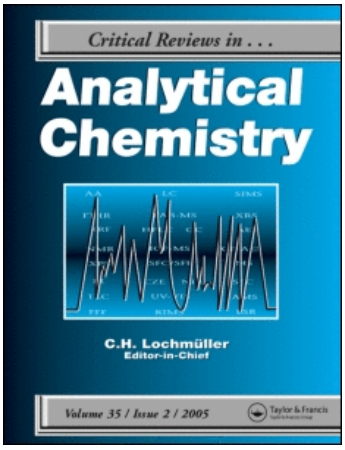

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF VITAMIN A COMPOUNDS

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I. INTRODUCTION

Vitamin A compounds, both natural and synthetic (collectively called retinoids), are important in the maintenance of the integrity of epithelial structures. The metabolism of natural retinoids such as retinol, retinal, retinoic acid, and retinyl esters has been studied extensively, and the metabolites have been determined by several techniques. The analytical techniques that were commonly used in the past were column chromatography, thin-layer chromatography (TLC), and gas liquid chromatography. However, the high-performance liquid chromatographic (HPLC) technique has proven to be by far the method of choice for the determination of retinoids. The determination of natural retinoids is facilitated by their inherent chemical properties, such as their intense absorption in the near ultraviolet (UV) region and the ability of several retinoids to fluoresce. These properties are often advantageous, particularly in the on-line determination of these compounds; an example is by UV in the HPLC technique. Since retinoids are unstable in the presence of light and air, special precautions must be taken in their extraction from and determination in biological materials.

In this review, we present several aspects of the methodology involving the extraction, separation, and quantitation of natural retinoids with special reference to the use of modern HPLC technique for the determination. We also describe briefly the other major chromatographic methods that were used in the past and are still being used for the determination of retinoids.

II. PREPARATION OF REFERENCE STANDARDS

Pure reference retinoids are important both for standardizing various chromatographic systems as well as for use in determining the recoveries of the retinoids during extraction from tissues. Authentic reference compounds such as *all-trans* retinol, *all-trans* retinal, and *all-trans* retinoic acid are available commercially. Retinyl esters with long and short chain fatty acids can be prepared synthetically by published procedures.[1] A mixture of isomers of

retinaldehyde and retinoic acid can be prepared by irradiation of appropriate *all-trans* retinoid and subsequent purification by HPLC. The *cis-isomer* of retinol can be prepared by reduction of the corresponding *cis* retinaldehyde by sodium borohydride. The concentrations of the retinoids of interest should be determined spectrophotometrically using the extinction coefficients reported by several investigators,[1,2] with either ethanol (for retinol, retinal, retinoic acid) or hexane (for retinyl esters) as the solvent.

A. Solubilities in Various Solvents

The solubility properties of naturally occurring retinoids in various solvents are of prime importance in selecting the right solvent for their extraction from tissues as well as for preparation for HPLC. Unfortunately, due to the diversity in the polarity of the metabolites of retinol, no single solvent has yet been described for the extraction of polar and nonpolar metabolites of retinol. Roberts et al.[3] have studied the solubilities of retinol and retinyl esters in various solvents and solvent mixtures. They have found that increased amounts of chloroform in mixture with methanol resulted in maximal solubilization of both retinol and retinyl esters. However, for polar metabolites of retinol and retinoic acid, absolute methanol appears to be the solvent of choice for solubilization.[2]

B. Storage

Standard retinoids in powder form should be stored at −70°C in a desiccator. Precaution should be taken when warming the compounds from −70°C to room temperature. Retinol in the powder form absorbs moisture; therefore, the vials containing retinol should be opened under dry conditions. Solutions of the retinoids can be stored under nitrogen in degassed, distilled methanol (polar retinoids) or in hexane (retinyl esters). Radioactive retinoids can also be stored under similar conditions at −70°C for at least 6 months.[4]

Since retinoids are not stable, it is advisable to separate and quantitate by HPLC on the same day they are extracted. However, if the determination cannot be conducted on the same day that the biological materials are collected, they must be frozen and stored at −70°C. Retinoids are stable[5] in the frozen liver. No differences in the concentrations of retinoids in liver were noted, for example, over a 6-month period.[6]

III. HANDLING, STORAGE, AND ANALYSIS

Since all retinoid compounds are photolabile in normal light, all operations should be carried out in a room with yellow light (ceiling lights, preheat rapid-start bug-a-ways 40 watt, T12 bulb, medium bipin base, Westinghouse). Retinoid compounds are relatively very stable under this light.

The handling and storage of biological materials for the determination of retinoids have been presented in detail.[5] Plasma or serum can be stored in brown plastic tubes at −70°C without degradation of retinoids. However, if retinol, retinyl esters, and the polar metabolites of retinol are being measured, it is advisable to store the tissues at −70°C under nitrogen after lyophilization to prevent the possible hydrolysis of retinyl esters. In the case of cells and plasma, lyophilization of the materials is not recommended. Lyophilization produces decomposition products which can be demonstrated by HPLC.

IV. EXTRACTION AND PREPARATION OF TISSUES FOR CHROMATOGRAPHY

Several methods have been reported[7-10] for the extraction of retinoids from tissues. However, an extraction method must be evaluated for recovery of the retinoid of interest before selecting the method for use in an experimental study. Also, the extraction method selected

should take advantage of the solubility characteristics of the retinoid being investigated. For example, retinol and its esters can be extracted easily from tissue homogenates with anhydrous ether,[11,12] but retinoic acid cannot be quantitatively recovered using this solvent.[3] Similarly, a chloroform:methanol extraction method, first developed by Bligh and Dyer,[7] will effectively extract retinyl acetate into the organic phase but will leave many of its metabolites in the aqueous layer.[2]

The direct extraction of lyophilized tissues originally reported by Ito et al.[2] appears to be the best general method for the complete extraction of a wide variety of retinoids with minimal production of artifacts. The method consists of homogenizing the tissues at 4°C in two parts water containing 1 mg of ascorbic acid or *n*-propylgallate/ml as an antioxidant and lyophilizing the resulting homogenate. The lyophilized tissues can then be extracted with a number of different organic solvents, all containing butylated hydroxytoluene (100 μg/ml), with the choice of the solvent depending on the polarity of the retinoids of interest.[3,13,14] Recently, Bhat and Lacroix[1] reported the complete extraction of polar and nonpolar metabolites of retinol from the lyophilized tissues using 99% methanol in water and then with hexane solvents. As mentioned earlier, the lyophilization method is not suitable for the measurement of retinoids in plasma. Therefore, when measuring levels of retinoids in plasma, the modified method of Thompson et al.[15] could be used. Other solvents used for the extraction of retinol and its esters from serum include petroleum-ether:dichloromethane:isopropanol,[16] hexane:methylenechloride:isopropanol,[17] and ethanol:0.01 *M* phosphate buffer, pH 5.4.[18] Although the lyophilization has been used in the extraction of retinoid metabolites from cells, a recent report[19] indicates nonspecific breakdown of retinoids during the extraction from certain types of cells. The direct extraction of cells with butanol/acetonitrile originally reported by McClean et al.[20] is suitable for the extraction of retinoid metabolites from embryonal carcinoma cells.

The final extract can be evaporated to dryness using a rotary evaporator and redissolved in an appropriate solvent for HPLC. Absolute methanol has been used to solubilize the residue and as a final solvent for HPLC for the determination of polar retinoids. When simultaneously measuring polar and nonpolar retinoids present in the tissue extracts, chloroform:methanol (1:1) was found to be suitable to prepare the extract for injection into HPLC. Several solvent mixtures[3] have been reported to dissolve the final residue of tissue extract containing retinol and retinyl esters.

V. SEPARATION BY CONVENTIONAL METHOD

Conventional chromatography with materials such as alumina, silicic acid, and Sephadex LH-20 and TLC have been used for the separation and quantitation of a variety of retinoids. In addition, ion-exchange chromatography,[21,22] chromatography with a calcium phosphate matrix,[23,24] and paper chromatography have often been used. In all of the above techniques, the retinoids were determined either by UV absorption (310 to 350 nm) or fluorescence.

