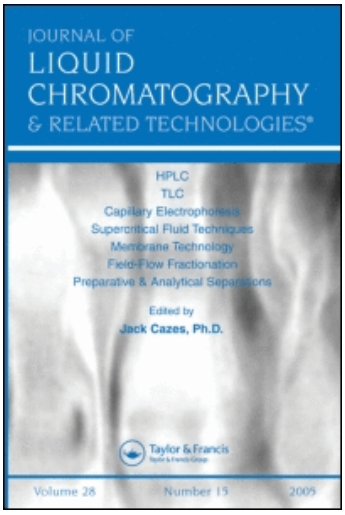

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The Reversed-Phase HPLC Behavior of Retinyl Esters

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THE REVERSED-PHASE HPLC BEHAVIOR OF RETINYL ESTERS

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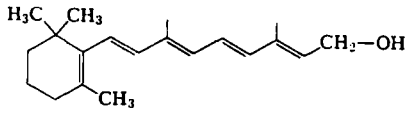
ABSTRACT

Fifteen retinyl esters were synthesized, including a number of esters containing naturally occurring polyunsaturated fatty acids. The esters were purified by HPLC and their chromatographic behavior on reversed phase HPLC was studied. A plot of Log k' versus % water in the eluting solvent for each ester was very close to linear in the range from 1% to 3.5% water in methanol. Retinyl esters containing a monounsaturated fatty acyl group had an effective carbon number (ECN) about 1.85 less than the corresponding ester with a saturated acyl group with the same chain length. Thus the monoenes were relatively difficult to separate from esters with saturated acyl groups with two fewer carbons. Retinyl esters with polyene acyl groups showed decreased ECNs of: 3.32-3.47 for dienes; 4.64-4.84 for trienes; and 5.92 for the one tetraene tested. From these data it is expected that even chain length trienes would chromatograph very close to the corresponding saturated odd chain ester with five fewer carbons. The usefulness of ECN is discussed. Retinyl arachidonate (R20:4) was not separated from both and retinyl palmitoleate (R16:1) and retinyl myristate (R14:0), using any one condition. Under the chromatography conditions used, the complex lipids eluted soon after the solvent front. Thus the Folch extract of liver or plasma could be used directly for analysis of retinyl esters monitored at 325 nm without preliminary separation from other lipids.

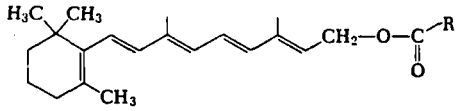
INTRODUCTION

There has been a considerable amount of interest in the separation and quantitation of retinyl esters in recent years (1-8). It is presumed that any naturally occurring fatty acid is a potential donor of the fatty acyl moiety of retinyl esters found in tissues. For example, Ross (6) has recently shown that milk retinyl esters contain a variety of fatty acyl groups including the short chain (8-14 carbons) fatty acids that characterize milk lipids. Many of the potential retinyl esters have not been synthesized or are not a part of any one study.

This paper reports the synthesis of fifteen retinyl esters, many of which are derivatives of naturally occurring polyunsaturated fatty acids that have not been reported, and the study of their behavior on an HPLC reversed phase column.



Retinol



Retinyl ester

MATERIALS AND METHODS

All fatty acyl chlorides were from NuChek Prep, Elysian, MN. Retinol and retinyl acetate were from Sigma Chemical Co., St. Louis, MO. HPLC solvents were from Fisher, Dallas, TX. Molecular sieve was from J.T. Baker, Phillipsburg, N.J.

Synthesis of Retinyl Esters.

Retinyl esters were synthesized by a modification of the method of Bhat (5). Pyridine was dried over activated Molecular sieve Type 4A, 8-12 mesh. Retinol (15-20 mg) was dissolved in 0.2 mL anhydrous pyridine in a 1 mL reacti-vial (Pierce Chemicals, Rockford, IL). About 15-20 uL of the appropriate fatty acyl chloride was added and an immediate precipitation of the fatty acyl pyridinide occurred. The sample was shaken, purged with nitrogen and heated at 65 ^{o}C for 2.25 hr. The pyridine was evaporated under nitrogen and the retinyl ester was extracted from the reaction mixture with 1 mL of hexane. Undissolved material was removed by centrifugation and the residue was extracted with an additional 1 mL of hexane. The clear yellow hexane extracts were combined and the solvent was evaporated under nitrogen. The resulting crude retinyl ester was dissolved in 1 mL of ethanol and subjected directly to HPLC analysis. Reaction yields were not rigorously determined but were estimated at about 50%. In cases in which the synthetic ester had been previously reported they gave retention times and relative retention times very similar to the literature values (5). New synthetic esters were tentatively identified, prior to isolation, by peak size and expected retention times. Their identities were established by isolation in pure form by preparative HPLC followed by mass spectral identification. All retinyl esters were purified by application of the crude reaction mixture to a 25 cm x 4.6 mm Ultrasphere ODS, 5 u column (Rainin, Woburn, MA) or a 25 cm x 10.0 mm Alltech ODS column (Alltech Associates, Deerfield, IL).

