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GRADIENT REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NATURALLY OCCURRING RETINOIDS

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SUMMARY

A gradient reversed-phase high-performance liquid chromatographic technique is described for the facile separation and quantitation of the naturally occurring retinoids: retinoic acid, retinol, and retinyl esters. An octadecylsilane column (Waters μBondapak C_{18}) is used, with gradient elution from methanol—water (80:20) (solvent A) to 70% or 100% methanol—tetrahydrofuran (50:50) (solvent B) at 2.0 ml/min; detection is by absorbance at 325 nm. Analysis can be completed, with return to starting conditions, in 25—30 min. The method is inherently flexible: retinyl esters can be eluted as a group, with little resolution, by gradient to 100% solvent B, or mostly resolved by gradient to 70% solvent B; separation of retinoids more polar than retinoic acid can be achieved by use of greater proportions of water in solvent A. The separation of vitamin A compounds from extracts of human, rat, and pig liver and from rat kidney by this technique is described.

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INTRODUCTION

Vitamin A and its derivatives (retinoids) occur in nature as compounds having a wide range of polarities, from the very non-polar vitamin A esters to retinoic acid and its water-soluble derivatives (retinoyl β-glucuronide, retinotaurine, etc.). Because of this wide range of polarities, the analysis of retinoids in biological tissues provides an excellent example of the "general elution problem" in liquid chromatography, the separation in a single chromatographic run of compounds having a range of polarities. Previous workers [1—7] have used reversed-phase high-performance liquid chromatography (HPLC) with a variety of isocratic, discontinuous step, and gradient elution eluents to separate these retinoids, but these techniques seemed to us either to lack resolution, to lack flexibility, or to require long analysis times. We propose here a gradient reversed-phase HPLC system which provides good resolution among most of the classes of retinoids found in biological tissues, is readily adapted to suit the analysis of retinoids required, and is relatively rapid.

MATERIALS AND METHODS

Gradient reversed-phase HPLC

Retinoids were analyzed by gradient reversed-phase chromatography on a 10-μm octadecylsilane column (μBondapak C_{18}, 30 cm × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.) preceded by a guard column (8 cm × 2 mm I.D.) packed with Co:Pell ODS (Whatman Chemical Separation, Clifton, NJ, U.S.A.). Isocratic elution with solvent A, methanol—water (80:20) for 5 min at 2.0 ml/min was followed by a linear gradient to an appropriate percentage (70% or 100%) of solvent B, methanol—tetrahydrofuran (50:50) over a 10-min period; isocratic elution with this final solvent composition continued for 5—10 min, in order to elute retinyl esters. A linear gradient over 5 min back to solvent A restored the system for the next sample. Reagent grade, filtered solvents (0.5-μm Fluoropore filters, Millipore, Bedford, MA, U.S.A.) were used. Waters Assoc. Model 6000A and M45 pumps were controlled by a Waters Model 660 solvent programmer; sample introduction was via a Waters Model U6K loop injector. Detection was by ultraviolet absorption at 325 nm (Model LC-75 detector, Perkin-Elmer, Norwalk, CT, U.S.A.) with quantitation by electronic integration (Model 3390A integrator, Hewlett-Packard, Palo Alto, CA, U.S.A.). A standard curve of integrator peak area versus microgram quantity of vitamin A (retinol and the retinyl component of retinyl palmitate) was prepared and confirmed by daily chromatography of standards. For additional confirmation of the identity and purity of retinoids eluted by this chromatographic system, a photodiode-array spectrophotometric detector (SPD-M1A, Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used to determine absorption spectra of individual peaks. All separations were conducted at ambient temperature (20—25° C).

Retinoid standards

All-*trans* retinoic acid, retinol, retinal, retinyl acetate, and retinyl palmitate were purchased commercially (Sigma, St. Louis, MO, U.S.A.); when necessary

they were purified by reversed-phase HPLC essentially as described above. [11,12-^{3}H] All-*trans* retinyl acetate was graciously provided by Hoffmann-La Roche (Basel, Switzerland). Anhydroretinol was prepared by acid-catalyzed dehydration of retinol [8]. The long-chain fatty acyl esters of retinol (i.e., retinyl myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate, and arachidate) were prepared by reaction of retinol with the corresponding acyl chlorides [9]. Synthetic 4-oxo-retinoic acid and retinoyl β-glucuronide were generously provided by Dr. Arun Barua. Solutions of standards were prepared in acetonitrile (to avoid hydrolysis and transesterification) containing 0.1% butylated hydroxytoluene (BHT).

