carotene was first dissolved in a small amount of carbon tetrachloride and then brought to volume with ethanol.

High-performance liquid chromatography

A Beckman Model 112 solvent delivery pump, a Beckman Model 420 controller, and a Beckman Model 340 organizer were used with an Altex Model 210 injector fitted with a 20-μl or 100-μl sample loop (Beckman, Berkeley, CA, U.S.A.). Detection was performed with a Kratos Model FS-970 liquid chromatography fluorometer and a Kratos Model GM-970 monochromator (Kratos Analytical, Ramsay, NJ, U.S.A.). A Varian Model CDS 111 integrator (Varian, Palo Alto, CA, U.S.A.) was used to quantitate peak areas. A Leeds and Northrup chart recorder with a vari-span scaler (Leeds and Northrup, North Wales, PA, U.S.A.) was used to record the relative fluorescence intensity. A reversed-phase μBondapak C_{18} (Microsorb ODS, 5-μm particle size) stainless-steel column, 150 × 4.6 mm I.D. long (Ranin Instrument, Woburn, MA, U.S.A.) was used as stationary phase. A stainless-steel 50 × 3.9 mm I.D. guard column (Supelco, Bellefonte, PA, U.S.A.) was also employed.

Fluorescence excitation and emission wavelengths of retinol were established by an Aminco scanning spectrophotofluorometer (American Instrument, Silver Springs, MD, U.S.A.). The excitation maximum for retinol in methanol was found at 348 nm and emission was 470 nm. Methanol was used as the mobile phase. A flow-rate faster than 2.5 ml/min was found to decrease reproducibility. Therefore, 1.5 ml/min was selected as the optimum rate. The detector was set at 0.01 attenuation.

Plasma samples

Human plasma samples were obtained from a local blood supplier and from volunteers for the evaluation of the accuracy, reproducibility, recovery, and applic-

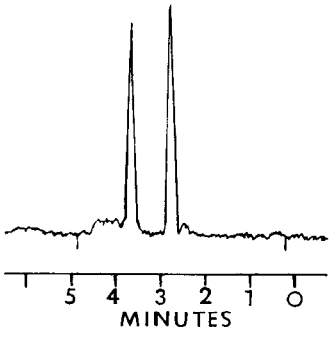
ability of the method. For routine analysis, 100 μl of plasma was first deproteinized with 2 volumes of ethanol containing retinol acetate as the internal standard. The samples were swirled in a Vortex mixer extracted with 1 ml of hexane, and centrifuged at 600 *g* for 1 min. Aliquots of 0.6–0.8 ml of hexane were evaporated under nitrogen. The contents of each tube were redissolved in 0.1–0.5 ml methanol and a portion was injected in triplicate into the column. Saponification was found to be unnecessary.

Retinol, dissolved in ethanol, was added to plasma samples to give 14.5–385 μg/100 ml (or 1.7–48.7 ng per injection) to determine the recovery and accuracy of the method. The samples were prepared as above. The possible interference of high β-carotene levels on retinol measurement was investigated by adding aliquots of a freshly prepared β-carotene solution to the plasma samples. β-Carotene was added to give a 60–1000 μg/100 ml increase in plasma concentration. The normal plasma carotene concentration is approximately 85 μg/100 ml with a range of 20–200 μg/100 ml[17].

To determine the lower limit of plasma retinol that could be accurately measured, 10, 5, 4, 3, 2, and 1 μl plasma were added with capillary syringes to 50 μl ethanol containing 25 ng of retinol acetate as an internal standard. The mixture was extracted with 0.5 ml of hexane, aliquots were evaporated and redissolved in 50 μl of methanol.

RESULTS

A typical chromatogram from a plasma extract to which a known amount of retinol acetate had been added as internal standard is shown in Fig. 1. Separation of retinol and retinol acetate was very good and no interfering peaks were found in the chromatogram under the experimental conditions. With a methanol flow-rate of 1.5 ml/min, the retention times for retinol and retinol acetate were 2.6 and 3.4 min, respectively. Retinol acetate had approximately 70% of the fluorescence of retinol.