Fifteen retinyl esters have been synthesized: Retinyl laurate (R12:0), retinyl myristate (R14:0), retinyl pentadecanoate (R15:0), retinyl palmitoleate (R16:1 n-7), retinyl palmitate (R16:0), retinyl heptadecanoate (R17:0), retinyl stearate (R18:0), retinyl oleate (R18:1 n-9), retinyl linoleate (R18:2 n-6), retinyl 6,9,12-octadecatrienoate (R18:3 n-6), retinyl eicosanoate (R20:0), retinyl 11,14-eicosadienoate (R20:2 n-6), retinyl 8,11,14-eicosatrienoate (R20:3 n-6), retinyl arachidonate (R20:4 n-6) and retinyl 13,16-docosadienoate (R22:2 n-6). This set of standards has many of the retinyl esters of the n-6 series of

polyunsaturated fatty acids found in hydrolysates of tissue lipids and represents many of the major potential donors of fatty acyl groups for the formation of retinyl esters.

Identification of Synthetic Retinyl Esters by Mass Spectrometry.

Mass Spectra of samples purified by HPLC were obtained using a Finnigan 4021 Mass Spectrometer operating in the chemical ionization mode (CI) using isobutane as the reagent gas and 70 ev as the ionizing potential. The mass spectra of the retinyl esters gave identifying high mass ions corresponding to $(M+H+CH_4)^+$, $(M-H)^+$, $(M)^+$, $(RCOOH+H)^+$ and $(Retinol-OH)^+$ where M is the molecular ion of the retinyl ester and RCOOH is the fatty acid portion of each ester.

Extraction of Vitamin A and Retinyl Esters from Biological Samples.

Rats were sacrificed by cervical dislocation and livers were quickly removed, minced by a razor blade and the portions were randomized to prevent variation of analyses due to localization of storage of vitamin A (9). Samples were extracted by the Folch method (10) as follows: Samples of liver were quickly weighed and homogenized in 20 volumes of chloroform-methanol (2:1). In most cases an ethanol solution of retinyl laurate (20 ug/g liver) was added to the homogenate as an internal standard. (Preliminary analysis had shown that retinyl laurate (R12:0) was absent from liver extracts.) After addition of 0.2 volumes of 0.1% NaCl solution, the chloroform layer was isolated and an aliquot, equivalent to 0.1 g of liver, was carefully measured. The chloroform was carefully evaporated to dryness in such a way as to assure that the lipid was restricted to the bottom of the tube. The sample was dissolved in 100 uL of chloroform and 40 uL was used for HPLC analysis.

HPLC of Retinyl Esters.

HPLC was performed on a Hewlett-Packard 1084B Liquid Chromatograph equipped with a variable wavelength detector.

Standard mixtures of retinyl esters were prepared from the purified synthetic compounds and used to determine their retention times under isocratic conditions at various compositions of water in methanol ranging from 1% to 3.5% (typically 2% or 3%). Samples were injected onto a 25 cm x 4.6 mm Ultrasphere ODS, 5 u column. Flow was 1.5 mL/min and detection was at 325 nm, the absorption maximum of retinol and retinyl esters.

Samples from liver were total lipid extracts as described above. A maximum of 40 uL of sample, equivalent to 40 mg liver, was injected because higher levels of chloroform produced asymmetrical peaks. This quantity of sample gave adequate peak sizes for accurate integration of peak areas. Minor peaks were normally amplified by attenuation changes in their region of the chromatogram. It was shown by monitoring the chromatogram at 205 nm, that the neutral lipid and phospholipids eluted soon after the solvent front under the chromatography conditions used and did not interfere with retinol and retinyl esters monitored at 325 nm. Therefore, it was unnecessary to separate polar lipids from retinol and retinyl esters prior to these HPLC analyses.

The capacity factor k' is defined as $(R_T - R_0)/R_0$ where R_T is the retention time of the component and R_0 is the time it takes an unretained component to pass through the column. The R_0 used was the time of the first detector response on the chromatogram (1.8 min).

HPLC of Retinol in Rat Plasma Samples.