A. Alumina Chromatography

Alumina chromatography has been used extensively to separate retinol from its esters.[25-27] This technique is also recommended for the initial separation of retinol isomers from their esters.[28] It has also been reported to be capable of separating retinaldehyde from retinoic acid.[8,29] The method uses water-deactivated alumina as an adsorption matrix. The retinoids are eluted with 5% ethyl ether in hexane, 15% benzene in hexane, 2% acetone in hexane (retinyl esters), or 3% dioxane in hexane (retinyl palmitate and retinal).[30-32] Recoveries of retinol and retinyl esters from the column vary from 85 to 96%.[29,32] However, the method does not allow the separation of some retinyl esters nor of the polar metabolites of natural retinoids.[33,34]

B. Silicic Acid Chromatography

Silicic acid adsorption chromatography uses heat-activated silicic acid. The retinoids are eluted from the column with various amounts of diethyl ether in Skelly B (2 to 20% v/v) and 20% methanol in diethylether. The retinoids that have been separated using this method were retinol, retinaldehyde, and retinoic acid and its more polar metabolites; recoveries ranged from 60 to 100%,[35-37] but extensive breakdown and production of artifacts of retinoids may occur.[38,39] Therefore, silicic acid chromatography is no longer recommended for the separation of retinoids.

C. Sephadex LH-20 and DEAE-Cellulose Chromatography

Ito et al.[2] first reported the application of liquid-gel partition chromatography to the separation of a spectrum of natural retinoids (solvent system: chloroform — skelly solve B — methanol in the proportion 65:35:0). They reported quantitative recovery of retinyl esters, retinaldehyde, retinol, and retinoic acid with little or no breakdown. Since then, Sephadex LH-20 chromatography has been employed in a number of studies involving the separation of retinoic acid metabolites before injection into HPLC.[40,41] The limitation of this technique is its inability to separate various retinyl esters from each other and retinaldehyle[2,42] and the isomers of retinoids.

DEAE-cellulose chromatography has been used for the separation of charged metabolites of retinol such as retinoic acid and the phosphate ester of retinol from the glycosylated form.[43,44] A gradient of ammonium acetate (1 to 50 m*M*) in 99% methanol has been used to elute these retinoids from the column. Recently, this method has been used for the initial separation of isomers of retinoic acid, before separation by HPLC.[28]

D. Thin-Layer Chromatography (TLC)

Most of the TLC in vitamin A research has been performed on silica gel plates, although alumina-coated plates have been used.[29,44a,45] The mobile phase generally consists of a high percentage (85 to 97%) of a nonpolar solvent (hexane, petroleum ether, benzene, cyclohexane) and a small percentage (3 to 15%) of a more polar solvent (ethylether, acetone, chloroform, ethyl acetate, or methyl heptenane). After separation, the retinoids can be detected by exposing the plate to iodine vapors or charring with strong acids. Specific methods such as fluorescence under UV light or spraying with a saturated solution of antimony trichloride in chloroform or with trifluroacetic or trichloroacetic acid have been used widely.[46]

The application of TLC to the separation of a variety of retinoids has been reported. This includes separation of (1) retinol isomers;[47] (2) the retinyl esters, retinol, retinaldehyde, retinoic acid, and anhydroretinol;[35,48,49] (3) polar metabolites of retinoic acid;[22,35,50] and (4) phosphate esters of retinol and retinoic acid.[44,51] Furthermore, TLC is still being used for a quick check of radiochemical purity of retinoids.[52] Although the separation of retinoids by this technique is rapid, extensive destruction of certain retinoids on the TLC plate has been reported.[52,53]

VI. HPLC

HPLC is highly recommended for the separation, identification, and quantitation of retinoids. Several review articles describing this technique have been published.[5,31,54,55] HPLC has several advantages: (1) rapid and nondestructive determination at room temperature, (2) highly reproducible separation after several injections, (3) separation of closely related compounds such as isomers of retinol and retinoic acid, (4) quantitative recovery of retinoids from the column, and finally (5) high sensitivity with capability of determining nanogram levels of many retinoids.

A variety of column packing materials and mobile phases have been used for vitamin A determination. For normal-phase chromatography, silica packings are generally used. In the reverse-phase mode, most separations are performed on octadecyl columns. The mobile phase for normal phase chromatography is usually *n*-hexane or ether containing low percentage of a more polar solvent such as *n*-propanol, methylene chloride, or dioxane. In reverse phase chromatography, a mixture of water or buffers with alkanols or acetonitrile are the most common eluents.

Due to characteristic absorption of retinoids in the UV region, the retinoids can be determined by on-line UV absorption (310 to 350 nm). The determination limit with UV absorption is generally greater than 2 ng. The second method of determination depends on the fluorescence of certain retinoids. By using a 470-nm emission filter with excitation at 325 nm, retinol and its esters can be easily determined at levels less than 1 ng of retinol. Retinaldehyde and retinoic acid, however, show only weak fluorescence at these wavelengths. Radioactively labeled retinoids can be measured by the collection of fractions from the column and measuring the radioactivity in a standard scintillation counter or by using a flow-through counter.

A. Normal- and Reverse-Phase HPLC

Normal-phase HPLC has been successfully employed for the separation of closely related retinoids that are not highly polar, such as isomers of retinol, retinyl esters, or retinaldehyde (Table 1). As is apparent from Table 1, silica is the most popular adsorbent used in this type of HPLC. The active sites on the silica surface (the "silanol") are weakly acidic, and therefore, basic substances are strongly retained or are irreversibly adsorbed. Care should be taken not to exceed pH 9, as the silica may start to dissolve. Water should be eliminated from the mobile phase when underivatized silica is used as a normal-phase packing, as silica may crystallize. The major problem with the normal-phase HPLC for the determination of retinoids is the gradual decrease in column efficiency because of the irreversible adsorption of lipids present in the biological materials to the column matrix.[56] Therefore, prepurification of the biological materials is essential before applying the extract to the column.[28]

Some of the problems associated with silica columns can be overcome by using bonded-phase column packing with nitrile group. These packings are compatible with both the normal- and reverse-phase modes and are less sensitive to the interfering lipids.[4] Normal-phase chromatography is suitable for the separation of a variety of isomers of vitamin A (Table 1).

Reverse-phase HPLC is perhaps the most popular technique for the separation of polar and nonpolar retinoids. This type of HPLC has several advantages over normal-phase HPLC: (1) it is not sensitive to the presence of water or lipids in the biological materials. Therefore, tissue extracts can be applied directly to the column without prepurification; (2) unlike normal-phase HPLC, in reverse-phase HPLC solvent gradients that cover a range of polarity can be used and the system takes a relatively short time to re-equilibrate with the mobile phase; (3) in reverse-phase HPLC, the more polar compounds elute from the column first, whereas in normal-phase chromatography, a strongly polar metabolite would bind strongly to the silica.

Systems used in the reverse-phase HPLC for the determination of many retinoids are summarized in Table 2. Reverse-phase HPLC is widely used for the study of the metabolism of retinoic acid to its polar metabolites. The selection of the column and the mobile phase depends upon the type of retinoid of interest. Since the retention behavior of a solute on a reverse-phase system depends on hydrophobic interaction, a variety of bonded-phase packing material can be used for the separation of retinoids.

B. Separation of Natural Retinoids

The separation of retinoids having a wide range of polarity such as retinoic acid, retinol,