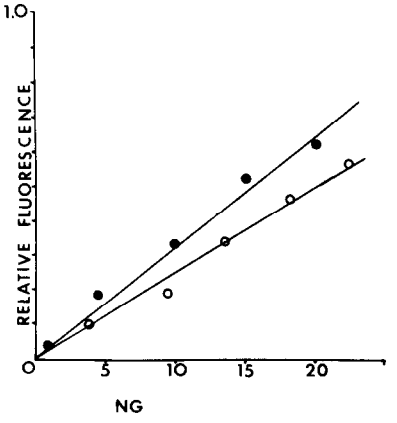


Fig. 1. Chromatogram of plasma extract containing retinol acetate as an internal standard. Retention times: 2.6 min for retinol and 3.4 min for retinol acetate. Conditions: 15 cm C_{18} reversed-phase column; 1.5 ml methanol/min; fluorescence excitation at 348 nm and emision at 470 nm.

Fig. 2. Standard curves of retinol (●) and retinol acetate (○). See Fig. 1 for conditions.

TABLE I

RECOVERY OF ADDED RETINOL FROM PLASMA

Plasma volume (ml)	*Retinol added (ng)*	*Retinol found (ng)*	*Recovery (%)*
0.1	0.0	38.7 ± 2.5*	–
0.1	14.5	54.6 ± 5.5	102.6
0.1	36.2	77.6 ± 1.8	103.6
0.1	72.4	111 ± 5.9	100.1
0.1	154	193 ± 8.9	99.8
0.1	385	424 ± 18	99.9

* Mean ± standard deviation; 6 replicates each.

The results of the recovery test for retinol added to 0.1 ml plasma are summarized in Table I. An average of 100–104% retinol was recovered over the concentration range of 14.5–385 μg/100 ml.

The standard curves for retinol and retinol acetate are shown in Fig. 2. The relationship between relative fluorescence intensity and concentrations of both retinol and retinol acetate was found to be linear up to about 50 ng. The calibration curve for retinol was $y = 0.328$, $r = 0.9966$, and $y = 0.236$, $r = 0.9982$ for retinol acetate. A linear relationship was also found between the ratio of peak areas of retinol/retinol acetate. The calibration curve (Fig. 3) can be used to determine plasma retinol after obtaining the ratio of peak areas.

The effect of β-carotene on the measurement of retinol is shown in Table II. β-Carotene added at concentrations of 62.5–1000 μg/100 ml was found to have no significant effect on retinol measurement.

The lower limit of detection for retinol in plasma was found to be about 0.5 ng. Plasma levels of retinol can be accurately measured by employing a sample as small as 1 μl although a better coefficient of variation can be obtained with samples of 5 μl or more. The recovery of the internal standard for the very small sample sizes was between 85–100% (Table III).

TABLE II

EFFECT OF β-CAROTENE ON PLASMA RETINOL ANALYSIS

Plasma (ml)	*Added β-Carotene (ng)*	*Retinol found (ng)*	*Recovery (%)*
0.1	0	34.6 ± 4.1*	–
0.1	62	36.0 ± 3.1	104
0.1	125	34.6 ± 3.1	100
0.1	250	34.7 ± 2.1	100
0.1	500	36.2 ± 4.2	105
0.1	1000	34.9 ± 2.3	101

* Mean ± standard deviation; 6 replicates each.