Preliminary results showed that retinyl esters were not present in lipid extracts of the plasma of rats fasted 18 hr prior to collection. Thus different chromatography conditions were adopted in order to retain retinol and the internal standard, retinyl acetate, on the column longer. The solvent used was 88% methanol and 12% water. This gave a retention time of about 10 min for retinol and about 30 min for retinyl acetate at 1.5 mL/min on the Ultrasphere column. If desired, faster analyses could be achieved by reducing the amount of water in the eluting solvent.

Quantitation of Retinol and Retinyl Esters.

The molar absorbances of several retinyl esters were calculated from the $\epsilon^{1\%}$ values at 325 nm given by Bhat (5) and used as follows: R12:0, 49,340; R14:0, 49,200; R15:0, 49,750; R16:0, 49,770; R16:1, 49,370; R17:0, 49,370; R18:0, 48,110; R18:1, 49,220; R18:2, 49,410; retinol, 52,000. The average value for the esters was 49,262 ± 466 (S.D. less than 1%). Thus the absorbance per unit weight depends only on the average molecular absorbance and the relative molecular weights of the reference ester to the sample ester when measured at 325 nm. When the internal standard method was used the equation was:

$$ug_s = Area_s/(Area_{is}/ug_{is} \times M_{is}/M_s),$$

where ug_s is ug of sample in the peak, $(Area)_s$ is the area of the sample, $(Area)_{is}$ is the area of the internal standard, ug_{is} is the ug of internal standard added to the total sample (or aliquot) and M_{is} and M_s are the molecular weights of the internal standard and the compound in the sample peak, respectively. Aliquoting factors are automatically taken into account if the areas due to sample and internal standard are all referred to the total sample, otherwise aliquoting factors are required.

In the case of plasma samples where retinyl acetate was the internal standard and retinol was being measured, it is necessary to multiply the term M_{is}/M_s by $\epsilon_{retinol}/\epsilon_{is}$ in order to take into account the difference in the absorbancies of the retinyl ester internal standard and retinol. When retinyl acetate was the internal standard the term on the right in the denominator was 1.211 [(328/286) x (52,000/49,262)].

If an internal standard was not used, the equation was: $ug_s = (Area_s \times \text{Aliquot Factor})/(Area_r/ug_r \times M_r/M_s)$ where $Area_r/ug_r$ is area per ug and M_r is the molecular weight of a reference compound. For example, when R12:0 was the reference, the value $(Area_r/ug_r)$ was 273,360 area units/ug (on our chromatograph) as determined from a standard curve. Thus to calculate the quantity of R16:0 the denominator was 244,192 [273,360 x (469/525)]. Since esters reported here have the same absorbancy coefficients, esters

with higher molecular weights than the reference compound have lower values for Area/ug than the reference.

A computer program, written in Turbo Basic (Bourland International) was developed to output the quantitative data in the form shown in TABLE 1. The program can readily be adapted to other uses by persons with some experience in computer programming. A listing of the program is available upon request. If the request includes the type analyses to be done, the authors will make suggestions as to how to modify the program. In its current state of revision there is some but not extensive documentation.

The program asks for input of the chromatography conditions, retention times and peak areas. It uses the chromatography conditions to select a set of relative retention times which are used to identify peaks. Once peaks are identified, it calculates quantities of the compound in each peak using stored values of the factors in the equations above. When all entries have been made, it calculates the percent of total composition for each entry. Finally it lists the retention time windows used for each of the standards. This feature is helpful when the retention time of a peak is just outside these limits and allows one to manually identify a peak. Opportunity to edit any input retention time or area is available in the program. The program is very useful for a number of reasons: 1) a quantitative table is set up automatically and calculations do not require personal use of a calculator, 2) the identification feature not only confirms the identity of a peak but helps one keep track of the internal consistency of the performance of the system on a day to day basis, 3) if the column efficiency is deteriorating and the relative retention times no longer hold, the program will indicate so by not being able to identify peaks, 4) there is full editing capability of all entry values. The program recalculates/re-identifies peaks at each correction. 5) all needed information and data is presented on each sample for future reference, 6) the format is such that it is easy to relate particular data to a particular chromatogram, particular experiment etc. 7) one gets a screen presentation of the results and has the option to print the presentation.

