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Note

Rapid preparation of anhydroretinol and its use as an internal standard in determination of liver total vitamin A by high-performance liquid chromatography

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High-performance liquid chromatographic (HPLC) assays of serum retinoids often utilize non-physiological esters of retinol (e.g. retinyl acetate, retinyl propionate, retinyl heptadecanoate) as internal standards to correct for losses during sample work-up and chromatography [1—6]. The saponification procedure for total tissue vitamin A [7], however, naturally renders esters unsuitable as standards in that assay. Alternative methods of quantifying recovery, such as the recovery of radioactivity from added labeled retinol, are limited to cases where radioactive retinoids are not used in the experimental design.

An internal standard must share chemical and physical properties with the compounds of interest, so as to co-purify during sample preparation procedures. In addition, it must fulfil the criteria set up for chromatography and detection [8]. We report, here, the rapid production of anhydroretinol (AR) and demonstrate its applicability as an internal standard for total tissue vitamin A determination by HPLC.

EXPERIMENTAL

All-*trans*-retinoids were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC solvents were from Burdick and Jackson (Muskegon, MI, U.S.A.). [11,12-^{3}H]retinyl acetate was supplied by the Chemoprevention Program, Chemical and Physical Carcinogenesis Branch of the National Cancer Institute (Bethesda, MD, U.S.A.). Other chemicals and reagents were reagent grade or better.

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HPLC was performed using an Altex 110A pump and a 15 cm × 4.6 mm I.D., 10-μm μBondapak C_{18} reversed-phase column (Beckman, Fullerton, CA, U.S.A.) with pure methanol at a flow-rate of 2.0 ml/min. Detection was at 325 nm by a Hitachi Model 140 variable-wavelength UV—VIS spectrophotometer equipped with a 10-μl flow cell, and peak heights were measured by a Hewlett-Packard 3390A integrator. The same spectrophotometer, without the flow cell, was used for UV spectra. Radioactivity was counted in a Beckman LS2800 beta-counter.

AR was produced by passing 1 ml of a hexane solution of retinyl acetate through a silica Sep-Pak (Millipore, Milford, MA, U.S.A.) washed previously with 5 ml of hexane. AR was eluted in a 3-ml volume of 2% diethyl ether in hexane following a 3-ml hexane wash. AR was also produced from retinol by the method of Dunagin, Jr. and Olson [9]. Prior to use, the AR solution was evaporated to dryness under nitrogen, and was reconstituted in ethanol.

Tritiated retinyl palmitate was prepared by HPLC of a hexane extract of a homogenate of liver from a rat previously fed [11,12-^{3}H]retinyl acetate, and collection of the peak eluting at the retention time of authentic retinyl palmitate.

For determination of recovery of AR relative to retinol, various amounts of retinol were added to a constant amount of AR, and the mixtures were either injected directly onto the liquid chromatograph or were diluted in water, saponified in an ethanolic solution of potassium hydroxide containing pyrogallol (Sigma) and extracted according to Thompson et al. [7]. The hexane extracts obtained by the latter treatment were then dried under nitrogen, reconstituted in ethanol and injected. AR and [^{3}H]retinyl palmitate were also added to rat liver homogenates (1:6 in water) and the mixtures processed for total vitamin A [7]. Chromatograms were run through the retention time of retinyl palmitate to veryify that complete hydrolysis had occurred.

RESULTS

Identification of anhydroretinol

When [11,12-^{3}H]retinyl acetate in hexane was applied to a silica Sep-Pak cartridge and eluted successively with 3-ml volumes of hexane and 2% diethyl ether in hexane, the 2% diethyl ether in hexane fraction contained a compound with a retention time of 3.0 min on HPLC. This compound was not present when the retinyl acetate solution was dried and injected without passage over silica. Since the compound had a retention time close to that of retinol, it was collected for further analysis. Its UV spectrum is shown in Fig. 1. Absorbance maxima are observed at 387 and 367 nm, with a prominant shoulder at 350 nm, in agreement with previous reports for AR [10, 12]. Electron-impact mass spectrometry indicated a molecular ion of mass 268 with an interpretive peak at *m/z* 253, representing the loss of a methyl group. Chemical-ionization mass spectrometry, similarly, indicated a parent ion of mass 268. These findings are in agreement with published mass spectrometric data for AR [13]. Finally, the compound had the identical retention time as AR produced from retinol, according to Dunagin, Jr. and Olson [9]. The absorption spectrum of the

