Vitamin A metabolism: analysis of steady-state neutral metabolites in rat tissues

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Abstract High-performance liquid chromatography systems were developed to rapidly separate retinol from its esters, analyze the total spectrum of neutral vitamin A compounds, and purify retinyl esters to homogeneity. Chemical ionization mass spectrometric techniques were used to identify vitamin A compounds; these techniques are also applicable to quantification of tissue vitamin A compounds. These methods provide rapid and sensitive techniques for separation and quantification of neutral retinol metabolites. Their utility was demonstrated by analysis of vitamin A metabolites in rat tissues under steady-state conditions. Tissue specificity was noted for the concentrations of retinol and its long-chain fatty acid esters, the ratio of retinol to retinyl esters, and the fatty acid composition of retinyl esters. Quantitatively minor amounts of several neutral polar retinol metabolites were detected, but neither 13-*cis*-retinol nor 4-hydroxyretinol was observed in vivo as metabolites of retinol in kidney.—**Williams, J. B., B. C. Pramanik, and J. L. Napoli.** Vitamin A metabolism: analysis of steady-state neutral metabolites in rat tissues. *J. Lipid Res.* 1984. **25:** 638–645.

Supplementary key words retinol • retinyl ester • high-performance liquid chromatography • mass spectrometry

Retinyl esters are the form of vitamin A absorbed into intestinal lymph (1, 2), and stored in liver (3, 4); most of the newly absorbed vitamin A in tissues is in the form of retinyl esters, predominantly of saturated fatty acids (5). Recently, several HPLC systems have been developed to expedite study of tissue retinyl esters (6–8). In contrast, although retinol metabolism has been investigated (9), application of powerful HPLC methods to the study of the entire spectrum of neutral vitamin A metabolites in tissues during the steady-state has been limited. The present study reports versatile HPLC systems for separation and quantification of retinol and retinyl esters, mass spectrometric techniques for the positive identification of retinyl esters, and new chromatographic methods for analysis of neutral polar retinol metabolites. Their utility for analyzing steady-state neutral retinol metabolites is demonstrated.

EXPERIMENTAL PROCEDURES

General

HPLC was performed with a Waters Associates Model ALC/GPC 204 liquid chromatograph equipped with a Model 660 solvent programmer and an additional M6000A pump. Radially-compressed reverse-phase (RC-ODS) columns (0.8 cm × 10 cm, 10-μM particles unless otherwise noted, ODS) and the semi-preparative normal-phase column (1 × 30 cm) were obtained from Waters Associates. The analytical normal-phase column (0.46 cm × 25 cm, Zorbax-Sil) was purchased from DuPont. The semi-preparative reverse-phase column (ODS-2, M9, 0.94 × 25 cm), and the analytical reverse-phase column (ODS-3, 0.46 × 25 cm) were obtained from Whatman. Internal standards were detected at 340 nm. Solvents were glass-distilled and filtered through a 0.45-μM filter. Mass spectra were obtained with a Finnigan Model 4021 mass spectrometer coupled to an INCOS 2000 Data System. PCI spectra were obtained while heating the solids probe from ambient temperature to 300°C over a 10-min period at an ionizer temperature of 250°C. Methane was used as the reagent gas at a source pressure of 4.2×10^{-5} torr. EI spectra were obtained at 70 eV. Radioactivity was measured with a Beckman Model LS-330 liquid scintillation counter equipped with an automatic external standardization system. Samples were counted at an efficiency of 28% in 8 ml of Triton X-100–toluene 1:2 containing 6 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazoyl)]benzene per liter. Ultraviolet absorbance spectra were obtained in ethanol

Abbreviations: HPLC, high-performance liquid chromatography; PCI, positive-ion chemical ionization; EDTA, ethylenediaminetetraacetic acid; ODS, octadecylsilane; EI, electron impact; RC-ODS, radially-compressed octadecylsilane.

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with a Beckman Model 25 recording spectrophotometer. All studies were conducted under yellow light.