Table 1
CHROMATOGRAPHY OF RETINOIDS ON NORMAL-PHASE SYSTEMS

Retinoid	Packing material	Eluent composition	Ref.
Retinol isomers	Micropak Si 10	Petroleum ether: dichloromethane: isopropanol (80:19.3:0.7 v/v)	77
	Zorbax CN + partisil ODS (in series)	*n*-Hexane: 2-octanol (99%)	4
	Zorbax SIL	*n*-Hexane:ethylacetate: dichlromethane (83.2:7.5:9.3)	79
	Si 60	*n*-Hexane: dioxane (95:5)	56
	Si 60	*n*-Hexane: methylethylketone (90:10)	80
	μ Porasil	*n*-Hexane: ether (80:20)	59
	Zorbax SIL	*n*-Hexane: dichloromethane:isopropanol (300:200:1.5)	81
Retinal isomers	Zorbax SIL	*n*-Hexane: diethylether (88:12)	82
	μ Porasil	Petroleum ether: diethylether (98:2)	83
	μ Bondapak CN	Hexane: ether (99:1)	85
	Si 60	*n*-Hexane: dioxane (97:5:2.5)	56
	Sperisorb	Petroleum ether: diethylether (90:10)	86
	μ Porasil	Gradient, hexane: diethylether (99.6: 0.4 to 80:20)	59
	Jasco pack Silica	Petroleum ether: diethylether (92:8)	87
	Shimadzu Silica	*n*-Hexane-diethylether (88:12)	88
	μ Porasil	*n*-Hexane: diethylether (98:2)	84
Retinyl ester isomers	μ Porasil	Hexane: diethylether (98:2)	89
	Corasil	*n*-Hexane: dioxane (99.9:0.1)	90
	Si 60	*n*-Hexane: dioxane (99.9:0.1)	56
	Si 60	Hexane: methyl *t*-butylether (99:1)	91
	Lichrosorb aloxt	Isooctane-diisopropylether (98:2)	65
Retinoic acid metabolites (isomers)	Partisil	Dichloromethane: glacial acetic acid (99.5:0.5)	9
	Partisil	Hexane: tetrahydrofuran (88:12)	92
	Partisil	Hexane: 2 propanol (99.5:0.5)	93
	RSIL	Petroleum ether: acetic acid acetonitrile (99.5:0.3:0.2)	94
	Zorbax NH_2	Acetonitrile: methylenechloride (90:10 + 10 m*M* acetic acid)	28
Retinyl phosphate and mannosyl retinyl phosphate	μ Porasil	Gradient, *n*-hexane: isopropanol: water (42.9:57.1:0 to 39:52:9)	95

retinal, and retinyl esters is best achieved by reverse-phase HPLC. A partisil ODS column appears to be suitable for the separation of a mixture of natural retinoids (Table 1). The typical separation of four retinoids on a Partisil ODS-2 column is shown in Figure 1. In this system, the retinoids are eluted from the column with acetonitrile:1% ammonium acetate (80:20); the application of reverse-phase HPLC to the metabolites of retinol in experimental animals was clearly demonstrated by Bhat et al.[72] (Figure 2 and Figure 3). However, complete separation of retinyl esters was not achieved on this column. Recently, the separation of natural retinoids, including a variety of retinyl esters, has been achieved on a Zorbax TMS column (Figure 4). This HPLC method can be applied to study the metabolism of retinol in tissues (Figure 5). However, a gradual decrease in the efficiency of a Zorbax TMS column has been observed due to the accumulation of lipids. This will sometimes decrease the retention time of retinyl esters. Nevertheless, the method seems to be ideal for study of the metabolites of retinol, such as retinoic acid and retinyl esters, in the tissues after the administration of radioactive retinol. This HPLC system will separate these metabolites of retinol.

Table 2
CHROMATOGRAPHY OF RETINOIDS ON REVERSE-PHASE SYSTEMS

Retinoid	Packing material	Eluent composition	Ref.
Mixture of natural retinoids	Partisil ODS-2	Acetonitrile: 1% ammonium acetate (80:20)	67
	Partisil ODS	Step gradient acetonitrile: water (55:45) to acetonitrile: water (98:2)	68
	Partisil ODS	Step gradient acetonitrile: 10 m*M* potassium phosphate (pH 7.2) (37:63 to 54:46) to acetonitrile: water (98:2)	60
	Zorbax TMS	Step gradient acetonitrile: 10 m*M* ammonium acetate (55:45) to acetonitrile: water (90:10)	96
	μ Bondapak C_{18}	Methanol: 0.01 *M* sodium acetate (80:20)	61
Retinol and retinyl esters	Ultrasphere ODS	Methanol: water (98:2)	1
	Supelcosil LC-8	Step gradient acetonitrile: water (88:12 to 98:2)	73
	Vydac ODS	Acetonitrile: water (65:35)	81
	Permaphase ODS	Gradient, water to methanol	97
	Permaphase ODS	Isopropanol: ethanol: water (56:16:28)	98
	μ Bondapak C_{18}	Methanol: water (95:5)	99
	Zorbax ODS	Acetonitrile: methylenechloride (70:30)	100
	RISL C_{18}	Methanol 58.9 × 10^{-3} *M*[Ag+]	77
Retinoic acid and metabolites	Partisil 10 ODS	Methanol: water (71:29)	52
	μ Bondapak C_{18}	Methanol: 0.01 *M* ammonium acetate (71:29)	61
	Partisil ODS 2	Gradient, acetonitrile: 1% ammonium acetate (2:98 to 75:25)	67
	Sperisorb ODS	Gradient, acetonitrile: 1% ammonium acetate (25:75 to 55:45)	101
	Lichrosphere	Methanol: water: acetic acid (89.7:10:0.2)	77
	Partisil ODS	Methanol: 0.02 *M* ammonium acetate (50:50) to methanol :0.10 *M* ammonium acetate (90:10)	102
	Partisil ODS-2	Methanol: water (80:20)	74
	Partisil ODS-2	Methanol: water (90:10)	103
	Spherisorb ODS	Acetonitrile: 1% ammonium acetate (48:52)	104
	Lichrosorb C_{18}	Acetonitrile: 1% ammonium acetate (80:20)	18
	μ Bondapak C_{18}	Methanol: 10 m*M* ammonium acetate (60:40)	55
	Spherisorb ODS	Acetonitrile: 0.01 *M* ammonium acetate (80:20, pH 6.7)	101
Retinyl phosphate and mannosyl retinyl Phosphate	Partisil ODS	Acetonitrile: 1% ammonium acetate (55:45)	68
	Partisil ODS	Acetonitrile: 10 m*M* phosphate buffer pH 7.2 (37:63)	60

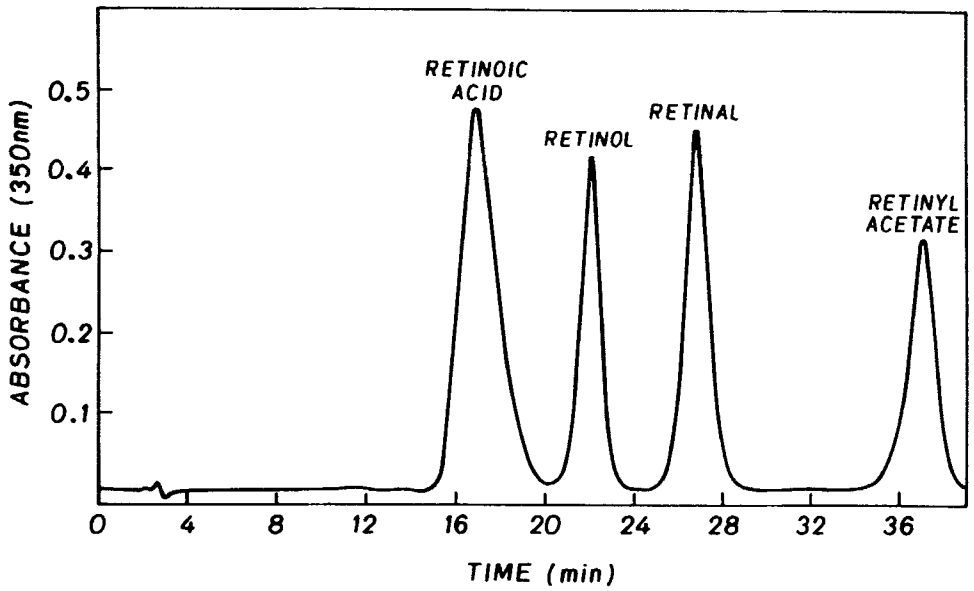


FIGURE 1. Chromatogram of four standard retinoids. Column, partisil-10-ODS-2, 25 cm × 4.6 mm i.d.; mobile phase, acetonitrile : 1% ammonium acetate (80: 20); flow rate, 1.1 ml/min. (From Frolik, C. A., Tavela, T. E., and Sporn, M. B., *J. Lipid Res.*, 19, 32, 1978. With permission.)