TABLE 1

Computer Program Output for Retinyl Ester Analysis

Retinol and Retinyl Esters in Control-1 Rat Liver
Sample Number C-1 Replicate Number 1 Date 7/22/88
Chromatogram No. 121
Ret. Time of R16:0 31.03 % Water in Methanol 0.0
Int. Std. 20 ug/g tissue Int. Std. Area 144000

No.	Ret.Time	Area	Identity	Total ug	Percent
1	3.41	51660	Retinol	6.17	2.81
2	22.58	19136	Retinyl 16:1	2.96	1.35
3	24.41	19264	Retinyl 18:2	3.13	1.43
4	25.51	11200	Retinyl 15:0	1.69	0.77
5	31.03	1222000	Retinyl 16:0	189.99	86.58
6	36.37	8122	Retinyl 17:0	1.30	0.59
7	44.00	86720	Retinyl 18:0	14.20	6.47
			Totals	219.45	100.00

CL	R_T Span	CL	R_T Span	CL	R_T Span
Retinol	2.8-3.7	12:0	14.6-15.6	14:0	21.1-22.0
16:1	21.8-22.8	18:2	23.7-24.6	15:0	25.0-26.0
16:0	30.6-31.5	18:1	30.9-31.8	17:0	35.9-36.8
18.0	43.9-44.9				

RESULTS

Reversed Phase HPLC of Synthetic Retinyl Esters.

Fifteen retinyl esters were synthesized and their chromatographic behavior on an Ultrasphere ODS column was studied using methanol-water combinations as the eluting solvent. FIGURES 1-3 show plots of the Log k' versus % water in the eluting solvent for three series of retinyl esters. It will be noted that graphs with sets of near straight and parallel lines are generated.

In FIGURE 1 the esters are grouped as the set of standards most commonly used by investigators interested in analysis of retinyl esters extracted from biological sources (5,6). The Log k' is essentially linear with solvent composition and the lines

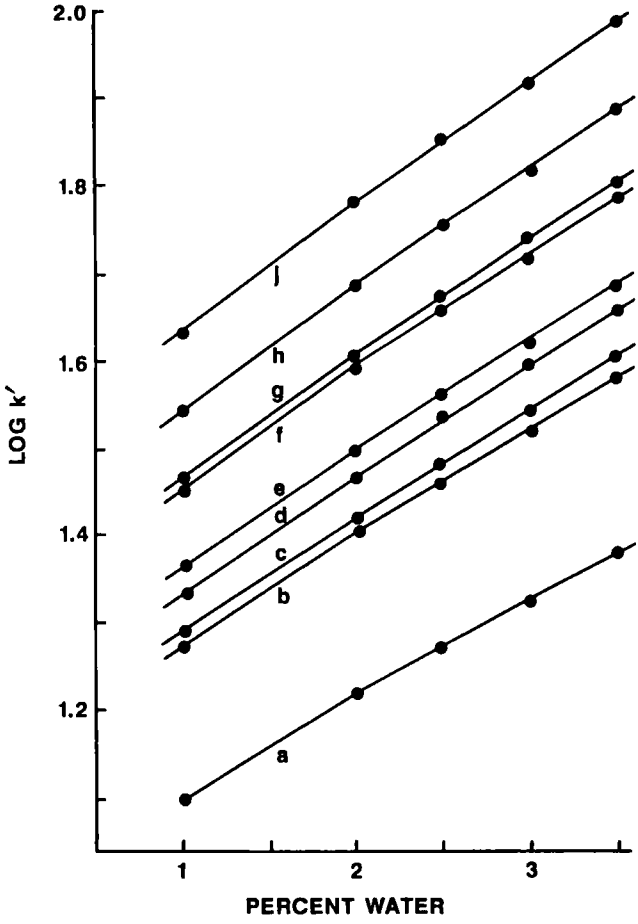


FIGURE 1. Effect of percent solvent composition on Log k' of retinyl esters. a, R12:0; b, R14:0; c, R16:1; d, R18:2; e, R15:0; f, R16:0; g, R18:1; h, R17:0; j, R18:0. See Methods for chromatography conditions.