Extraction of biological tissues

Female Sprague-Dawley-derived rats (Holtzman, Madison, WI, U.S.A.) were maintained on a vitamin A-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) supplemented with a daily oral dose of 100 μg retinyl acetate in corn oil. At six weeks of age each rat was given 0.57 μCi (26 μg) [11,12-^{3}H] all-*trans* retinyl acetate by intragastric infusion in corn oil. Seven days later the animals were sacrificed under diethyl ether anesthesia, and the liver and kidneys were removed, weighed, and frozen. For analysis, each tissue was first thawed and then ground thoroughly by mortar and pestle with 2—3 times its weight of anhydrous sodium sulfate, and then was extracted with dichloromethane [10]; the liver extract was diluted to 50 ml and the kidney extract diluted to 25 ml with dichloromethane. Aliquots of these extracts were added to 0.1 ml of 0.1% BHT in ethanol and evaporated under a gentle stream of argon, then dissolved in 0.1 ml of 2-propanol plus 0.05 ml dichloromethane for injection onto the chromatograph. Fractions (2 ml) of the HPLC effluent in 10 ml Biofluor liquid scintillation cocktail (New England Nuclear, Boston, MA, U.S.A.) were counted in a liquid scintillation counter (LS 7500, Beckman Instruments, Irvine, CA, U.S.A.); counts were corrected for efficiency of counting by use of an external standard.

A small portion (20 mg) of human liver obtained by needle biopsy (autopsy sample from a normal subject) was similarly extracted and the extract dissolved in 0.1 ml of 2-propanol [11]. Liver from a two-month-old pig was obtained at slaughter, and representative portions were analyzed similarly. All manipulations were carried out under yellow light (Westinghouse F40 Gold fluorescent lamps).

Saponification and acid-catalyzed dehydration of liver extracts

Small volumes (0.3 ml) of the dichloromethane extract of rat liver were evaporated just to dryness. For saponification of the sample, 1 ml of 10% methanolic sodium hydroxide was added and hydrolysis was allowed to proceed overnight at room temperature. After addition of 1 ml water, the non-saponifiable lipid was extracted with hexane; the hexane extracts were then evaporated and the residue was dissolved in 2-propanol for injection onto the chromatograph. For acid-catalyzed dehydration of the liver extract, the concentrated sample was dissolved in 1 ml of 10% ethanolic hydrochloric acid, overlaid with 1 ml hexane, and left overnight at room temperature [8]. After addition of 1 ml aqueous 1 *M* sodium hydroxide, the lipids were