Compounds

[11-^{3}H]Retinal (1.9 Ci/mmol) and [11-^{3}H]retinyl acetate (1.4 Ci/mmol) were obtained from the Chemopreventive Program, Division of Cancer Cause and Prevention, National Cancer Institute. 13-*cis*-Retinoic acid and 4-oxoretinoic acid were provided by Dr. Beverly Pawson, Hoffman-La Roche Inc., Nutley, NJ. [11-^{3}H]Retinol was prepared from [11-^{3}H]retinal as described previously (10). Its purity was assessed with a RC-ODS column eluted with water–methanol 15:85. The acyl chlorides of fatty acids were purchased from Nu-Chek Prep, Elysian, MN.

Synthesis of retinyl esters

Retinol (200 mg) was dissolved in benzene (5 ml) and pyridine (0.5 ml); the appropriate acyl chloride (100 mg) was added, and the reaction was allowed to proceed at 25°C for 10 min. Water and diethyl ether were added, the phases were separated, and the organic phase was washed with water and dried over sodium sulfate. The solvent was evaporated under diminished pressure. Each residue was applied to an alumina column (activity III, 1.5 × 7 cm) equilibrated with hexane, and was eluted with hexane–ethyl acetate 9:1. The recovered esters were applied to a semi-preparative normal-phase HPLC column eluted with tetrahydrofuran–hexane 2:98 at a flow rate of 5.0 ml/min. Retinyl esters were stored in hexane at −20°C.

Synthesis of retinol derivatives

Anhydroretinol was prepared by treatment of retinol with dilute HCl in methanol. It was purified by HPLC as described for retinyl esters. 13-*cis*-Retinol, 4-hydroxyretinol, and 5,6-epoxyretinol were obtained by reduction of the appropriate methyl esters with lithium triethylborohydride. 13-*cis*-Retinol was eluted at 24 ml from a RC-ODS column with water–methanol 13:87 at a flow rate of 2.0 ml/min. 4-Hydroxyretinol and 5,6-epoxyretinol were eluted from the same column with water–methanol 20:80 at 12 and 22 ml, respectively. 13-*cis*-Retinol and 5,6-epoxyretinol were then applied to a normal-phase analytical HPLC column and eluted at 19 and 39 ml, respectively, with acetone–hexane 7:93 at a flow rate of 2.0 ml/min. 4-Hydroxyretinol was applied to the same normal-phase HPLC system, but after 48 ml the solvent was changed to acetone–hexane 1:1. 4-Hydroxyretinol eluted at 58 ml. 13-*cis*-Retinol and 4-hydroxyretinol had absorbance maxima at 325 nm. 5,6-Epoxyretinol had absorbance maxima at 325 and 310 nm (11, 12). The EI mass spectrum of 13-*cis*-retinol was indistinguishable from that of all-*trans*-retinol (13). The mass spectrum of 5,6-epoxyretinol was similar to a spectrum previously reported (12): m/z 91 (relative intensity 100), 302 (43), 284 (20), 271 (44). The mass spectrum of 4-hydroxyretinol had a molecular ion at m/z 302 (relative intensity 12), and peaks at m/z 284 (25), 91 (88), 105 (79), 119 (100), 145 (57), and 157 (35) among others typical of vitamin A compounds (13, 14).

Animals

Sprague-Dawley rats (150 g, Charles River Breeding Laboratories) were maintained on a 12-hr light-cycle and were fed a stock diet ad libitum (Purina rat chow). Each of ten rats was administered oral doses of [11-^{3}H]retinol (2 μg each, 3.1×10^7 dpm) in corn oil (0.1 ml) daily for 5 days. Three additional rats similarly received [11-^{3}H]retinyl acetate (5 μg, 4.8×10^7 dpm) for 6 days. The animals were killed (9 AM) 24 hr after the final dose under ether anesthesia by cardiac exsanguination with EDTA-treated syringes. Tissues were removed and placed in ice-cold saline. Liver and kidney were trimmed of fat and connective tissue. Intestinal mucosa was scraped from the muscularis. Blood was centrifuged to obtain plasma. The retinoids were extracted, and the neutral fractions were obtained by DEAE-Sephadex A-25 chromatography as described (10, 15). Aliquots in triplicate were measured for radioactivity. The extracts were stored in hexane (20 ml) at −70°C.

A control extraction was done in which [11-^{3}H]retinol was added to a liver homogenate. HPLC analysis of the extract showed that at least 95% of the radioactivity was recovered as [^{3}H]retinol.