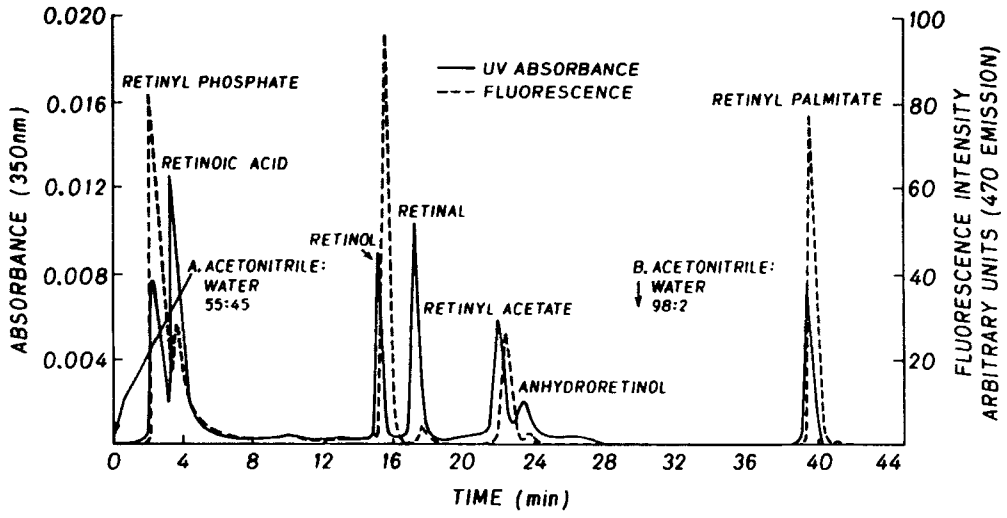


FIGURE 2. Reverse-phase HPLC separation of four standard retinoids. Column, partisil-10-ODS-2, 25 cm × 4.6 mm i.d.; eluting solvent, acetonitrile: water (55/45) containing 1% ammonium acetate and acetonitrile: water (98:2). (From Bhat, P. V., De Luca, L. M., and Wind, M. L., *Anal. Biochem.*, 102, 243, 1980. With permission.)

A modification of this method was recently reported by Sundaresan[57] in which baseline separation of retinol, retinyl acetate, and retinyl palmitate was successfully achieved on Zorbax TMS column (4.6 mm i.d. × 15 cm) employing an isocratic mobile phase of acetonitrile: water (85:15) (Figure 5C). This rapid HPLC method was applied to quantitate retinol and retinyl esters (mainly retinyl palmitate) in liver as well as in serum or plasma of the rat.

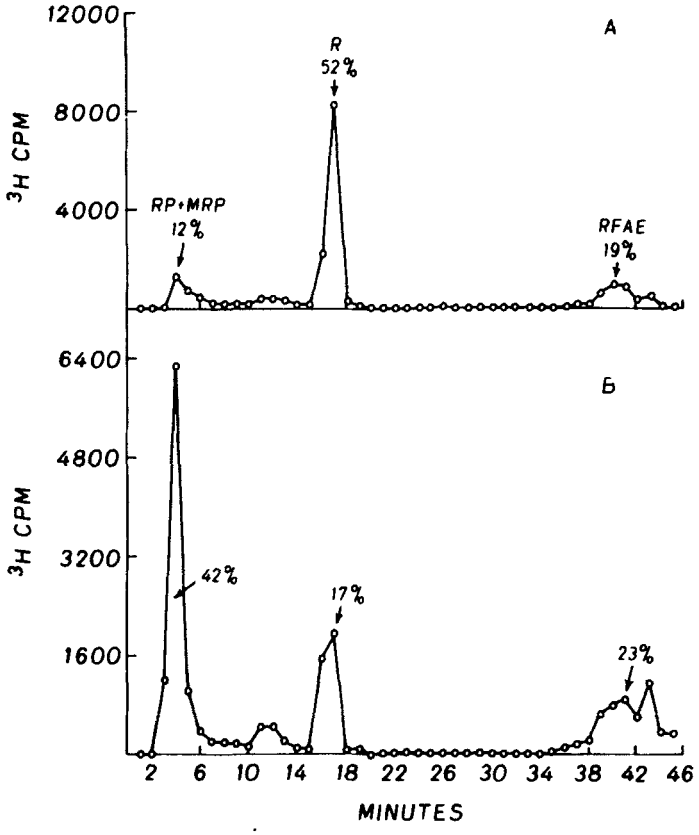


FIGURE 3. (A) Reverse-phase HPLC of the mouse intestinal metabolites of [1-^{3}H] retinol. This pattern was obtained 3 h after the intubation of the label. (B) Reverse-phase HPLC of the mouse intestinal metabolites of [1-^{3}H] retinol. This pattern was obtained 24 h after the intubation of the label. RP: retinyl phosphate; MRP: mannosyl retinyl phosphate; R: retinol; RFAE: retinyl fatty acyl esters; column and other conditions same as in Figure 2. (From Bhat, P. V., DeLuca, L. M., and Wind, M. L., *Anal. Biochem.*, 102, 243, 1980. With permission.)

C. Chromatography of *cis-trans* Isomers of Retinol, Retinal, Retinoic Acid, and Retinyl Esters

The isomers of retinoids can be separated by normal-phase chromatography (Table 1). The four isomers of retinol (11-*cis*, 13-*cis*, *all trans*, and 9-*cis*) can be separated on Zorbax CN and Partisil ODS columns connected in series, using two solvent systems (Figure 6). The advantage of this system is the use of bonded phase packing which allows relatively rapid column re-equilibration when a change in mobile phase is required. This HPLC method has been successfully applied to the study of *cis-trans* isomerization of retinol in the biological systems. However, clean-up of the tissue extract on the alumina column has been recommended before separation by HPLC.[28] Although use of the silica column has been reported for the separation of isomers of retinol, its application to the isomers present in the tissue extract has not been described.[58,80]

Normal-phase HPLC has been used exclusively for the separation of a variety of isomers of retinaldehyde. Since enzymatic *cis-trans* isomerization of retinaldehyde occurs in the visual cycle, most of the published methods on the separation of retinal isomers were developed for the study of *cis-trans* isomerization in the eye. The common solvent mixture used in the separation of retinal isomers consists of *n*-hexane or petroleum ether with a low

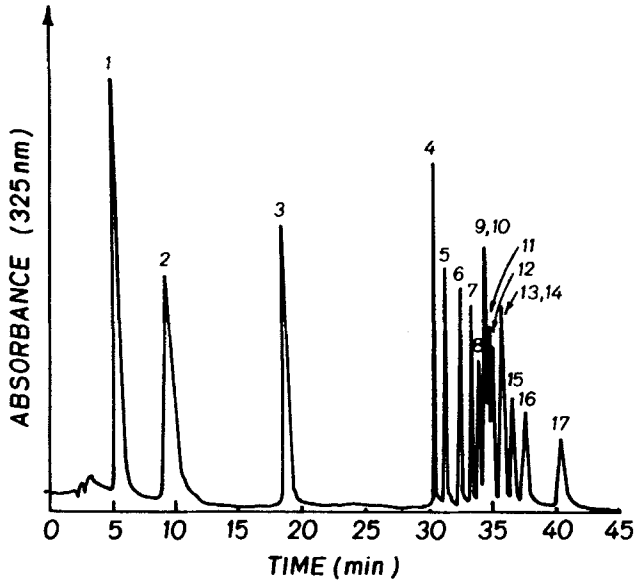


FIGURE 4. HPLC separation of standard retinoids on Zorbax TMS column, 25 cm × 4.6 mm i.d. Peaks 1: 5,6-epoxy retinoic acid; 2: *all-trans* retinoic acid; 3: *all-trans* retinol; 4: retinyl propionate; 5: retinyl hexanoate; 6: retinyl decanoate; 7: retinyl laurate; 8: retinyl linolinate; 9: retinyl myristate; 10: retinyl palmitoleate; 11: retinyl linoleate; 12: retinyl pentadecanoate; 13: retinyl palmitate; 14: retinyl oleate; 15: retinyl heptadecanoate; 16: retinyl stearate; 17: retinyl arachidonate. (From Bhat, P. V. and La croix, A., *Fed. Proc.*, 44, 771, 1985. With permission.)

percentage of more polar modifiers (Table 1). An example of a baseline separation of four isomers of retinal is illustrated in Figure 7. In this system hexane:ether (87.5:12.5) was used as the mobile phase, and the separation of retinal isomers was achieved on a μ-porasil column. The same investigators[59] reported the chromatography of retinal oximes on Micropak Si-5 with *n*-hexane: dioxane (90:10) and on Lichrosorb 10 μ with *n*-hexane: dioxane (90:10) (Table 1). Derivatizing retinal with hydroxylamine gives the quantitative extraction of retinal in the form of retinal oxime from the tissues without any alteration of isomeric configuration. However, the formation of *syn-* and *anti*-retinal oximes of each isomer poses a problem for the quantitative determination of individual isomers of retinal.