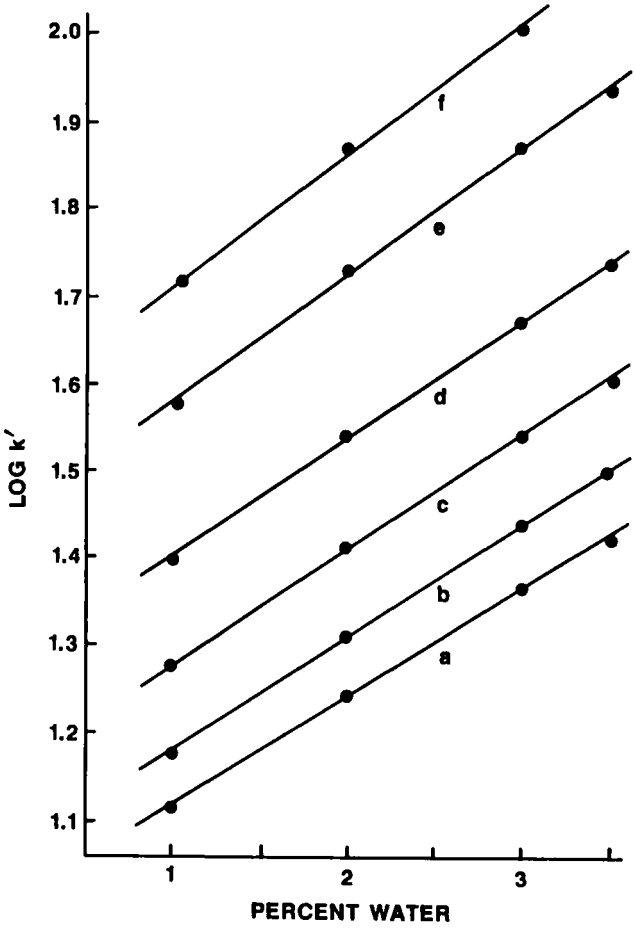


FIGURE 2. Effect of percent solvent composition on Log k' of retinyl esters. a, R18:3; b, R20:4; c, R20:3; d, R20:2; e, R22:2; f, R20:0. See Methods for chromatography conditions.

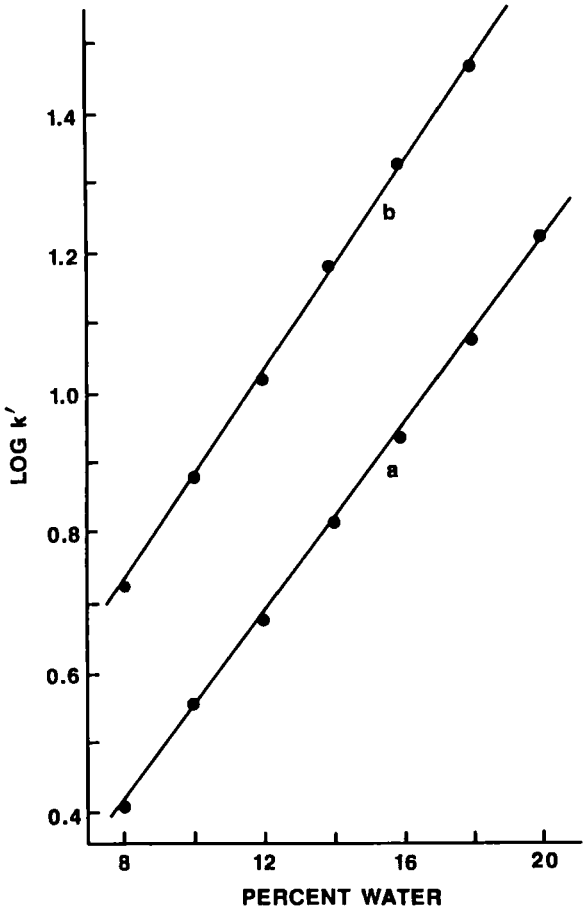


FIGURE 3. Effect of percent solvent composition on Log k' of retinol (a) and retinyl acetate (b). See Methods for chromatography conditions.

TABLE 2

Retention Times of Retinyl Esters as a Function of Solvent Composition.[a]

Retinyl Ester	Retention Times (Min) 1% Water	2% Water	3% Water	3.5% Water
R12:0	19.31	25.79	32.12	35.94
R18:3	25.28	33.27	43.45	49.42
R14:0	28.19	38.96	49.56	55.99
R20:4	28.98	38.65	50.98	58.26
R16:1	29.03	40.33	51.81	58.80
R18:2	31.86	44.75	58.11	66.27
R15:0	34.25	48.10	61.80	70.09
R20:3	35.80	48.46	64.51	73.99
R16:0	41.73	59.53	77.20	87.84
R18:1	42.68	61.11	80.15	91.61
R20:2	47.07	64.73	87.01	100.15
R17:0	50.91	73.77	96.58	110.14
R18:0	62.29	91.46	120.91	138.26
R22:2	69.42	98.53	134.71	156.10
R20:0	94.90	136.23	186.50	215.36

[a] Chromatography conditions were 1.5 mL/min on 4.6 mm x 250 mm, 5 u, Ultrasphere ODS column, using Methanol and Water for elution. Values represent typical runs.

for these compounds are approximately parallel in the solvent range from 1-3.5% water in methanol. The retention times for various esters at various solvent compositions are given in TABLE 2. Some of the lines, such as R14:0 vs R16:1 and R16:0 vs R18:1, are close together, but they never cross in the solvent range tested. Baseline resolution of R14:0 and R16:1 occurs at 3% water or above. Resolution of R16:0 and R18:1 is acceptable but not complete at 3 or 3.5% water.