Analysis of neutral metabolites

Unless otherwise stated, samples were prepared for HPLC by removing the solvent under a stream of nitrogen, dissolving the residue in ethanol, filtering the solution through a 0.45-μm filter, and reducing the final volume to less than 0.5 ml. For the determination of their specific activity, retinol and retinyl esters were isolated with a semi-preparative reverse-phase HPLC column eluted with water–2-propanol 30:70 for 14 min followed by 2-propanol at a flow rate of 4.0 ml/min (HPLC System 1). Radioactivity was measured in triplicate for the retinol and retinyl esters in each tissue. The mass of vitamin A in the retinol and retinyl ester fractions was determined in triplicate with the trifluoroacetate assay (16), except for intestinal mucosa, which was done in duplicate. The tissue concentrations of retinol and retinyl esters was calculated assuming a tissue density of 1 g/ml.

Neutral retinoids were analyzed by HPLC with retinol, retinal, anhydroretinol, and retinyl esters included as internal standards. HPLC was performed with a RC-ODS

(5-μm particles). Samples were eluted with a linear gradient of water–methanol 15:85 to methanol over 20 min at a flow rate of 2.0 ml/min (HPLC System 2). Fractions (1 ml) were collected and measured for radioactivity. The proportions of retinyl palmitate and retinyl oleate were determined after rechromatography over a semi-preparative normal-phase column eluted with tetrahydrofuran–hexane 0.1:99.9 at a flow rate of 5.0 ml/min (HPLC System 3). Fractions (0.8 ml) were collected after recycling the sample once for a total of two passes.

Analysis of neutral polar retinol metabolites from kidney

Aliquots of the kidney extract obtained from [^{3}H]retinyl acetate-dosed animals were analyzed in HPLC System 2. Combined fractions 20–27 (retinol) and 3–19 (polar fraction) were obtained. For determination of the amount of 13-*cis*-retinol, the retinol fraction was rechromatographed on an analytical normal-phase HPLC column eluted with acetone–hexane 7:93. For analysis of polar retinol metabolites, the polar fraction was rechromatographed with unlabeled 5,6-epoxyretinol and 4-hydroxyretinol standards on an analytical normal-phase column eluted with acetone–hexane 15:85 at a flow rate of 2.0 ml/min. To determine the effects of our extraction methods on polar retinol metabolites, pure all-*trans*-[11-^{3}H]retinol was added to a kidney homogenate. The homogenate was extracted and analyzed as above. The amount of radioactivity comigrating with 13-*cis*-retinol, all-*trans*-retinol, 5,6-epoxyretinol, and 4-hydroxyretinol was corrected for total sample recovery.

Isolation of retinyl esters for mass spectrometry

Aliquots of the neutral metabolites extracted from kidney were chromatographed in HPLC System 2 without internal standards, and individual retinyl esters were collected. Each was rechromatographed in HPLC System 3. As a final purification step, each retinyl ester was chromatographed over an analytical reverse-phase column eluted with dichloromethane–acetonitrile 3:97 at a flow rate of 3.0 ml/min.

RESULTS

HPLC System 1, developed for the rapid separation of retinol and total retinyl esters, achieved substantial retention of retinol (approximately 5 column volumes) and eluted the retinyl esters together (**Fig. 1**). The specific radioactivities of retinol and the combined retinyl esters in each tissue were determined from these fractions (**Table 1**). Despite the differences in total radioactivity, the specific radioactivities of the retinol and retinyl ester fractions in most tissues were similar. Kidney, however, had a two-