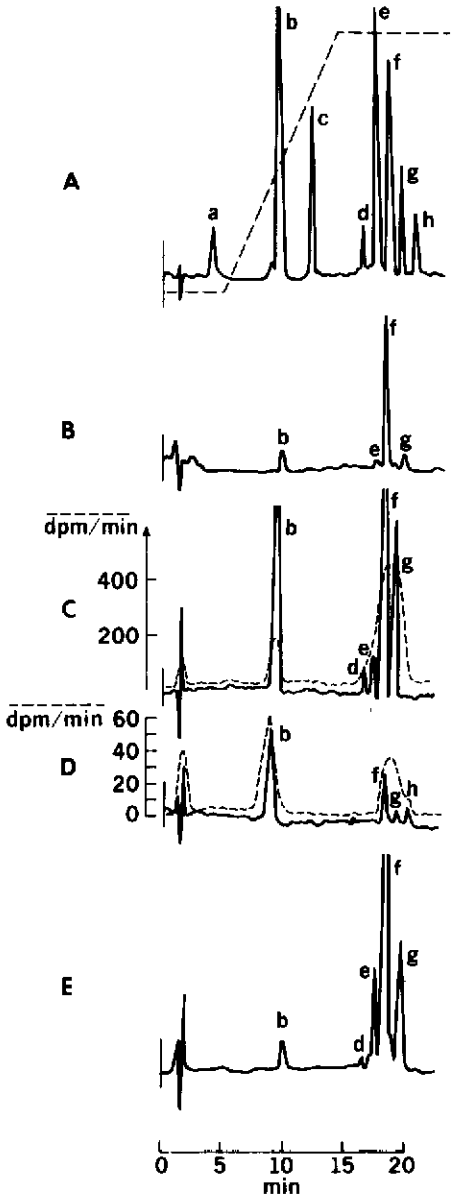

Fig. 1. Gradient reversed-phase HPLC of retinoids. (A) Analysis of retinoid standards. —, absorbance at 325 nm; - - - -, change of solvent composition from 0% solvent B to 70% solvent B. For other chromatographic conditions see Table I. (B) Analysis of vitamin A compounds in an extract of pig liver (20 mg sample). (C) Analysis of vitamin A compounds in an extract of liver from a rat given an oral dose of [^{3}H]retinyl acetate. A 2-ml aliquot of a total of 50 ml extract (equivalent to 0.4 g liver) was analyzed. —, Absorbance at 325 nm; - - - -, radioactivity. (D) Analysis of vitamin A compounds in an extract of kidneys from a rat given an oral dose of [^{3}H]retinyl acetate. A 10-ml aliquot of a total of 25 ml extract (equivalent to 0.96 g kidney) was analyzed. —, Absorbance at 325 nm; - - - -, radioactivity. (E) Analysis of vitamin A compounds in an extract of human liver (20 mg sample). Peaks: a = retinoic acid; b = retinol; c = retinyl acetate; d = retinyl linolenate; e = retinyl myristate plus retinyl palmitoleate plus retinyl linoleate; f = retinyl palmitate plus retinyl oleate; g = retinyl stearate; h = retinyl arachidate.

extracted with hexane, and the hexane extracts concentrated and dissolved in 2-propanol as described above.

RESULTS

Separation of retinoids

The separation of retinoid standards by this gradient reversed-phase HPLC system is shown in Fig. 1A. The major classes of naturally occurring retinoids are clearly separated: retinoic acid, retinol, and long-chain fatty acyl retinyl esters, thus allowing rapid quantitation of these classes of retinoids in biological tissue extracts. Gradient elution to 70% solvent B (i.e., to a final solvent composition of methanol—tetrahydrofuran—water of 59:35:6) allows resolution of most of the commonly occurring retinyl esters, with the following exceptions: retinyl myristate, palmitoleate, and linoleate were unresolved, as were retinyl palmitate plus oleate. Identity of these retinoids in biological tissue extracts was confirmed by their co-elution with authentic standards. Fig. 1 B—E shows the application of this chromatographic system to extracts of several biological tissues: porcine liver, rat liver and kidney, and human liver.

Quantitation of retinoids

Using the detector and integrator described above, the standard curve of peak area versus micrograms of vitamin A (retinol and the retinyl component of retinyl palmitate) was linear up to 5 μg, and curvilinear but usable up to 20 μg. Amounts less than 10 ng were not reproducibly detectable and quantifiable with this detector and integrator. Reproducibility was determined by repetitive injections of retinol and retinyl esters standards (Table I).

TABLE I

CHROMATOGRAPHIC CONDITIONS

Column	μBondapak C_{18} (octadecylsilane), 30 cm × 3.9 mm I.D.
Mobile phases	Solvent A: methanol—water (80:20) Solvent B: methanol—tetrahydrofuran (50:50)
Flow-rate	2.0 ml/min
Gradient conditions	5-min isocratic solvent A, followed by linear gradient over 10 min to 70% or 100% solvent B, held at this composition for 5—10 min; 5-min linear gradient back to solvent A
Detector wavelength	325 nm
Temperature	Ambient
Sensitivity	To 10 ng retinol or retinyl ester
Reproducibility (retinol and retinyl ester standards)	Within-day, standard error of mean = 2.7% (n = 5) Between-day, standard error of mean = 1.6% (n = 10)

Saponification and dehydration of liver extracts

After saponification of the liver extract, retinyl esters disappeared with a concomitant increase in the area of the retinol peak (Fig. 2B). Acid-catalyzed dehydration of liver extract, on the other hand, abolished the peak due to retinol, while giving rise to peaks having retention times similar to that of anhydroretinol, 14 min (Fig. 2C).