There are several reports in the literature on the separation of isomers of retinoic acid.[60-62] Most of these studies describe the separation of 13-*cis* from that of *all-trans* retinoic acid. The methylesters of isomers of retinoic acid are better separated than the underivatized retinoic acid on the reverse-phase system.[63] The separation of several isomers of methyl retinoate obtained after the irradiation of *all-trans* methyl retinoate on a Partisil ODS-2 column eluted with methanol:water (85:15) is shown in Figure 8. However, the application of this method to biological systems has not been described. Recently, Bhat and Lacroix reported[28] a simple and rapid separation of 11-*cis*-, 13-*cis*-, *all-trans*-, and 9-*cis*-retinoic acid on a Zorbax NH_2 column (Figure 9). The method allows the study of *cis-trans* isomerization of retinoic acid in tissues (Figure 10). However, the authors suggested that the tissue lipid extracts be cleaned up on a DEAE column before applying them to the Zorbax NH_2 columns.[28]

Few reports describe the separation of some of the fatty acid esters of retinol isomers.[64,65] The separation of isomers of retinyl esters has been studied by Alvarez et al.[64] A typical

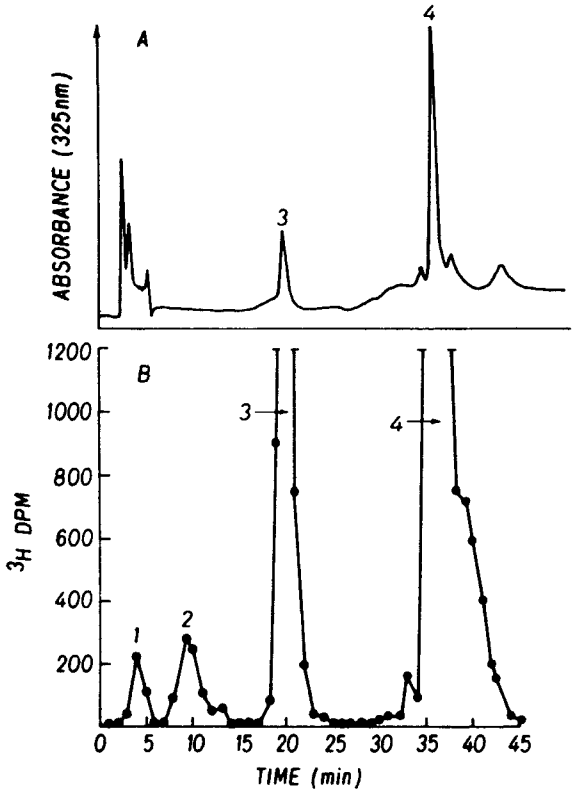


FIGURE 5. (A) HPLC analysis of vitamin A metabolites from liver of the rat maintained on vitamin A-deficient diet. The rat was sacrificed at 9 d after the injection of 11 μCi (157 μg) of [11, 12-^{3}H] retinol. Detection of ultraviolet-absorbing peak at 325 nm. (B) Radioactive metabolites formed from [11,12-^{3}H] retinol. Peaks 1: a mixture of polar metabolites of retinol; 2: *all-trans* retinoic acid; 3: *all-trans* retinol; 4: mixture of retinyl esters. (From Bhat, P. V. and Lacroix, A., *Fed. Proc.*, 44, 771, 1985. With permission.) (C). Chromatogram of a standard mixture of retinol, retinyl acetate, and retinyl palmitate. Zorbax TMS column (15 cm × 4.6 mm i.d.). Mobile phase, isocratic acetonitrile: water (85: 15). Flow rate, 1.2 ml/min. Sample size, 80 to 250 ng in 20 μl of chloroform: methanol (1:1). (From Sundaresan, P. R., *Fed. Proc.*, 44, 545, 1985. With permission.)

separation of 13-*cis*, 11-*cis*, 9-*cis*-, and *all-trans*- retinyl stearate is shown in Figure 11. Again, the separation was possible on a normal-phase HPLC system (5 μ ultrasphere Si). The authors have successfully applied this technique to the determination of retinyl esters in ocular tissues. The 11-*cis* isomer of retinyl esters was present in significant amounts only in ocular tissues. The application of HPLC methods to the separation of complex mixtures of retinyl esters in tissues other than the eye has not been described.

In working with isomers of vitamin A, many precautions should be taken in the isolation, separation, and derivatization for their chemical characterization. As mentioned earlier, vitamin A derivatives undergo extensive isomerization under fluorescent light and heat; there is always a possibility of nonspecific isomerization. For example, mild derivatization procedure should be used for preparation of methyl esters of retinoic acid. Gentle treatment with an ethereal solution of diazomethane at 4°C is sufficient to quantitatively methylate retinoic acid without any nonspecific isomerization of the compound. Since normal-phase

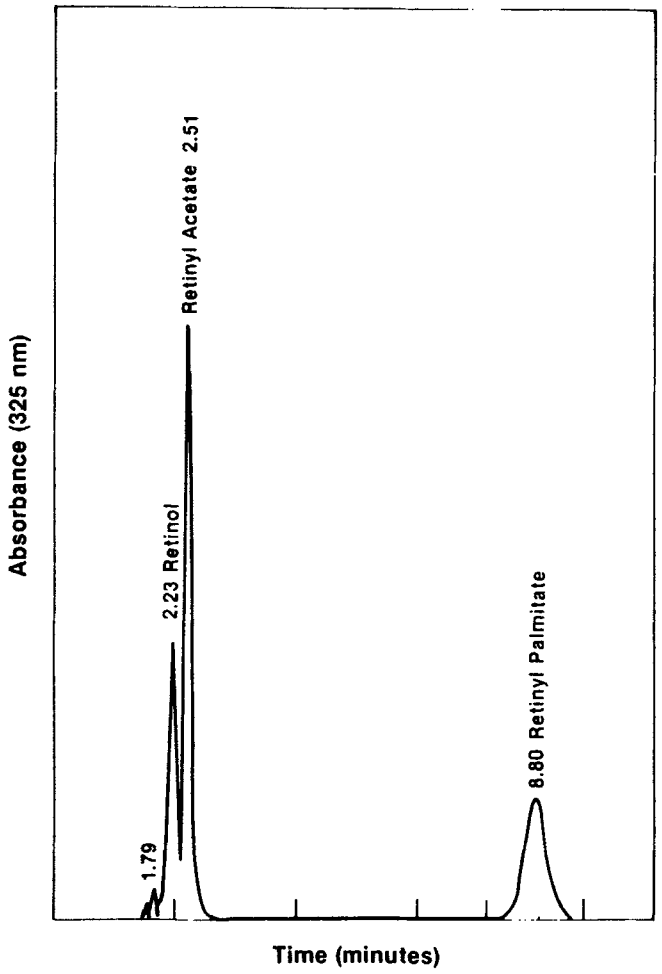


FIGURE 5C

HPLC is commonly used for the separation of a variety of isomers of vitamin A, use of a precolumn or sample purification by another mild technique, such as by an alumina, DEAE, or Sephadex LH-20 column, is highly recommended. If radiolabeled precursors of vitamin A are used for the study of *cis-trans* isomerization, it is advisable to analyze the tissue lipid extract containing the radiolabeled isomers with "cold" standard isomers of the appropriate retinoids.