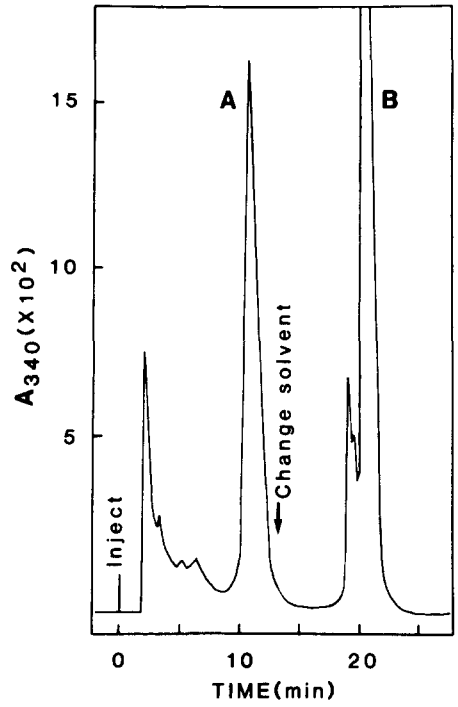

Fig. 1. Separation of retinol from retinyl esters by HPLC. An aliquot of the neutral metabolites extracted from liver was eluted through a semi-preparative reverse-phase column with 2-propanol–water 70:30 for 14 min, followed by 2-propanol at a flow rate of 4.0 ml/min (HPLC System 1). The area between 38 and 52 ml was collected for retinol analysis, and the area between 74 and 88 ml was collected for retinyl ester analysis. Neutral retinoids from plasma, kidney, and mucosa were analyzed similarly.

fold higher specific radioactivity of vitamin A compounds than did the other tissues. The amount of retinol present relative to its esters was tissue-dependent.

Resolution of a broad spectrum of neutral vitamin A compounds was achieved with HPLC System 2 (**Fig. 2**). Retinol was substantially retained and, except for retinyl palmitate and retinyl oleate, the major esters were resolved. Retinyl palmitate and retinyl oleate were resolved by HPLC System 3 (**Fig. 3**). Application of these methods to several tissue extracts confirmed differences in the tissues' retinol content and in the ratio of retinol to retinyl esters. The fatty acid composition of the retinyl esters was also tissue-specific (**Table 2**). The limits of detection of these methods are dependent upon the specific radioactivities of the vitamin A compounds dosed. In this case, lower limits of 75 ng of retinol and 125 ng of retinyl palmitate were quantified radiometrically.

PCI mass spectrometric methods were applied for verification of retinyl ester structure. The PCI mass spectrum of retinyl palmitate is exemplary (**Fig. 4**). The spectrum had a parent peak at m/z 525, produced by proton addition to the molecule. Cleavage of the fatty acyl group yielded two peaks at m/z 239 and 285, representing the fatty acyl group (R'CO-), and the retinyl fragment

TABLE 1. Neutral vitamin A compounds in tissues of rats fed a stock diet

Tissue	Total Neutral [^{3}H]Vitamin A	Specific Radioactivity		Concentration	
		Retinol	Retinyl Esters	Retinol	Retinyl Esters
	dpm × 10^{-4}/*g tissue*[a]	*dpm* × 10^{-7}/*μmol*		*μM*	
Liver	232 ± 24	1.8	2.3	9.8	86.9
Kidney	59 ± 7	4.1	5.3	3.9	7.4
Mucosa[b]	6 ± 0.8	2.3	3.9	0.2	1.3
Plasma	2 ± 0.2[c]	1.9	—[d]	1.1	—[d]

Values are the mean ± SD of triplicate determinations from the combined tissues of ten rats. Each rat received [11-^{3}H]retinol (7.2 nmol, 3.1 × 10^7 dpm) daily for 5 days.

[a] There was a 7–10% error in determining radioactivity.

[b] Small intestinal mucosa.

[c] dpm/ml.

[d] Plasma retinyl esters were below the limit of detection of the trifluoroacetate assay.

(RCH_2O-), respectively. Conversion of the retinol portion of the molecule to anhydroretinol and protonation of anhydroretinol by the reagent gas provides the base peak at *m/z* 269 (anhydroretinol plus H^+). Concomitant elimination of the fatty acid portion provides the peak at *m/z* 255 ($R'CO_2$-). Alternatively, a McLafferty-type rear-

