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Note

Separation of vitamin A and retinyl esters by reversed-phase high-performance liquid chromatography*

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Vitamin A is important in testicular development, maintenance of spermatogenesis and regulation of epithelial differentiation[1]. Retinoic acid can support testosterone biosynthesis in rat testes, but not spermatogenesis[2]. Therefore, the metabolism of vitamin A and the biological activity of its metabolites have become a subject of considerable interest and methods to separate the various metabolites of vitamin A are continually being developed and improved. Careful use of high-performance liquid chromatography (HPLC) has made possible the efficient separation and detection of natural retinoids and their metabolites without significant isomerization and/or degradation of these compounds. A number of geometric isomers of retinoic acid and methyl retinoate have been separated by reversed-phase HPLC[3]. Frolik *et al.*[4] developed a reversed-phase system which separated retinoic acid, retinol, retinal and retinyl acetate. Recently, another reversed-phase HPLC separation of polar retinoids and retinyl esters was reported using a single, isocratic elution with a three step, discontinuous gradient and a high solvent flow-rate, but without baseline separation of retinal[5]. Further, the isolation, separation and identification of carotenoids and retinoids have been summarized in a recent rewiew[6]. In the present report, we describe a simple, reversed-phase procedure for the separation of retinoic acid, retinol, retinal, retinyl acetate and retinyl palmitate. The direct application of this procedure to study the metabolism of all-*trans*-[11-^{3}H]retinyl acetate in rat testis is demonstrated.

MATERIALS AND METHODS

Chemicals

All-*trans*-retinoic acid and all-*trans*-[11-^{3}H]-retinyl acetate (1.41 Ci/mmole) were obtained from Dr. W. E. Scott, Hoffmann-La Roche (Nutley, NJ, U.S.A.) and Chemoprevention Program, Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, MD, U.S.A.), respectively. Retinol, retinal, retinyl acetate, butylated hydroxytoluene (BHT) and retinyl palmitate were purchased from Sigma (St. Louis, MO, U.S.A.). Glass-distilled residue-free solvents (Burdick and

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Jackson Labs, Muskegon, MI, U.S.A.) were used for extractions and HPLC. The water used was deionized, filtered over charcoal and glass distilled.

High-performance liquid chromatography

HPLC was performed on a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatography system consisting of a Model 660 solvent programmer, a Model 440 absorbance detector, Model 6000 A solvent delivery systems and a 6-port sample injector (Valco, Houston, TX, U.S.A.). A reversed-phase octadecylsilane (ODS) column was used (Partisil PXS, 10/25, ODS-3, 10 μm, Whatman, Clifton, NJ, U.S.A.). The column (25 cm × 4.6 mm I.D.) was protected by a guard column packed with ODS, 30–39 μm, 7 cm × 2.1 mm I.D. The column was eluted with acetonitrile–1% ammonium acetate (70:30, v/v) at a flow-rate of 1.0 ml/min for 40 min, then a linear gradient to 98% acetonitrile for 15 min followed by a 65 min elution with acetonitrile–1% ammonium acetate (98:2 v/v) at a flow rate of 1.5 ml/min.

All-*trans*-[11-^{3}H]retinyl acetate was purified on a 25 cm × 4.6 mm I.D. Partisil PXS, 10/25, ODS-2 column (Whatman) using a methanol–0.01 *M* acetic acid (85:15, v/v) mixture at a flow-rate of 1.0 ml/min (retention time, 60 min).

Preparation of testicular sample for analysis

Male Sprague-Dawley Holtzman rats (Charles River Breeding Labs, Wilmington, MA, U.S.A.) weighing 400–450 g were used. The rats were lightly anaesthesized with diethyl ether and the purified all-*trans*-[11-^{3}H]retinyl acetate (1 μCi in 50 μl of ethanol/testis) was injected into the testes. Rats were killed by cervical dislocation under light diethyl ether anaesthesia 6 h post injection. Testes were removed, rinsed with ice cold 0.9% NaCl, and after removing the tunica albuginea, the testes were immediately homogenized in five volumes of cholorform–methanol (2:1, v/v) containing 50 μg/ml BHT, using a Sorvall Omni-Mixer (Sorvall, Newton, CT, U.S.A.). Homogenates were filtered under vacuum on a Buchner funnel through Whatman No. 1 filter paper and a 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). The samples were concentrated under a stream of nitrogen, dissolved in a known volume of methanol, an aliquot was taken and the radioactivity was counted.

A portion of the sample, usually $8 \cdot 10^4$–$10 \cdot 10^4$ dpm, was used for the separation of retinyl acetate metabolites by HPLC. All operations with retinyl acetate were carried out under gold light or in the dark.

Determination of radioactivity

Fractions (1 min) were collected from the column in 7-ml vials and 4 ml of scintillation fluid (Insta-gel, Packard, Downers Grove, IL, U.S.A.) was added to each vial. The radioactivity was measured and corrected for quenching with a Packard Model PO-1 PRIAS liquid scintillation spectrometer.