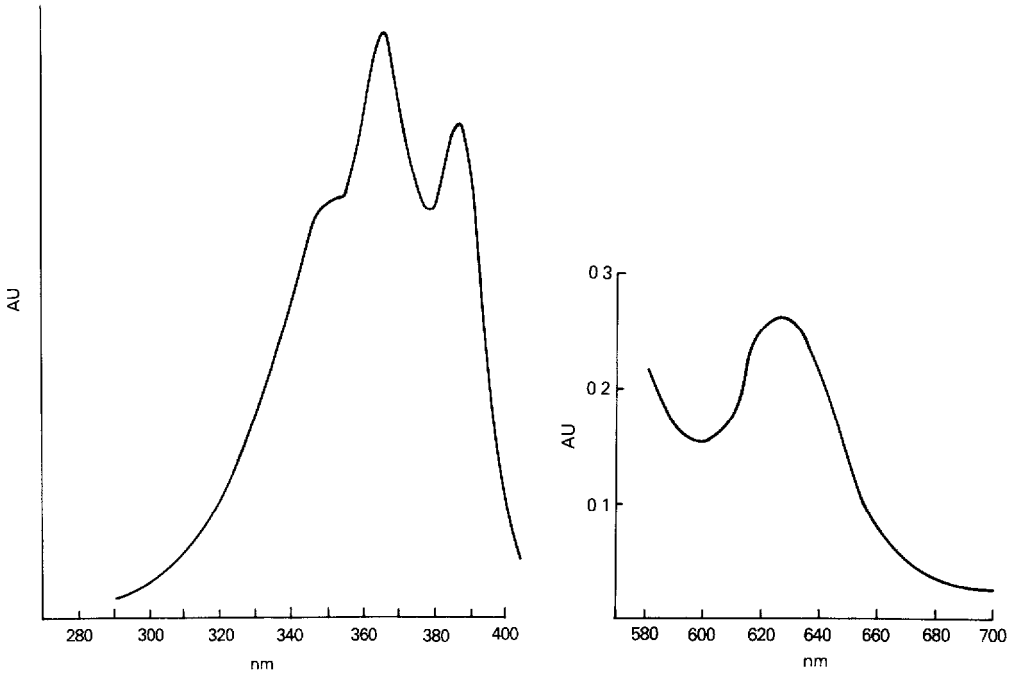

Fig. 1. Ultraviolet spectrum of the compound with a 3.0-min retention time. The absorbance of the compound eluting at 3.0 min was measured against methanol in 5-nm increments from 260 to 400 nm. The absorbance maxima at 387.5 and 367 nm were resolved to 0.5-nm increments.

Fig. 2. Antimony trichloride chromagen spectrum of material eluting at 3.0 min. The material eluting at 3.0 min was evaporated to dryness under nitrogen and reconstituted in chloroform. A 22% antimony trichloride in chloroform solution was added and the absorbance was measured against chloroform in 5-nm increments between 580 and 700 nm. The absorbance maximum at ca. 620 nm is characteristic of AR.

chromagen produced by reaction with antimony trichloride (Fig. 2) suggests AR over the vitamin A_2 isomer containing an additional unsaturation in the cyclohexene ring [10]. The typical yield of AR from retinyl acetate is only of the order of 15%, but the product is chromatographically pure. The acid—alcohol dehydration of retinol [9] followed by HPLC is better suited for production of large quantities of AR.

Utility of AR in HPLC assay of tissue total vitamin A

When various amounts of retinol were chromatographed, with a constant amount of AR, the ratios of peak height of retinol to AR were highly correlated (0.996) with the mass ratio of the compounds. Linearity was studied over a twenty-fold variation in mass ratio, and the regression line passed through the origin.

Table I shows the ratios of retinol to AR peak heights in mixtures of varying mass ratios of retinol to AR either injected directly or saponified and extracted prior to HPLC. The mean of the ratios in the processed samples was 1.04 ± 0.04 times that of samples directly injected, indicating stoichiometric recovery of these two compounds.

TABLE I

COMPARISON OF THE RATIOS OF RETINOL PEAK HEIGHT TO AR PEAK HEIGHT IN MIXTURES DIRECTLY INJECTED OR PROCESSED FOR TOTAL VITAMIN A

Mixture*	Peak-height ratio		Initial ratio**
	Initial	Final	Final ratio
5·25	0.66	0.64	1.03
15 25	2.53	2.34	1.08
30:25	4.91	4.73	1.04
45:25	7.21	6.65	1.08
5:40	0.54	0.50	1.08
10:40	0.96	0 93	1.03
20:40	1.90	1.85	1.03
40:40	3.72	3.77	0.99
60:40	5.56	5.61	0.99

*Volumes of retinol and AR (in μl) of two preparations.
**For all values, the mean ± S.D. of initial ratio to final ratio is 1.04 ± 0.04.