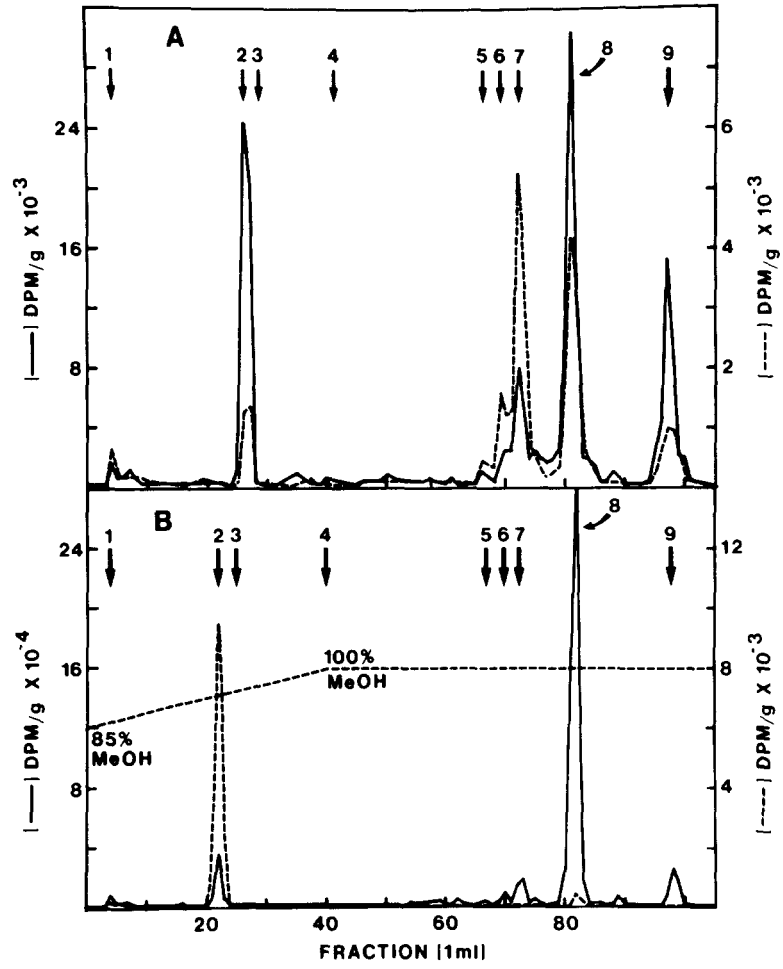

Fig. 2. HPLC analysis of neutral vitamin A compounds in rat tissues. Samples were analyzed on a RC-ODS column (5-μm particles) eluted with a linear gradient of water–methanol 15:85 to methanol over 20 min at a flow rate of 2.0 ml/min (HPLC System 2). The data are expressed as dpm/g of tissue per fraction. Internal standards are: 1, 5,6-epoxyretinol; 2, retinol; 3, retinal; 4, anhydroretinol; 5, retinyl docosahexaenoate; 6, retinyl palmitoleate; 7, retinyl linoleate; 8, retinyl palmitate and retinyl oleate; 9, retinyl stearate; A, small intestinal mucosa (– – –); kidney (——); B, plasma (– – –); liver (——).

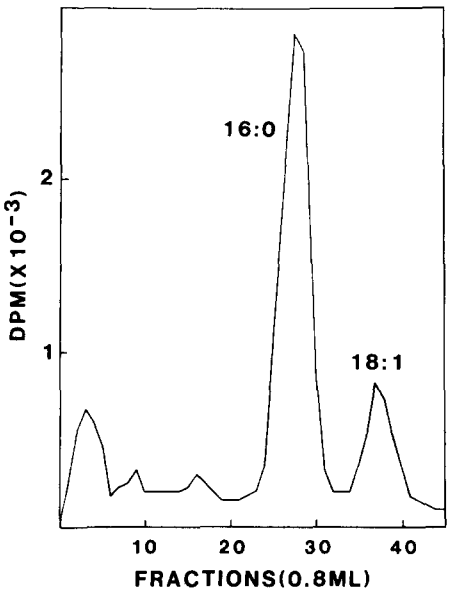

Fig. 3. Separation of retinyl palmitate and retinyl oleate by normal-phase HPLC. Retinyl palmitate and retinyl oleate collected from HPLC system 2 (Fig. 2) were eluted from a semi-preparative column with tetrahydrofuran–hexane 0.1:99.9 at a flow rate of 5.0 ml/min (HPLC system 3). The sample was recycled once for a total of two passes. Designations are: 16:0, retinyl palmitate; 18:1, retinyl oleate.