Since retinyl esters can perhaps be formed from any biological fatty acid, we decided to synthesize esters that could be formed from a number of different naturally occurring polyunsaturated fatty acids. FIGURE 2 shows the chromatographic behavior of a series of retinyl esters whose acyl groups are

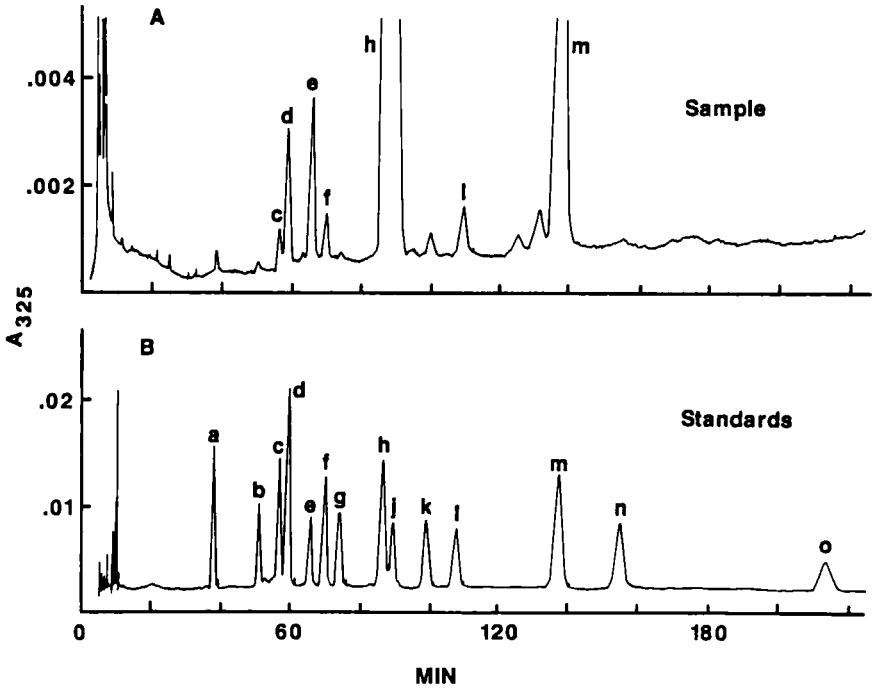


FIGURE 4. HPLC chromatogram of retinyl esters. A, Sample of whole lipid from rat liver. B, Synthetic standards. a, R12:0; b, R18:3; c, R14:0; d, R16:1 and R20:4; e, R18:2; f, R15:0; g, R20:3; h, R16:0; j, R18:1; k, R20:2; l, R17:0; m, R18:0; n, R22:2; o, R20:0. See Methods for chromatography conditions.

unsaturated (n-6) except R20:0. As can be seen, the compounds elute in the order R18:3 n-6, R20:4 n-6, R20:3 n-6, R20:2 n-6, and R22:2 n-6 and show a linear relationship between Log k′ and % water in methanol. When the compounds of this set were mixed with those of FIGURE 1, they could all be resolved at 3% water or higher except R16:1 and R20:4 or R14:0 and R20:4 (see FIGURE 4B and TABLE 2).

Retinol elutes from this column at much higher % water in methanol. FIGURE 3 shows Log k′ vs % water in methanol is linear for retinol and retinyl acetate. The retention times of retinol

ranged from 6.4 min (8% water) to 31.6 min (20% water) at 1.5 mL/min, while the corresponding values for retinyl acetate were 11.3 min and 78.4 min, respectively.

A plot of the Log (relative retention time x 10) for each retinyl ester derived from a saturated fatty acids versus carbon chain length of the acyl moiety, gave a straight line (not shown). The plot was linear for each solvent composition using the data presented in TABLE 3. The resulting lines were used to determine the values of the slopes (m) and the Log (RRT x 10)-intercepts (b) as given in TABLE 2. The values of all effective carbon numbers (ECNs) were then calculated from the equation using the appropriate constants. As can be seen in TABLE 3, the calculated values for the saturated acyl retinyl esters gave values very close to the unit values with standard deviations of ECN ranging from 0.01 to 0.10 and with an average deviation of 0.026. The standard deviations of the ECN values for the unsaturated acyl retinyl esters ranged from 0.03 to 0.10 with an average deviation of 0.070.

TABLE 3 also shows the change in ECN values of the unsaturated retinyl esters relative to the ECNs of the saturated esters with the same chain length. For convenience, the TABLE lists the retinyl esters that are difficult to separate from each particular unsaturated ester.

Analysis of Retinol and Retinyl Esters in Liver and Plasma.