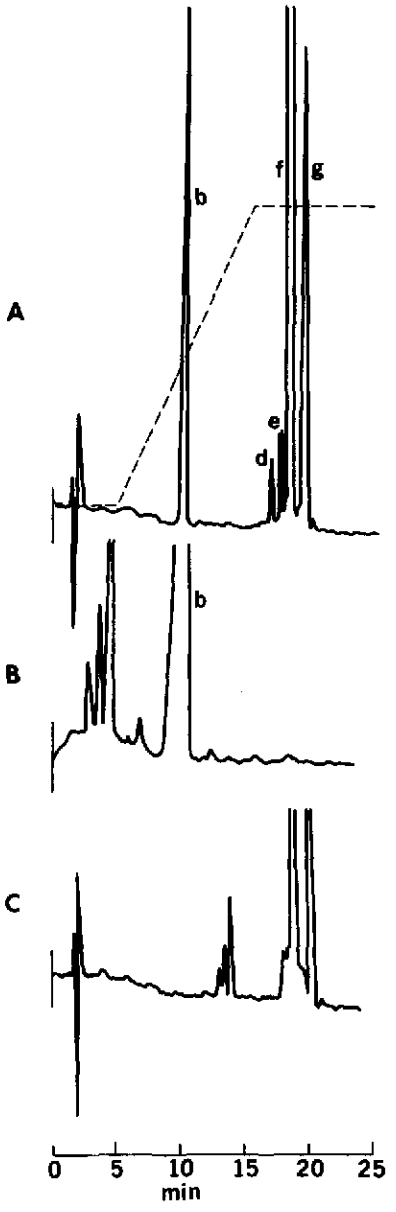

Fig. 2. Saponification and acid-catalyzed dehydration of liver extracts. (A) Gradient reversed-phase HPLC of rat liver extract (0.3 ml of 50 ml extract, equivalent to 0.06 g liver). (B) Analysis of saponified rat liver extract. (C) Analysis of acid-catalyzed dehydrated rat liver extract. Chromatographic conditions as in Fig. 1.

Three-dimensional recording of chromatogram

The three-dimensional recording of absorption—wavelength—time as given by the photodiode-array spectrophotometric detector (Fig. 3) shows the presence of other light-absorbing eluates, in addition to the retinoids. However, none of these (with the exception of carotenoids in the human liver extract, retention time 20.5 min) co-elutes with retinol or retinyl esters.

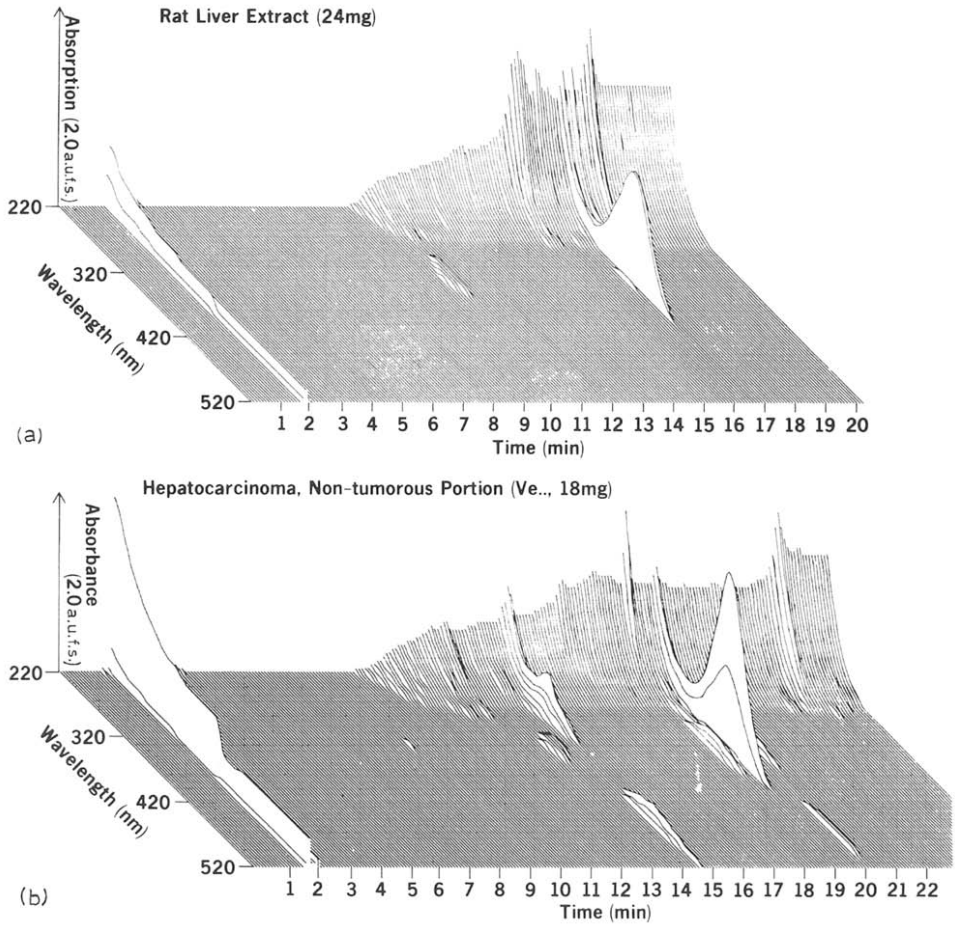

Fig. 3. Three-dimensional chromatograms of gradient HPLC separation of Vitamin A compounds from liver extracts. (A) Analysis of rat liver extract (equivalent to 24 mg liver). Isocratic elution for 5 min with 100% solvent A, followed by a linear gradient over 10 min to 100% solvent B, continued at this solvent composition for 5 min. Retention time for retinol, 10.7 min; retention times for long-chain retinyl esters, 16.4—17 min. (B) Analysis of human liver extract (18 mg liver). Isocratic elution for 5 min with 100% solvent A, followed by a linear gradient over 10 min to 70% solvent B, continued at this solvent composition for 10 min. Retention time for retinol, 10.9 min; retention times for long-chain acyl retinyl esters, 18.6—21.2 min; retention time for hydrocarbon carotenoids, 20.3 min.