D. Separation of Polar Metabolites

A number of reverse-phase HPLC systems are available for the separation of polar metabolites of retinol, particularly for studying the metabolism of retinoic acid. Many HPLC column packing materials (Table 2) are available. The separation of retinoids for the study of the *in vivo* metabolism of [11^{3}H] retinyl acetate to polar metabolites in liver tissue is illustrated in Figure 12. Frolik et al.[5] used a reverse-phase HPLC method for the study of tritiated retinoic acid conversion to 4 hydroxy- and 4 keto-retinoic acid in hamster tracheal organ culture (Figure 13). Several investigators have used an HPLC technique for the purification of the metabolites of retinoic acid for structural studies using either mass spectrometry or nuclear magnetic resonance spectroscopy.[40,41,66]

The bonded-phase column materials used in the determination of polar metabolites of

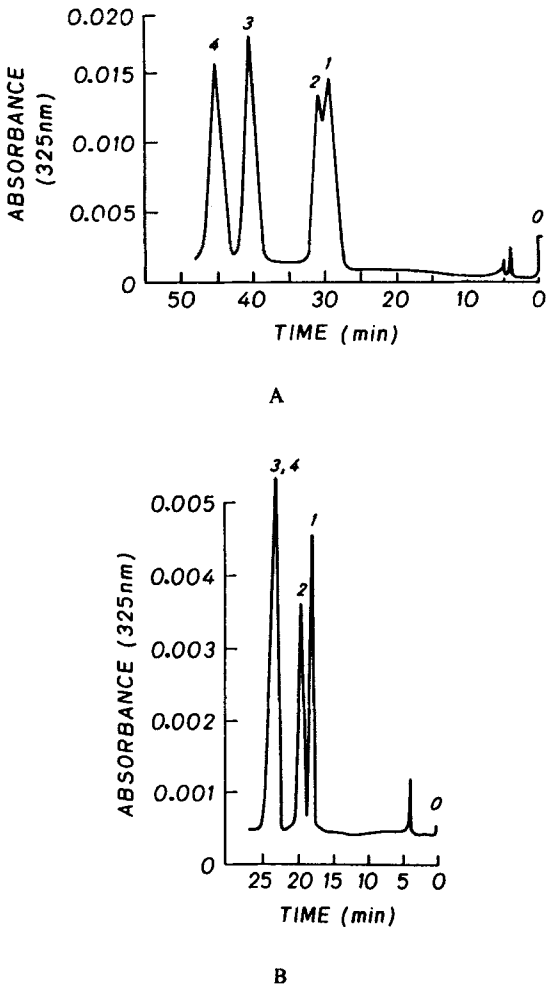


FIGURE 6. (A) Chromatogram of a standard mixture of *cis-trans* isomers of retinol. Partisil-10-QDS (25 cm × 4.6 mm i.d.) and Zorbax CN 5 μm (25 cm × 4.6 mm i.d.) columns connected in series. Mobile phase, 1% of 2 octanol in hexane. Flow rate, 2.0 ml/min. Sample size, 400 to 500 ng in 20 μl of methanol. Peaks 1: *11-cis*-retinol; 2: *13-cis*-retinol; 3: *9-cis*-retinol; and 4: *all-trans*-retinol. (B) Separation of standard mixture of four isomers of retinol. Conditions same as in Figure 6A except the composition of the mobile phase, 5% dioxane in hexane. (From Bhat, P. V., Co, M. T., and Lacroix, A., *J. Chromatogr.*, 260, 129, 1983. With permission.)

retinol and retinoic acid are octadecylsilane, octyl-silane, and trimethylsilyl-silane, a mixture of either acetonitrile: water or methanol: water has been used as the mobile phase. In addition, ammonium acetate has been used as an ion-suppressor of charged metabolites, which reduces the tailing of these compounds and also often facilitates the selective separation of closely related compounds by increasing the retention times.[67,68]

E. Quantitation of Different Species of Retinyl Esters

Earlier methods for the separation of retinyl esters either by reverse-phase paper chromatography[29,33,69,70] or by TLC[48,71] were abandoned due to breakdown and up to 50% losses of retinyl esters.

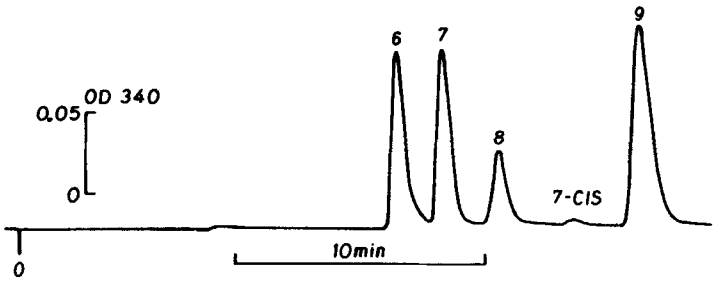


FIGURE 7. Isocratic chromatogram of five retinal isomers present in an irradiated solution of *all trans*/retinal in ethanol. Column, μ porasil, 25 cm × 4.6 mm i.d.; mobile phase, ethyl ether in hexane (12.5%, v/v); λ = 310 nm; flow = 0.6 ml/min.; peaks 6, *13-cis*; 7, *11-cis*; 8, *9-cis*; 9, *all-trans*. (From Bridges, C. D. B., Fong, S. L., and Alvarez, R. A., *Vision Res.*, 20, 355, 1980. With permission.)

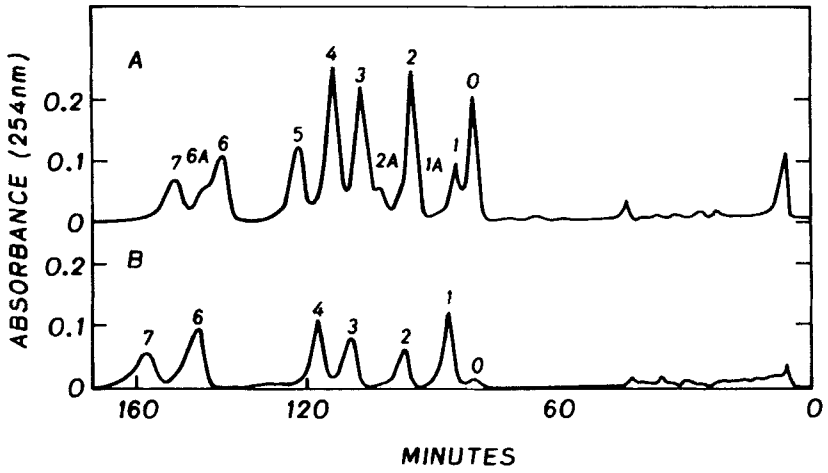


FIGURE 8. Chromatogram of an isomerate resulting from irradiation for 56 h of methyl *all-trans* retinoate in (A) dimethyl sulfoxide and (B) heptane. Column, Partisil-10-ODS-2 (10 μm; 25 cm × 4.6 mm i.d.); mobile phase, methanol: water (85: 15); flow rate 0.7 ml/min; peak (0), *13-cis* (photocyclized) methyl retinoate; peak (1), *9, 11, 13-tri-cis* methyl retinoate; peak (2), *11, 13-di-cis* methyl retinoate; peak (2A), *7, 13-di-cis* methyl retinoate; peak (3), *13-cis* methyl retinoate; peak (4), *9, 13-di-cis* methyl retinoate; peak (5), *11-cis* methyl retinoate; peak (6), *9-cis* methyl retinoate; peak (6A), *7-cis* methyl retinoate; peak (7), *all-trans* methyl retinoate. (From McKenzie, R. M., Hellwege, D. M., McGregor, H. L., Rockley, N. L., Riquetti, P. J., and Nelson, E. C., *J. Chromatogr.*, 155, 379, 1978. With permission.)

It is only recently, because of interest in the synthesis and utilization of complex short- and long-chain fatty acid esters of retinol in liver tissue and other organs,[72,73] that new HPLC methods were devised to separate and quantitate the retinyl esters in biological materials. Although several HPLC methods have been described for the separation of retinyl esters,[1,74,75] the quantitative application of the method to tissue extracts has been limited. De Ruyter and De Leenheer reported[76] an HPLC system capable of resolving individual long-chain esters of retinol on a reverse-phase column containing octadecylsilane groups as the bonded-phase with methanol as the mobile phase. However, the separation of retinyl oleate from retinyl palmitate was not achieved in this system. Later, using the same system, they were able to

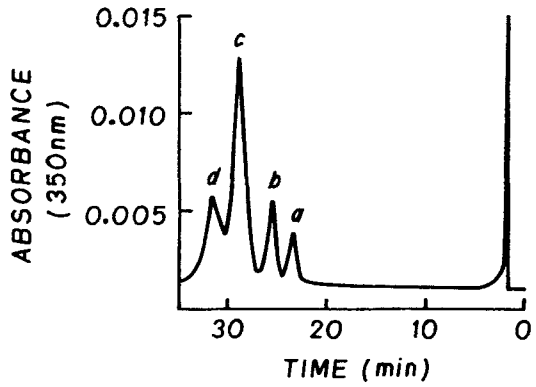


FIGURE 9. Separation of a mixture of *11-cis, 13-cis, 9-cis,* and *all-trans* retinoic acid. Column, Zorbax NH_2, 5 μm, 25 cm × 4.6 mm i.d. Mobile phase, acetonitrile/dichloromethane 90/10, containing 10 m*M* acetic acid. Flow rate, 2.0 ml/min. Peaks a, *11-cis* retinoic acid; b, *13-cis* retinoic acid; c, *9-cis* retinoic acid; and d, *all-trans* retinoic acid. (From Bhat, P. V. and Lacroix, A., *Methods in Enzymology,* Vol. 123, Chytil, F. and McCormick, D. B., Eds., Academic Press, Orlando, FL, 1986, 75. With permission.)