rangement, between the carbonyl oxygen and a proton on C-15 of the retinyl moiety would provide the fatty acid which gives the peak at m/z 257 after protonation by the reagent gas ($R'CO_2H$ plus H^+). Peaks at m/z 157, 145, 133, 119, 105, 107, 95, and 91 are similar to peaks observed in the EI mass spectra of retinol and retinyl acetate (13). The limit of detection for retinyl palmitate was 25 ng. Data from the PCI mass spectra of several retinyl esters are compiled in **Table 3.**

To obtain biological samples suitable for mass spectral analysis, retinyl esters from kidney were collected after chromatography through HPLC Systems 2 and 3. These esters were individually applied to a nonaqueous reverse-phase system (see Experimental Procedures). In this manner, sufficient quantities were obtained to permit mass spectral verification of the structures of retinyl palmitate, retinyl oleate, and retinyl stearate.

An analytical normal-phase column eluted with acetone–hexane 7:93 separated 13-*cis*-retinol (19 ml) and all-*trans*-retinol (25 ml). The retinol component obtained from analysis of a kidney extract in HPLC System 2 (fractions 20–27, Fig. 2A) was rechromatographed over this normal-phase system. 13-*cis*-Retinol was not present in the tissue extract of animals dosed with [^{3}H]retinyl acetate in amounts greater than the control extract (18% and 17% of total retinol, respectively).

An analytical normal-phase column was used to resolve polar retinol derivatives (**Fig. 5**). Analysis of the polar materials observed in HPLC System 2 (fractions 3–19, Fig. 2A) revealed similar peaks in the control and the extract of kidney from dosed animals (Fig. 5). The largest peak in both cases comigrated with 5,6-epoxyretinol. Neither contained significant amounts of radioactivity which comigrated with 4-hydroxyretinol. Two unidentified peaks were noted which eluted at 5 and 19 ml, but each was also present in the control.

DISCUSSION

Several HPLC systems, besides those reported here, are available for retinol metabolite analyses. de Ruyter and de Leenheer (6) used a silver-impregnated ODS column to resolve esters. Ross (7) circumvented the problems of impregnation with silver, and achieved complete resolution of ten different retinyl esters with an acetonitrile-based solvent during 60 min of eluting a C_8-derivatized column. The system probably could be modified to retain

TABLE 2. Compositions of retinol and its major esters present in tissues of male rats fed a stock diet

		Retinyl Esters[a]				
Tissue	Retinol	22:6	18:2	16:1	16:0 + 18:1 (% 16:0)[b]	18:0
		% of total tritium recovered in the neutral fraction				
Liver	8 ± 2	1 ± 1	3 ± 2	4 ± 1	72 ± 5 (91)	7 ± 0
Kidney	27 ± 1	1 ± 0	2 ± 1	8 ± 1	34 ± 1 (73)	21 ± 1
Mucosa[c]	8	3	8	35	26 (51)	10
Plasma[d]	93 ± 1				6 ± 1 (–)	

Retinol and retinyl esters were separated with HPLC system 2 (Fig. 2). Retinyl palmitate and retinyl oleate were separated with HPLC system 3 (Fig. 3).

[a] Designations are: 22:6, docosahexaenoate; 16:1, palmitoleate; 18:2, linoleate; 16:0, palmitate; 18:1, oleate; 18:0, stearate.

[b] Percent 16:0 in the mixture of 16:0 and 18:1 esters.

[c] Small intestinal mucosa values are from a single determination.

[d] Plasma retinyl esters other than combined 16:0 and 18:1 esters were below detection limits.