The HPLC chromatogram for a mixture of 15 retinyl ester standards is shown in FIGURE 4B. Under the conditions used all of the esters resolve with the exception of R20:4 and R16:1 (peak d). Peak d had an area corresponding to the sum of R20:4 and R16:1. As can be seen in FIGURE 4A, retinyl esters extracted from liver show retention time identity with R14:0 (c), R16:1 or R20:4 (d), R18:2 (e), R15:0 (f), R16:0 (h), R17:0 (l) and R18:0 (m).

The analysis of retinol and retinyl esters in rat liver and plasma is given in TABLE 4. The identities of the esters are tentative at this point and are in accordance with those reported in the literature (5). Results of analysis of rat plasma showed that retinol is present and retinyl esters are absent. These

TABLE 3

Effective Carbon Numbers of Retinyl Esters.[a]

Retinyl Ester	Average ECN[b]	Change in ECN[c]	Difficult to Separate from:
R12:0	12.01 ± .03	--	
R18:3	13.36 ± .10	4.64	
R14:0	13.98 ± .01	--	
R20:4	14.08 ± .10	5.92	R14:0; R16:1
R16:1	14.17 ± .03	1.83	R14:0; R20:4
R18:2	14.68 ± .05	3.32	
R15:0	14.99 ± .01	--	R20:3
R20:3	15.16 ± .09	4.84	R15:0
R16:0	16.00 ± .01	--	R18:1
R18:1	16.15 ± .03	1.85	R16:0
R20:2	16.54 ± .09	3.46	
R17:0	17.02 ± .02	--	
R18:0	18.05 ± .03	--	
R22:2	18.53 ± .07	3.47	
R20:0	20.05 ± .10	--	

[a] The Log (RRT x 10) was plotted versus the carbon chain length (ECN) of the acyl group of the saturated retinyl esters. Slopes (m) of the lines and Log (RRT x 10)-intercepts at zero ECN (b) were used in the equation: Log (RRT x 10) = (m) ECN + b, where m = 0.08484, b = 0.3574 at 1% water; m = 0.09035, b = 0.4475 at 2%; m = 0.09537, b = 0.5253 at 3%; m = 0.09722, b = 0.5545 at 3.5%. RRTs were calculated from R_T of R16:0.

[b] Average ± standard deviations of values from four equations above.

[c] Change in ECN is the difference between the chain length and the average ECN of unsaturated esters.

analyses gave very consistent results with standard deviations of 9.5% amongst animals and an average of 1.3% for duplicate sample injections.

DISCUSSION

The plot of Log k' versus % water in the eluting solvent was almost linear in the case of each ester. Studies of other homologous saturated and unsaturated lipids on reversed phase

TABLE 4

Retinol and Retinyl Esters in Rat Liver and Plasma.

Retinyl Ester[a]	Liver ug/g[b]	Liver % Total	plasma ug/dl
R16:1	3.8 ± 0.5	1.8 ± 0.3	n.d.[c]
R18:2	4.5 ± 1.1	2.2 ± 0.5	n.d.
R15.0	1.7 ± 0.2	0.8 ± 0.1	n.d.
R16:0	159.6 ± 24.9	77.5 ± 5.4	n.d.
R17:0	1.6 ± 0.5	0.8 ± 0.2	n.d.
R18:0	19.2 ± 5.6	9.3 ± 2.2	n.d.
Retinol	15.0 ± 5.7	7.5 ± 3.0	51.9 ± 4.9[d]

[a] Identities based on literature identifications (5).
[b] Values are the means and standard deviations for data from six animals.
[c] n.d. is not detected.
[d] The standard deviation amongst animals was 9.5%. The average standard deviation on duplicate HPLC sample injections was 1.3%.

columns have been done and linearity between Log k' and solvent compositions has been observed for methyl esters (11), free fatty acids (11) and phospholipids (12,13).

The effective carbon number (ECN) is especially useful because it allows one to evaluate the effect on the chromatographic behavior due to introduction of double bonds into a molecule. Patton (12) and Smith (13) have successfully used the ECN in the study of saturated and unsaturated phospholipids.

From our studies, introduction of one double bond into the acyl portion of a retinyl ester has the chromatographic effect of removing 1.83-1.85 carbons (TABLE 3). This makes it clear that the monoenes will be difficult to separate from the corresponding saturated compounds with two fewer carbons, and if resolution does occur, the monoene should elute slightly later than the saturated compound.

The three dienes, R18:2, R20:2, R22:2 gave average ECN values of 14.68, 16.54, and 18.53, respectively (TABLE 3). The decrease

of ECN due to two double bonds was 3.32, 3.46 and 3.47, respectively, which was slightly less than twice the effect (3.66-3.70) of introduction of one double bond. This means that the diene retinyl esters should separate well from and elute slightly later than the ester with the saturated acyl group with four fewer carbons.