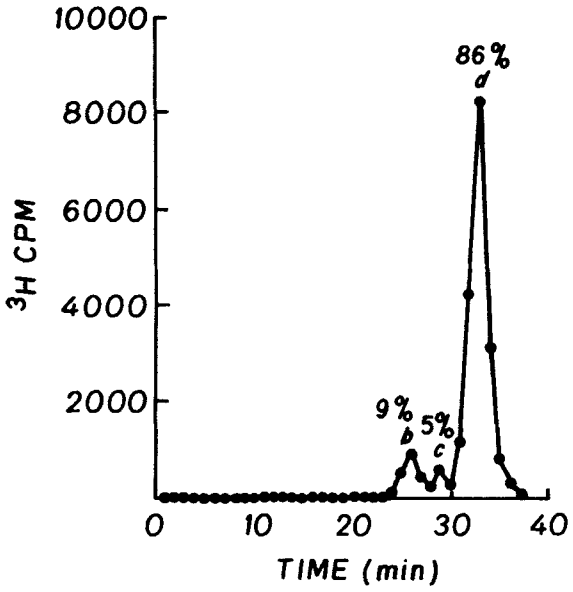


FIGURE 10. Occurrence of radioactive *13-cis* and *9-cis* retinoic acid in the liver tissue of a normal rat injected with pure *all-trans* [11-^{3}H] retinoic acid. The rat was sacrificed 30 min after the injection of the label and the labeled isomers were isolated and purified by DEAE-cellulose prior to injection onto HPLC. Column and other conditions are the same as in Figure 9. (From Bhat, P. V. and Lacroix, A., *Methods in Enzymology,* Vol. 123, Claytil, F. and McCormick, D. B., Eds., Academic Press, Orlando, FL, 1986, 75. With permission.)

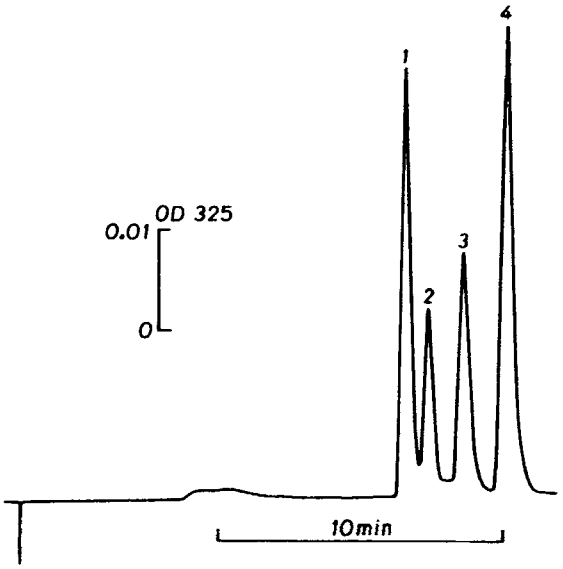


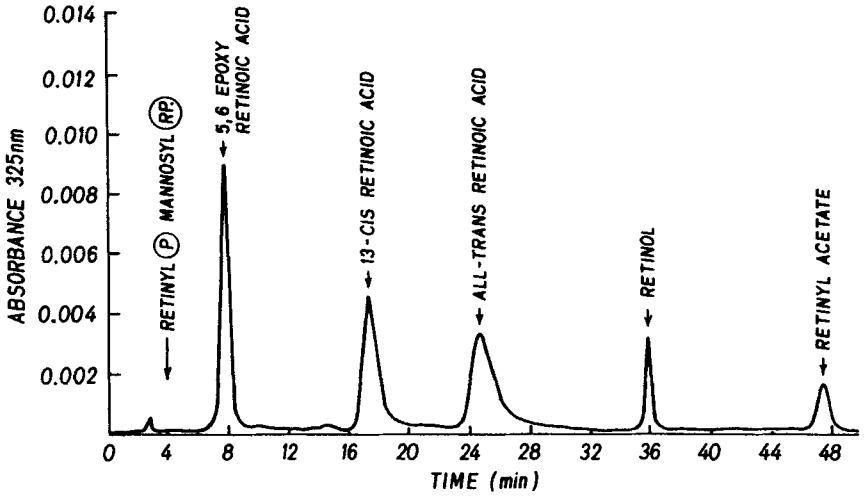
FIGURE 11. Retinyl stearate: separation of the *13-cis* (1), *11-cis* (2), *9-cis* (3), and *all-trans* (4) isomers. Column, ultrasphere Si, 25 cm × 4.6 mm i.d., mobile phase, diethylether: hexane (0.4% v/v); flow rate, 0.7 ml/min. (From Alvarez, R. A., Bridges, C. D. B., and Fong, S. L., *Invest. Ophthalmol. Vis. Sci.*, 20, 304, 1981. With permission.)

separate a variety of saturated and unsaturated retinyl esters by including silver ions in the mobile phase.[77] The separation of saturated and unsaturated retinyl esters has also been achieved using an octyl-substitute in reverse-phase columns developed with acetonitrile: water mixtures.[74] A detailed study of the use of HPLC in the separation and quantitation of retinyl esters in tissue extracts was described by Bhat and Lacroix.[1] They described the separation of a variety of naturally occurring retinyl esters on an ultrasphere-ODS column eluted with methanol: water mixtures (Figure 14). The determination limit was 40 pmol of various retinyl esters, and the method can be applied to the quantitative measurement of retinyl esters present in the tissues. The present authors have used chloroform: methanol (2:1) as the final solvent to solubilize tissue lipids before application onto the HPLC column. Use of the method with tritiated retinyl acetate to study of turnover of retinyl esters has been described by the same authors (Figure 15). Although the method is sensitive for the measurement of retinyl esters, it takes at least 80 min for their separation.

For quantitative measurement of retinyl esters, the choice of the internal standard must be limited to compounds not found in the biological materials and with molar extinction coefficients similar to that of retinol. The use of retinyl acetate, retinyl heptanoate, or retinyl propionate as internal standards has been reported.[16,74,78]

F. Conclusions

Modern chromatographic technique for the determination of retinoids is becoming focused on HPLC. For quantitative determination of retinoids, HPLC has become the method of choice, mainly because HPLC, particularly reverse-phase HPLC, offers maximum stability and minimum production of artifacts of these extremely labile compounds. HPLC separation of retinoids is rapid and can be conducted under very mild conditions at room temperature. Other techniques such as alumina chromatography, TLC, gas chromatography-mass spectrometry along with HPLC would greatly facilitate the separation and final identification of retinoids of interest present in the biological materials.



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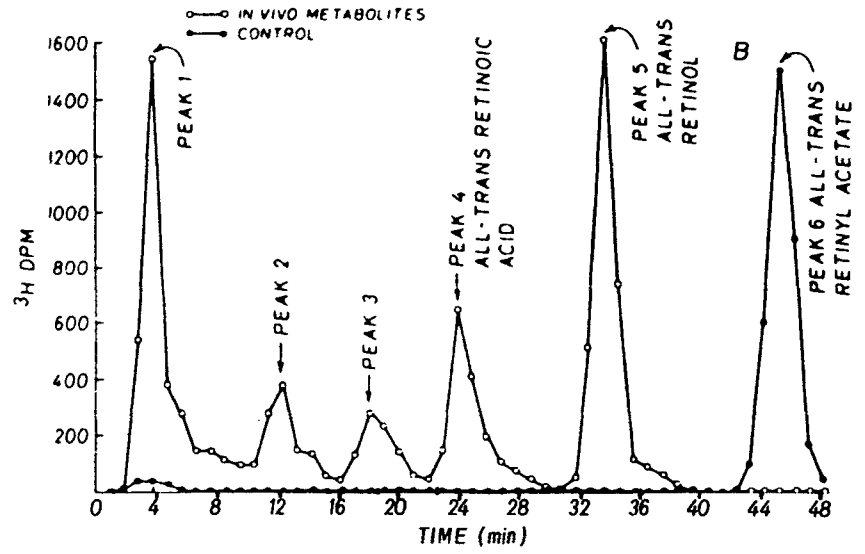

B

FIGURE 12. (A) Reverse-phase HPLC separation of a test mixture of standard retinoids on a 10 μm Partisil-10-ODS-2 column, 25 cm × 4.6 mm i.d. (B) Chromatography of *in vivo* formed radioactive vitamin A compounds in liver tissue of vitamin A-deficient rat. (From Bhat, P. V. and Lacroix, A., *Biochim. Biophys. Acta.*, 752, 451, 1983. With permission.)

