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Note

Separation of vitamin A compounds by thin-layer chromatography

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Several problems exist in the separation of vitamin A compounds. They are readily oxidized when exposed to air, are thermally labile, and are very light-sensitive[1]. Although methods including column chromatography on silica gel[2], partition and ion-exchange chromatography[3], high-speed liquid chromatography[4], gel chromatography[5], and gas chromatography[6,7] are employed in the separation of vitamin A compounds, thin-layer chromatography (TLC)[8–10] remains a convenient technique in terms of its simplicity and rapidity. However, none of the solvent systems used so far in TLC offers distinct one-dimensional separation of retinol and retinoic acid in the presence of retinal and the acetate and palmitate esters of retinol[8,9]. In addition, quantitation of various vitamin A compounds by TLC has been unsatisfactory[8–10].

MATERIALS AND METHODS

Chemicals

All chemicals, except those mentioned below, were obtained from Fischer Scientific (Fair Lawn, N.J., U.S.A.) and were analytical-reagent grade. They were used without further purification. Siliclad was purchased from Clay Adams (Parsippany, N.J., U.S.A.). The following *all trans* vitamin A compounds were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.): vitamin A acetate, retinol, retinal, and retinoic acid. Vitamin A palmitate was a gift from Hoffmann-La Roche (Nutley, N.J., U.S.A.), and butylated hydroxytoluene (BHT) was a gift from Ashland Chemicals (Columbus, Ohio, U.S.A.). The solvent system employed for TLC was acetone–light petroleum (b.p. 35.8–56.5°) (18:82, v/v).

Equipment

All glassware was soaked overnight in 2 *N* sodium hydroxide solution. After thorough rinsing with distilled water, the glassware was siliconized by immersion for

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1 min in a 1% solution of Siliclad in water and then rinsed several times with distilled water and dried for 3 h at 150°.

Pre-coated 0.25-mm-thick silica gel 60 F_{254} plates (5 cm × 20 cm) from EM Labs. (Elmsford, N.Y., U.S.A.) were employed. The plates were activated at 110° for 20 min, cooled to room temperature, then sprayed with diethyl ether containing 50 μg BHT/ml and left to dry in air.

Other equipment included: Büchi rotary evaporator (Brinkmann, Westbury, N.Y., U.S.A.); 5-μl microcapillaries (Brinkmann); cylinderical 23 cm × 6 cm developing chambers (Brinkmann); Whatman No. 1 filter paper (V.W.R. Scientific, Columbus, Ohio, U.S.A.); metabolic shaker (V.W.R.); Pyrex culture test tubes (16 mm × 125 mm) with screw caps (V.W.R.); ultraviolet (UV) lamp equipped with short (254 nm) and long (366 nm) wavelengths (Ultra-Violet Products, San Gabriel, Calif., U.S.A.); a spectrophotometer equipped with a digital readout (Gilford, Oberlin, Ohio, U.S.A.); and a double-beam scanning spectrophotometer (Carey, Monrovia, Calif., U.S.A.).

Procedure

A known amount of each vitamin A compound, varying from 1.5 to 2.5 mg/ml, was dissolved in chloroform–methanol (1:1) containing 50 μg BHT per millilitre. The compounds were spotted on the TLC plates either individually or in mixtures of known concentrations varying from 7.5 to 12.5 μg of each compound in a volume of 5 μl. The points of application were marked at 2 cm from the bottom edge of the plate, and the adsorbent layer was scored across the plate at a distance of 16 cm above the points of application. A blank lane was marked for use as a control in the quantitative determination.

The developing chambers were lined with Whatman No. 1 filter paper and allowed to equilibrate for 1 h with 25 ml of the developing solvent [acetone–light petroleum (18:82)]. The plates were placed in the developing chambers immediately after application of the samples, and developed by the ascending method in the dark at 8°. Time of development varied from 40 to 45 min.

Identification of the spots

The compounds were located by their fluorescence or absorption under UV light (long-wave, 366 nm).

Quantitative determination

Each spot was scraped from the plate and extracted for 20 min in a culture test tube with 12 ml of chloroform–methanol (1:1) containing 50 μg BHT per milliliter with the aid of a metabolic shaker. An equivalent amount of silica gel was extracted by the same procedure from the blank lane.

Each extract was centrifuged for 10 min, filtered through glass wool, and evaporated to dryness at 30° in a rotary evaporator. The residue was redissolved in 3.5 ml chloroform–methanol (1:1) containing 50 μg BHT per milliliter and determined quantitatively at the appropriate wavelength using the Gilford spectrophotometer.

Percent recoveries of the individual compounds were determined by comparison to the appropriate standard curves. The Carey double-beam scanning spectrophotom-

eter was employed to determine the maximum absorbance at the appropriate wavelength of each vitamin A compound at various concentrations. All operations were carried out under dim light.

RESULTS AND DISCUSSION

Table I presents the R_F values of the *all trans* vitamin A compounds as well as the percent recovery after development. Complete separation of the vitamin A compounds with 75–94% recovery was achieved by this one-dimensional TLC method.

TABLE I

TLC SEPARATION OF VITAMIN A COMPOUNDS

Solvent: acetone–light petroleum (18:82).

All-trans vitamin A compound	*Spot as observed under UV light (366 nm)*	*R_F values**	*$\lambda_{max.}$ (nm)*	*Extinction coefficient*** *($E^{1\%}_{1cm}$)*	*Recovery (%)*
Retinol palmitate	Bright yellow fluorescence	0.71 ± 0.009	332	758	94
Retinol acetate	Bright yellow fluorescence	0.54 ± 0.013	329	1385	75
Retinal	Dull black absorbance	0.40 ± 0.015	328	1275	84
Retinol	Bright yellow fluorescence	0.26 ± 0.003	328	1434	83
Retinoic acid	Dull black absorbance	0.21 ± 0.005	360	1257	80

* Mean value (± S.D.) of 15 observations.

** Compounds were dissolved in chloroform–methanol (1:1) containing 50 μg BHT per millilitre.

Although acetone–light petroleum (6:94) was used by John *et al.*[9] and 9–25% acetone in light petroleum was examined in preliminary experiments in the present study, the best separation of the five *all trans* vitamin A compounds without tailing was achieved with acetone–light petroleum (18:82).

The present method is simpler than that of Varma *et al.*[8] who employed a two-dimensional TLC system to separate retinoic acid from retinol. Furthermore, our procedure allows quantitation of vitamin A compounds, which was not possible by the TLC method employed by John *et al.*[9].

In the present method, the TLC plates were sprayed with diethyl ether containing BHT, an antioxidant which serves to reduce the oxidative decomposition of vitamin A compounds[11–16] and other pigmented natural products[17] that would otherwise occur during chromatography. Since BHT does not absorb UV light above 300 nm, it did not interfere with detection of the spots on the TLC plates nor with quantitation of the compounds following extraction from the silica gel.

CONCLUSIONS

The method described is a simple, rapid, and reproducible TLC procedure for the one-dimensional separation of *all trans* vitamin A compounds: retinol and its acetate and palmitate esters, retinal, and retinoic acid. The solvent system [acetone–light petroleum (18:82)] allows the complete separation of retinol and retinoic acid in the presence of retinal and the acetate and palmitate esters of retinol. This separa-

tion is not achieved with other solvent systems previously reported[8,9]. The recovery of various vitamin A compounds by our method was highly reproducible and varied between 75 and 94% depending on the compound.

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