RESULTS

Retinoic acid, retinol, retinal, retinyl acetate and retinyl palmitate were chromatographed individually or as a mixture by reversed-phase HPLC using acetonitrile–1% ammonium acetate as the mobile phase and Partisil PXS, 10/25, ODS-3 column. A typical HPLC profile is shown in Fig. 1. This chromatogram was

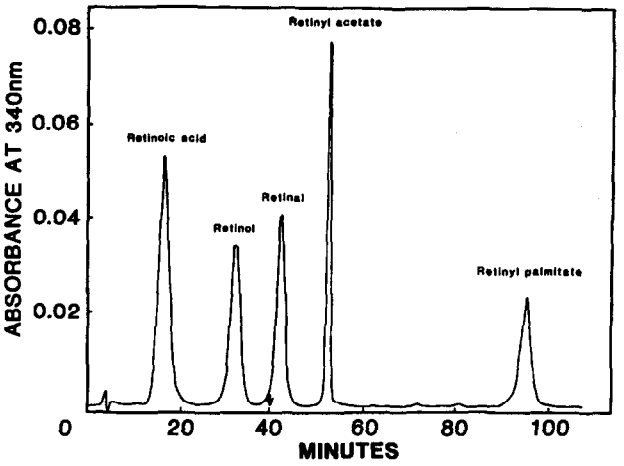

Fig. 1. HPLC profile of a mixture of retinoic acid, retinol, retinal, retinyl acetate and retinyl palmitate separated on a reversed-phase Partisil PXS 10/25 ODS-3 (25 cm × 4.6 mm I.D.) column and with acetonitrile–1% ammonium acetate (70:30, v/v) at a flow-rate of 1.0 ml/min for 40 min, then a 15 min linear gradient to 98% acetonitrile at a flow-rate of 1.5 ml/min. The quantity of each standard retinoid was 1.5 μg, sensitivity 0.1 a.u.f.s., at 340 nm. Changes in flow-rate (1.5 ml/min) and start of the gradient are indicated by arrow (↓).

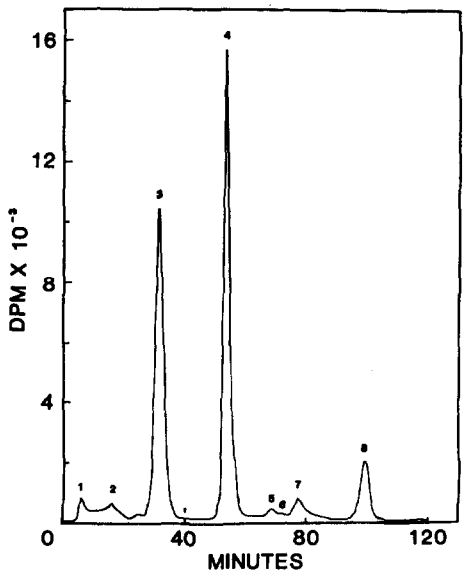

Fig. 2. HPLC profile of metabolites of all-*trans*.[11-^{3}H]retinyl acetate. The testicular metabolites were separated by HPLC as described in the legend of Fig. 1. Purified all-*trans*.[11-^{3}H]retinyl acetate (1 μCi) was injected into each testis in 50 μl of ethanol and rats were killed at 6 h. The peaks are: (1) polar metabolite; (2) retinoic acid; (3) retinol; (4) retinyl acetate; (5–7) unknown, and (8) retinyl palmitate.

obtained from a mixture of 1.5 μg each of the standard retinoid at 0.1 a.u.f.s. Each retinoid had a distinct retention time and was clearly separated from the other. The recovery of all vitamin A derivatives, based on radioactivity, was greater than 90%. In the metabolic studies, the testicular extract was separated by reversed-phase HPLC (Fig. 2). Eight radioactive peaks were observed. The average percent distribution of radioactivity of three individual experiments in the chromatographic fractions as labeled in Fig. 2 after 6 h were: (1) 3.3%; (2) 3.0%; (3) 37.5%; (4) 38.1%; (5) 1.4%; (6) 1.8%; (7) 5.5%; and (8) 9.4%. At 6 h the highest amount of radioactivity was in retinyl acetate, but it is evident that some was hydrolyzed to free retinol and some was esterified to retinyl palmitate. No retinal was detected.

DISCUSSION

The primary objective of this study was to develop an improved procedure for the simple, rapid, reliable separation of natural retinoids and retinyl esters. The separation of vitamin A and its esters has been previously reported by other workers[4–6]. Taylor[6] obtained reasonably good separation of retinoic acid, retinal, retinol and retinyl palmitate. However, the retinoic acid peak had a trailing edge. Polar metabolites would elute extremely close to or overlap with retinoic acid or would elute in the void volume. The method developed, utilizing reversed-phase HPLC, offers the advantages of high resolution, low solvent volume and high sensitivity. The system gives quantitative recoveries with little or no production of artifacts, a necessity for any study of retinoid metabolism. Furthermore, it is capable of separating some of the polar metabolites which elute from the column prior to retinoic acid. The system has wide applicability and with minor changes in solvent composition, it is possible to separate a wide range of natural retinoids, including isomers, short-chain, long-chain and polyunsaturated fatty acid esters of retinol. The flow-rate is fairly low (1 ml/min) and thus, use of a large volume and variety of solvents and number of steps is avoided.

As shown in Fig. 2, in addition to free retinol, retinyl acetate and retinyl palmitate peaks, a retinoic acid peak was also present, indicating the *in vivo* conversion of retinol into retinoic acid in rat testis. It was further noted that the amount of free retinol was greater than the retinyl palmitate: a major retinol ester of rat testis seminiferous tubules[7] whereas in liver, retinol is present predominantly as retinyl palmitate[8].

In conclusion, a reversed-phase HPLC separation procedure has been developed which can be of great use in the separation and identification of natural retinoids and their metabolites.

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