The trienes, R18:3 and R20:3, gave average ECN values of 13.36 and 15.16, respectively, corresponding to a decrease in ECN of 4.64 and 4.84 (TABLE 3). Thus even chain-length trienes may be difficult to separate from the odd chain saturated compounds with five fewer carbons. By extrapolation of this idea, it would be expected that R22:3 would elute very close to R17:0.

The only tetraene tested was R20:4 n-6 (retinyl arachidonate). Its average decrease in ECN was 5.92 (TABLE 3) giving it a chromatography property much like R14:0 and R16:1. Under the conditions tried, retinyl arachidonate could not be resolved from both R14:0 and R16:1 (see retention times in TABLE 2). We would predict that R22:4 would be difficult to separate from R16:0 and R18:1 on the basis of a decrease in ECN of 5.9 for tetraenes.

The enzyme systems involved in the synthesis of retinyl esters in various tissues of different species is currently under investigation in several laboratories. MacDonald has found an enzyme in the small intestine which utilizes lecithin as the acyl donor (14) and cellular retinol-binding protein, type II as the retinol donor. The enzyme apparently utilizes the sn-1-acyl moiety of lecithin as the acyl donor. Torma has found an Acyl-CoA:Retinol Acyltransferase in mouse epidermis (15). In rat liver, Ong (16) has found both an acyl-CoA-dependent and an acyl-CoA-independent enzyme that esterify retinol. Ball has similarly found an acyl-CoA:retinol acyltransferase in rat liver and mammary tumor microsomes (17). These latter workers were unable to form R20:4 when utilizing arachidonyl-CoA as the substrate.

The distribution of saturated and unsaturated fatty acids in tissue lipids varies with the species, the tissue and dietary conditions. However, the percent distribution of fatty acyl groups of retinyl esters does not match the percent distrubution of fatty acids in total lipids. But until more is known about the

tissue distribution and the number of enzymes involved in the biosynthesis of retinyl esters and their respective substrate specificities, one should assume that any naturally occurring fatty acid could be a precursor of retinyl esters. It is possible that retinyl esters derived from polyunsaturated fatty acids exist but have not been identified because they happen to elute in the vicinity of the even and odd chain saturated retinyl esters being used as HPLC standards.

The regularity of the HPLC behavior of the fifteen synthetic saturated and unsaturated retinyl esters reported here makes it possible to predict, with some degree of confidence, the chromatographic behavior of homologous unsaturated esters yet to be synthesized. We think this predictive element will alert investigators to the possibility that peaks attributed to certain odd chain esters may be unsaturated esters instead, and it will help in the selection of appropriate synthetic standards for HPLC.

The identities of liver retinyl esters in HPLC peaks in FIGURE 4A are tentative in some cases and are based on cochromatography with synthetic standards and are in harmony with literature identifications (5). Under the chromatography conditions used R16:1 and R20:4 did not separate (peak d). Other investigators have reported that R16:1 was present in liver lipid extracts (4,5) and rat milk (6), based on co-chromatography with authentic R16:1. However, no R20:4 standard was provided in those studies. The retinyl ester labeled R15:0 (peak f) is apparently not R20:3 n-6 (peak g), but it is still possible that it is R20:3 n-3 although Aveldano (11) reports that the methyl ester and the free fatty acid of 20:3 n-3 elutes from a reversed phase column slightly later than 20:3 n-6. We speculate that the ester labeled R17:0 could be one of the R22:3 series.

This study points out the need for investigators to identify the retinyl esters by more rigorous means than co-chromatography with synthetic standards. Even in cases where the esters have been identified by more rigorous criteria, it is possible that some peaks contain mixtures that include esters derived from unsaturated fatty acids. We think that any study that utilizes the identity of a retinyl ester as the basis for a conclusion will need to investigate its true identity in the species and tissue

studied. The identity of the naturally occurring retinyl esters in organs will be the topic of another study.

One could argue that it is not important to rigorously analyze and identify any retinyl ester but R16:0 and R18:0 which represent more than 90% of the total retinyl ester in liver. However, one only need remember how much has been learned about the important roles of polyunsaturated fatty acids in metabolic control mechanisms to dispel the idea. It would be interesting indeed if it turned out that unsaturated retinyl esters had very unusual and specific functions in which both moieties played a role in control mechanisms. There is precedence for such multiple roles being mediated by a single molecular structure. For example phosphoinositides provide diglyceride, inositol phosphates and arachidonate in the second messenger system of the plasma membrane. The diglyceride controls protein kinase C, inositol phosphates regulate calcium ions and arachidonate is converted to prostaglandins, thromboxanes and leukotrienes. In a yet to be understood way, they are involved in a sequence of events that ultimately leads to mitogenesis.

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