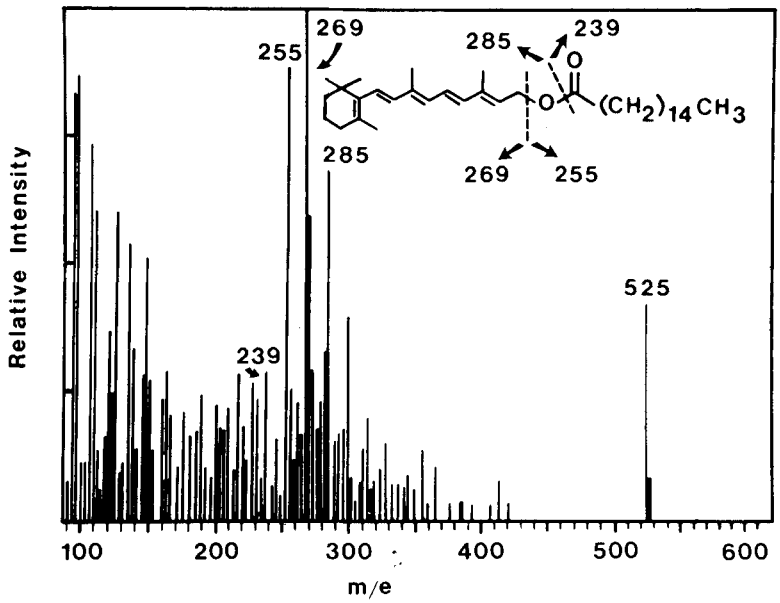

Fig. 4. PCI mass spectrum of synthetic retinyl palmitate.

retinol, but such modification would entail much longer analysis time to obtain the same retention volumes of retinol achieved by the methods reported here. Retinyl phosphate, retinol, retinal, and retinyl palmitate have been separated during a 45-min elution of an ODS column with an acetonitrile-based solvent system; but resolution of the fatty acid esters of retinol was not reported (17). Retinyl esters have also been separated with methanol-based systems during lengthy elutions (>80 min), but retinol and its polar metabolites were not resolved under these conditions (8). The present work provides versatile HPLC systems that resolve a wide variety of vitamin A compounds, including most esters, so that a complete spectrum of neutral tissue metabolites can be examined.

Retinyl esters, as a group, were conveniently separated from retinol in HPLC System 1. This reverse-phase system used 2-propanol as the major solvent, rather than methanol or acetonitrile. The use of 2-propanol is advantageous because it effects rapid elution of retinyl esters, but provides substantial retention of retinol (4 to 5 column volumes), ensuring its separation from more polar substances. The total neutral retinol metabolite pool was analyzed on a RC-ODS column with methanol as the major solvent (HPLC System 2). Polar material is retained longer, at a lower water composition, in methanol compared to 2-propanol-based systems. Methanol, therefore, allows good resolution of polar materials at higher flow rates, which facilitates examinations of polar metabolites. Moreover, optimal resolution of the individual retinyl esters is obtained in methanol-based systems.

HPLC systems based on radially-compressed columns are versatile. For example, the same RC-ODS column

TABLE 3. Positive-ion chemical ionization (PCI) mass spectral characteristics of retinyl esters

Peak[a]	Retinyl Ester					
	16:0[b]	16:1	18:0	18:1	18:2	22:6
	m/z (relative intensity)					
$(M + H)^+$	525 (44)	523 (3)	553 (3)	551 (3)	549 (4)	597 (2)
RCH_2O^+	285 (68)	285 (67)	285 (63)	285 (74)	285 (23)	285 (8)
(Anhydroretinol + $H)^+$	269 (100)	269 (100)	269 (100)	269 (100)	269 (100)	269 (100)
$R'CO_2^+$	255 (89)	253 (10)	283 (24)	281 (25)	279[d]	327 (3)
$(R'CO_2H + H)^+$	257 (26)	255 (32)	—[c]	283 (5)	281 (5)	329 (6)
$R'CO^+$	239 (29)	237 (27)	267 (25)	265 (42)	263 (15)	311 (3)

PCI mass spectra were obtained as detailed in Experimental Procedures with methane as reagent gas.

[a] M = RCH_2OCOR', where RCH_2O represents the retinyl alcohol portion, and R'CO represents the fatty acyl portion of the molecule. $(M + H)^+$ is the parent ion in the mass spectrum.

[b] For designations, see Table 2.

[c] This retinyl stearate fragment has *m/z* 285 and is superimposed on the RCH_2O^+ peak.

[d] This fragment was not detected.