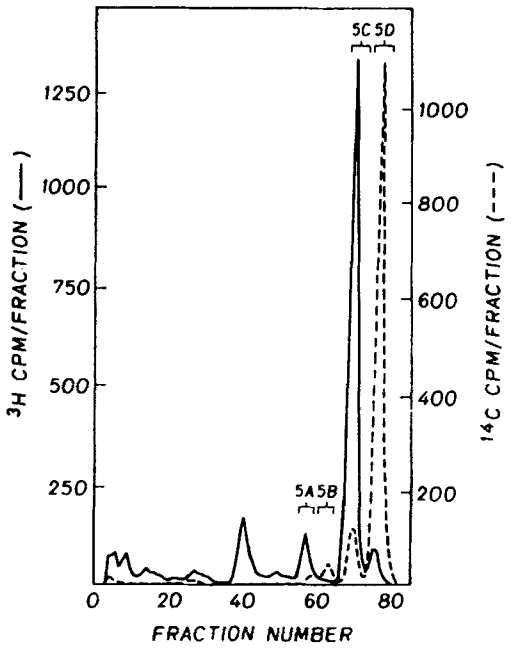

FIGURE 13. Chromatogram of metabolites of *all-trans* retinoic acid synthesized *in vitro* in a hamster liver supernatant system using *all-trans* [15-^{14}C] retinoic acid (----) or *in vivo* from hamster liver obtained 2 h after an intravenous 4 μg dose of *all-trans*-[10-^{3}H] acid (——). Column, Chromanetics ODS (5 μm), 25 cm × 4.6 mm i.d.; mobile phase, acetonitrile: acetic acid (0.1%) (42:58); flow rate, 1.2 ml/min.; peak (5A), *all-trans*-4-hydroxyretinoic acid(D3); peak (5B), unidentified; peak (5C), *13-cis*-4-oxo retinoic acid; peak (5D), *all-trans*-4-oxoretinoic acid (D4). (From Frolik, C. A. and Olson, J. A., *The Retinoids*, Vol. 1, Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds., Academic Press, Orlando, FL, 1984, 181. With permission.)

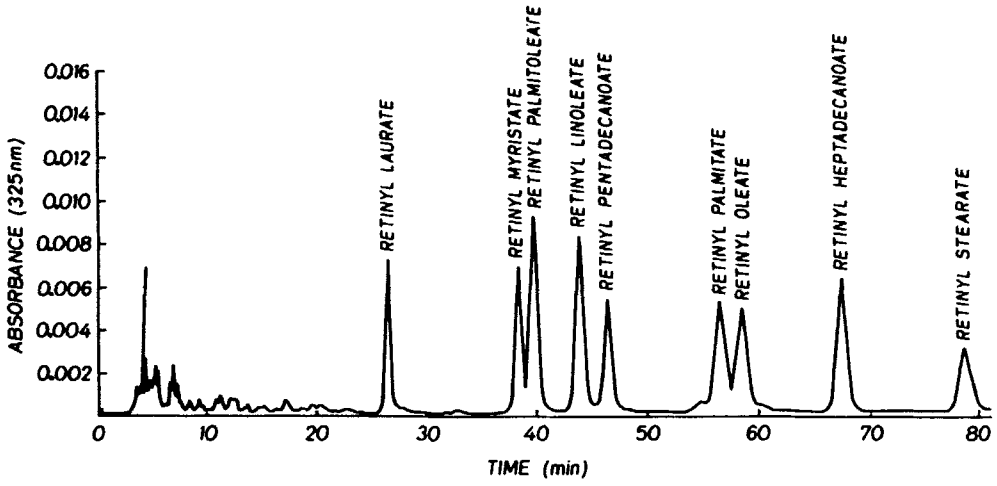

FIGURE 14. Separation of a mixture of nine standard retinyl fatty acyl esters (RFAE) on an ultrasphere ODS (5 μm) column, 25 cm × 4.6 mm i.d.; mobile phase, methanol: water (98:2). The quantity of each standard was 150 to 200 ng. (From Bhat, P. V. and Lacroix, A., *J. Chromatogr.*, 272, 269, 1983. With permission.)

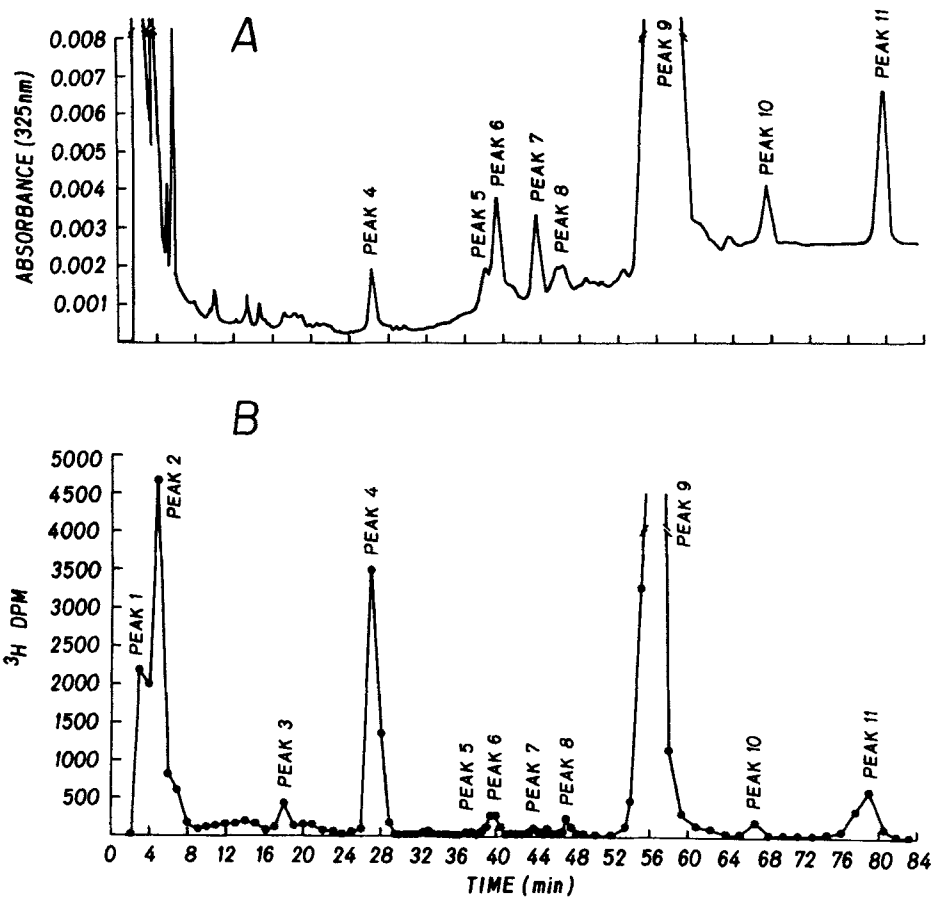

FIGURE 15. HPLC of lipid extract of normal rat liver. One rat was injected with 25 μCi of [11-^{3}H] retinyl acetate intraperitoneally and sacrificed after 24 h. An aliquot of the lipid extract (20 μl) was injected in chloroform: methanol (1:1) onto an ultrasphere ODS column (25 cm × 4.6 mm i.d.) and eluted with methanol: water mixtures. The UV was monitored at 325 to detect RFAE (A) and 0.5 or 1.0 min fractions were collected and counted (B). Peaks: 4, retinyl laurate; 5, myristate; 6, palmitoleate; 7, linoleate; 8, pentadecanoate; 9, palmitate; 10, heptadecanoate and 11, stearate, identified by cochromatography with standard RFAE. Radioactive peaks: 3, an unidentified ester of retinol; 2, retinol; and 1, a mixture of polar metabolites of retinol. (From Bhat, P. V. and Lacroix, A., *J. Chromatogr.*, 272, 269, 1983. With permission.)

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