TABLE II

COMPARISON OF AR TO [^{3}H]RETINYL PALMITATE AS AN INTERNAL STANDARD FOR TOTAL VITAMIN A FROM LIVER HOMOGENATE

	Retinol* (ng)	AR* (ng)	Retinol (dpm)	Retinol, mass corrected**	
				By AR	By dpm
Mean***	368	31	2440	475	457
S.D.	34	4.3	80	33	57
C.V.	9.3	13.7	3.3	7.0	12.5

*Using an $A^{1\%}_{1\,cm}$ of 1835.
**The amounts of internal standards added to each replicate were 40.0 ng of AR and 3000 dpm of ^{3}H-retinyl palmitate.
***$n = 4$.

Finally, Table II shows retinol and AR peak heights and retinol-associated radioactivity from chromatograms of extracts of saponified rat liver homogenates to which AR and tritiated retinyl palmitate had been added. The coefficient of variation (C.V.) for the mass of retinol was 9.3%, less than that of AR (13.7%) but more than that for recovered radioactivity (3.3%). When the retinol mass was normalized by the AR peak height, the C.V. was 7.0%, whereas, if normalized to recovered radioactivity, the C.V. was 12.5%. Final recoveries of retinol and AR averaged 80 and 77%, respectively.

Typical chromatograms of retinol extracted from saponified homogenates of liver from a vitamin A-deficient rat and a vitamin A-replete rat are shown in Fig. 3a and b, respectively. The internal standard is baseline-resolved in both cases. Fig. 3c and d, similarly, depict chromatograms of tissue extracts from these rats, but without added AR, and demonstrate the lack of interfering compounds at 325 nm. Other tissues assayed for total vitamin A also yielded no

Fig. 3. Chromatograms of hepatic vitamin A in vitamin A-sufficient and vitamin A-deplete rats. Liver homogenates were assayed as described in the Experimental section: (a) vitamin A-replete, AR added prior to saponification, (b) vitamin A-deficient, AR added; (c) vitamin A-replete; (d) vitamin A-deplete Retinol elutes at the position denoted by the asterisk, and has a capacity ratio of 1.0; AR, in the position of the arrow, has a capacity ratio of 2.8 There is negligible absorbance at 325 nm at the retention time of AR when AR was not added to the homogenates.

interfering compounds. During assay of adrenal gland total vitamin A, occasionally an unknown peak eluting at 2.5 min, which interfered with the AR peak, was observed.

The ratios of absorbances at 340, 315, 300 and 280 nm to that at 325 nm were 0.78, 0.86, 0.56 and 0.20, respectively, for both authentic all-*trans*-retinol and for the retinol peak collected from assay of liver total vitamin A. Thus, it is unlikely that tissue compounds other than retinol interfere in the quantitative determination of vitamin A in this chromatographic system.

DISCUSSION

AR fulfils the criteria for its use as an internal standard in the HPLC assay of tissue total vitamin A. It is easy to prepare, it is stable during saponification and it extracts stoichiometrically with retinol from the saponification mixture. Ratios of peak heights of retinol and AR at 325 nm are linearly related to their mass ratios despite the considerable bathochromic shift in UV absorbance spectrum relative to retinol due to the additional double bond in conjugation. The early, distinct elution of AR in this isocratic system and the minimal instrumentation required make the assay quick and inexpensive, while retaining the specificity and sensitivity characteristic of HPLC.

AR has been used in this laboratory to quantify total tissue vitamin A in

numerous tissues of rats at various levels of vitamin A status [11]. The results suggest that it may be useful in the assay of vitamin A in kidney, ovary, intestine, eye, placenta and various tissues of gestational day-19 fetuses [11]. Others have reported information suggesting that AR is not a retinoid metabolite ordinarily found in intestine [4] or by the action of mammary gland microsomes [6], although its production was reported in spontaneously transformed fibroblasts [12]. We suggest that the use of AR provides greater precision in vitamin A assay and may be especially useful when low levels of tissue vitamin A are expected or where concern about the potential for sample handling losses is great.

Finally, the production of AR from retinyl acetate by chromatography on silica should serve as a reminder of the lability of retinoids during sample handling and preparation.

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