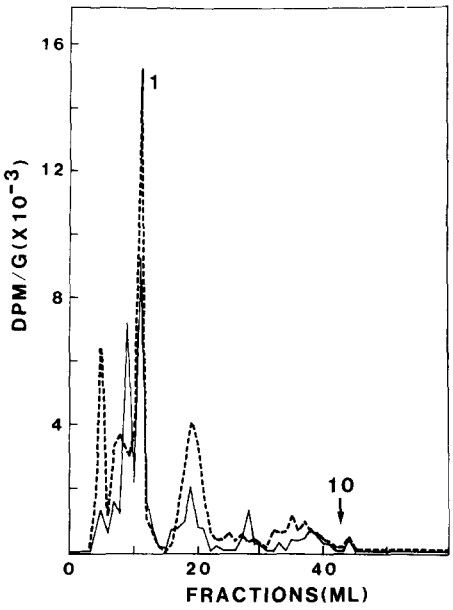

Fig. 5. Normal-phase HPLC of neutral polar materials derived from retinol. The polar fractions from HPLC system 2 (fractions 3–19, Fig. 2A) were rechromatographed on an analytical normal-phase column eluted with acetone–hexane 15:85 at a flow rate of 2.0 ml/min. The data are expressed as dpm/g of tissue per fraction, and represent comparable amounts of total radioactivity. Internal standards are: 1, 5,6-epoxyretinol; 10, 4-hydroxyretinol; control extract (——); kidney extract (– – –).

used in System 2, when eluted with 20% water in methanol–2-propanol 1:1 at 3.0 ml/min will resolve retinoic acid (5 min), retinol (13 min), and retinal (15 min); changing the solvent to methanol–2-propanol 1:1 after 17 min will elute retinyl acetate (21 min), retinyl linoleate (25 min), retinyl palmitate (27 min), and retinyl stearate (28.5 min). This modification allows rapid analysis (less than 30 min) of these compounds and may be especially useful for studying metabolism. A system of 25% water in 2-propanol, operated at a flow rate of 2.0 ml/min, will separate retinol (4 min), retinal (5 min), and when eluted with 2-propanol after 6 min, retinyl palmitate (12.5 min). This system would serve as an alternative to HPLC System 1 for rapid separation of retinol from its esters.

The PCI mass spectrometric methods allow structural verification of retinyl esters. Retinyl esters yield peaks at m/z 285 (retinol $-$ H^+) and 269 (anhydroretinol). The nature of the fatty acyl group is indicated by the molecular ion, and two characteristic fragments ($R'CO_2H_2^+$ and $R'CO^+$). This fragmentation pattern offers the opportunity to monitor only these five selected ions. Such selected ion monitoring, in conjunction with chromatographic analysis, provides positive identification, and sensitive quantification.

Equilibration of vitamin A doses with endogenous vitamin A requires 5–8 days in normal rats (18–20); small doses equilibrate as rapidly as 5 days (18). Consequently, our studies likely reflect steady-state metabolism of vitamin A. The equivalent specific activities of the tissue retinol and retinyl esters support this conclusion. Under these conditions, at least 90% of the neutral vitamin A in the tissues was present as retinol and retinyl esters; neutral polar metabolites of retinol were quantitatively minor. Although the ratio of retinol to its esters is probably determined in part by the dietary status and the age of the animal, the steady-state concentrations of retinol and its proportional contribution to neutral vitamin A were tissue-specific. The fatty acid compositions of retinyl esters during the steady-state were also tissue-specific. The latter results are in agreement with previous studies of retinyl ester composition in plasma, kidney, and liver, despite differences in dosage, administration, and experimental methods (3, 5).

Resolution of the polar neutral metabolites indicated peaks common to both the control and the kidney extracts. Some of the peaks were larger than those in the control (elution volume 5 and 19 ml) and may represent true polar neutral metabolites of retinol. Nevertheless, their quantitative contribution to the overall spectrum of steady-state metabolites is small, indicating rapid clearance or a low rate of synthesis. The quantities of 5,6-epoxyretinol and 13-*cis*-retinol in the control and the tissue extract were similar; no evidence was found of 4-hydroxyretinol. It seems, therefore, that in kidney, all-*trans*-retinol undergoes acylation to all-*trans*-esters, and that oxidation to retinoic acid occurs before *trans/cis* isomerization to provide 13-*cis*-retinoic acid (15, 21). Finally, our finding of substantial conversion of all-*trans*-retinol to 13-*cis*-retinol in our control samples, emphasizes the facility of the *trans* to *cis* isomerization during handling. This result stresses the need for careful controls when investigating the biological occurrence of retinol and retinoic acid isomers.

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