Recovery of retinol and retinyl palmitate from HPLC

[^{3}H]Retinol and [^{3}H]retinyl palmitate from a rat liver extract were purified by gradient reversed-phase HPLC as described above. Aliquots of these fractions were re-chromatographed and the appropriate fractions collected and counted by liquid scintillation. The recoveries of radioactivity in retinol (two samples, 45 ng each) and retinyl palmitate (two samples, 230 ng each) averaged 96% (91—100%) and 102% (100—104%), respectively.

DISCUSSION

Tetrahydrofuran was chosen as the third component of this solvent system because it is completely miscible with water and with methanol, thus avoiding any problems of solvent compatibility. This provides a flexible chromatographic system: the solvent gradient can be readily run to 100% solvent B, methanol—tetrahydrofuran (50:50) for the rapid elution of retinyl esters, or to a less non-polar composition [e.g., to 70% solvent B, methanol—tetrahydrofuran—water (59:35:6)] for improved resolution of retinyl esters. If determination of retinol and retinyl esters, but not of retinoic acid, is desired, the initial isocratic portion of the elution solvent program may be omitted and the gradient begun with 30% solvent B, methanol—tetrahydrofuran—water (71:15:14). If, on the other hand, resolution of vitamin A metabolites more polar than retinoic acid is desired, a more polar initial solvent composition (with inclusion of a low concentration of salt to decrease peak tailing of ionizable retinoids [2, 3] and a longer gradient may be used. For example, using an immediate 15-min gradient from methanol—water (70:30), [containing 0.1% ammonium acetate], to methanol—tetrahydrofuran (50:50) the uncorrected retention times are: 4-oxo-retinoic acid, 4.1 min; retinoyl β-glucuronide, 7.9 min; retinoic acid, 9.0 min; retinol, 11.3 min; retinyl acetate, 12.7 min; retinyl palmitate, 16.5 min. Alternatively, the gradient elution system of Silva and DeLuca [12], which uses a solvent gradient from 10 m*M* aqueous ammonium acetate—methanol (98:2) to methanol, might be easily used as the initial gradient, with elution by methanol—tetrahydrofuran as the latter component of the solvent gradient.

Because *cis—trans* isomers of each of the retinoid classes are not well separated by this elution technique, all the geometric isomers of each class can be quantitated as a single peak. Retinal is not satisfactorially resolved from retinol by this column and solvent combination. However, quantitatively important levels of retinal have not been shown in biological tissues other than the eye [13]; thus the lack of resolution of retinal from retinol does not appear to be a serious handicap in most studies of vitamin A metabolism.

Although isocratic elution methods have been developed to give complete resolution of long-chain fatty acyl esters of retinol [14—16], the gradient elution method described here has the advantage of producing sharper peaks, thus facilitating quantitation of retinyl esters in small samples. Although several pairs of retinyl esters are not resolved by this technique (e.g., retinyl oleate co-elutes with retinyl palmitate, and linoleate and palmitoleate with myristate), the ester profile from any given tissue appears to be characteristic of that tissue: human liver retinyl esters appear to have a fatty acid composition similar to that of rat liver and pig liver, but decidedly different from that of rat kidney or rat mammary tissue (cf. Fig. 1; ref. 17).

This method owes its high specificity for vitamin A to the fact that few other compounds in biological tissues have appreciable light absorption at 325 nm, and to the fact that all of these interferences (except β-carotene) are well separated chromatographically from retinol and retinyl esters. This separation is dramatically demonstrated by the three-dimensional chromatograms (absorption—wavelength—time) of tissue extracts (Fig. 3). The saponification

procedure applied to a liver extract confirms that there were no other compounds present contributing to the absorbance of the retinyl esters, and the dehydration procedure demonstrates that no other compounds contributed to the absorbance peak attributed to retinol in this extract. Chromatographic analysis of liver extracts from a rat previously given [^{3}H]retinyl acetate showed that the radioactivity co-eluted with peaks identified on the basis of retention times as retinol and retinyl esters.

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