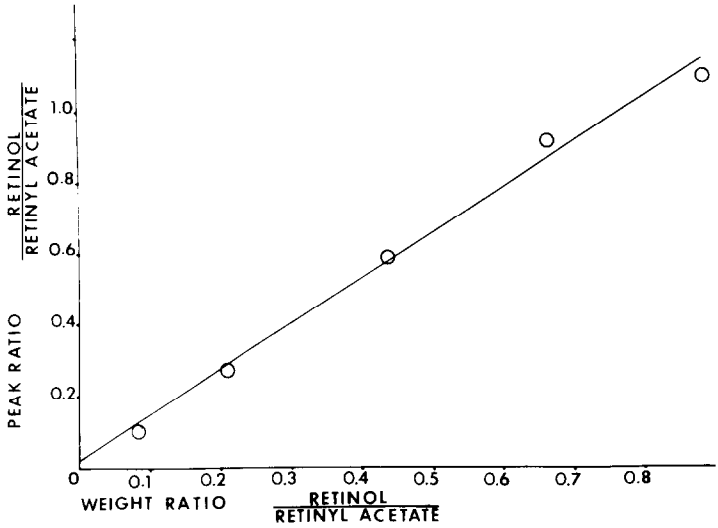


Fig. 3. Calibration curve of retinol/retinol acetate relative fluorescence *versus* weight ratio. $y = 1.32x$. See Fig. 1 for conditions.

DISCUSSION

The recent development of HPLC techniques has offered expanded flexibility as well as increased sensitivity and specific identification for compounds of medical and biochemical interest. The HPLC method with UV detection for vitamin A developed during recent years has been found to be much more sensitive, convenient, and reproducible than either colorimetric or fluorometric procedures[16]. However, the uneven baseline, typical of UV chromatograms for plasma samples limits its reliability for small amounts of retinol. Fluorescence detection of chromatographic eluates, on the other hand, permits the elimination of extra peaks and changing baselines with a significant increase in sensitivity. Also, compounds can be selectively

TABLE III

PRECISION AND LINEARITY OF SMALL PLASMA VOLUMES

Plasma (μl)	*Retinol found (ng)*	*Coefficient of variation (%)*	*Recovery (%)*
10	7.50 ± 0.36* (5)	4.8	98.8**
5	3.75 ± 0.41 (6)	11	99.1
4	2.60 ± 0.43 (6)	17	87.0
3	1.95 ± 0.35 (6)	13	95.7
2	1.59 ± 0.23 (5)	12	85.2
1	0.85 ± 0.20 (5)	25	92.8

* Mean ± standard deviation; number of replicates in parentheses. For all data, $r = 0.9968$, $m = 0.7480$ ng/μl, $b = -0.0702$ ng.

** Based on added retinol acetate internal standard.

analyzed by selecting specific wavelengths for excitation and emission. Therefore, by employing fluorescence detection the procedure outlined affords specific measurements of retinol that are approximately one order of magnitude better than assays with UV detection.

The colorimetric method with trifluoroacetic acid[9] has been widely utilized as a standard procedure for retinol measurement for a number of years. However, the colorimetric assay requires a correction factor of unknown accuracy for the carotenoids[14]. The average retinol content in the plasma of thirty normal adults when determined by the colorimetric procedure was 46.6 ± 11.3 μg/100 ml as compared to 48.7 ± 10.2 μg/100 ml when the HPLC–fluorescence method was used. The correlation coefficient between the two methods was found to be 0.795 ($p < 0.001$). However, the coefficient of variation from the same sample was much smaller for the HPLC method than for the colorimetric method.

The high recovery and good linearity for added retinol over a wide range (Table I), as well as freedom from interference by normal to high levels of β-carotene (Table II) demonstrate the accuracy and usefulness of the method. Carotene was not eluted from the column due to its insolubility in methanol. Repeated carotene injections and extended use of the column did affect the baseline slightly, but by washing it with tetrahydrofuran its performance was restored.

The described HPLC–fluorescence procedure for measuring retinol and its acetate is very rapid, sensitive, and accurate. It is more sensitive than the HPLC method with UV detection and may be applied to the analysis of other tissues and vitamin A analogues in samples of medical and pharmacological interest.

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