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SEPARATION AND ESTIMATION OF RETINYL FATTY ACYL ESTERS IN TISSUES OF NORMAL RAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatography system for the complete separation of naturally occurring retinyl fatty acyl esters (RFAE) is described. The sensitivity of the method allows the detection of as little as 40 pmol of the various RFAE. The procedure was applied to the separation and estimation of endogenous RFAE present in tissues of normal rats; in addition, the incorporation of [^{3}H]retinyl acetate into RFAE was also investigated. Retinyl palmitate is the major fatty acyl ester (79%) present endogenously in various tissues. However, eight other RFAE were also present in some tissues. At 24 hours after the injection of the label, radioactivity present in retinol and its metabolites was recovered mainly in liver tissue followed by kidneys, adrenals, lungs, intestine, trachea, testis, blood, heart and spleen. However, it was found that, in liver tissue, the specific radioactivity (dpm/nmol) of several RFAE was greater than that of retinyl palmitate (retinyl laurate 66-fold, retinyl pentadecanoate 5-fold, retinyl palmitoleate 4-fold).

INTRODUCTION

Retinol is stored in large quantities in liver as fatty acyl esters [1] and is released from the esters as and when it is required for its action. To study the biosynthesis and turnover of the various retinyl fatty acyl esters (RFAE), an effective and sensitive technique to separate and estimate the RFAE is required. In the past, the separation of RFAE was achieved either by reversed-phase paper chromatography [2] or by thin layers of silica gel [3, 4], and was quantitated by fluorometry [5]. However, the recoveries of RFAE by these techniques are poor, and losses up to 50% were reported [5]. De Ruyter and De Leenheer [6] developed a reversed-phase high-performance liquid chromatographic (HPLC) method for the simultaneous determination of retinol and

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retinyl esters and later modified the method to separate retinyl oleate from palmitate using silver ions in the mobile phase [7]. In the present paper we describe a fast and reliable HPLC technique for the separation and estimation of naturally occurring RFAE. The method has been applied for the determination of endogenous RFAE present in various tissues of normal rat.

MATERIALS AND METHODS

Chemicals

All-*trans*-retinol was a gift from Hoffmann-La Roche (Nutley, NJ, U.S.A.). The fatty acyl chlorides (99% pure) were purchased from Sigma (St. Louis, MO, U.S.A.). [11-^{3}H]Retinyl acetate (specific activity 2.9 Ci/mmol) was a gift from Dr L. De Luca, NIH and was purified by HPLC using an ODS-1 column [8] and found to be greater than 95% pure. Neutral alumina (Brockmann activity 1; 80—200 mesh) and HPLC grade solvents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Chemical synthesis of RFAE

Retinyl laurate, myristate, palmitoleate, linoleate, pentadecanoate, palmitate, oleate, heptadecanoate, and stearate were synthesized by reaction of all-*trans*-retinol with the corresponding acyl chlorides as described by Huang and Goodman [4], with slight modifications. To 10 μg of all-*trans*-retinol in a test tube were added 500 μl of anhydrous pyridine and 15 μg of the appropriate fatty acyl chloride. The test tube was warmed to 50—55°C under nitrogen for 1 h. Then the pyridine in the tube was completely evaporated and the contents were extracted with hexane. The hexane extract was concentrated to a small volume and directly applied to a 10% (v/w) water deactivated alumina column (10 cm × 1.5 cm I.D.). The column was first washed with hexane (20 ml). The fatty acyl esters were then eluted from the column using 2% acetone in hexane as the eluting solvent. Retinyl esters obtained from alumina column chromatography were further purified by HPLC on a Partisil ODS-1 column [8]. The yields of different esters of retinol varied between 75 and 95%.

Metabolic studies in normal rat tissues with [11-^{3}H] retinyl acetate

Sprague—Dawley rats (200—250 g) maintained on normal laboratory chow were injected intraperitoneally with 25 μCi (2.83 μg) of [11-^{3}H]retinyl acetate in 50 μl ethanol. Rats were sacrificed by cervical dislocation 24 h after the injection of the label. The tissues were immediately removed, washed with cold phosphate-buffered saline and lyophylized overnight.

Extraction of tissues

The lyophilized tissues were ground to powder with a pestle and mortar and extracted successively with 20 ml of 99% methanol per gram of tissue and with 50 ml of hexane. The combination of 99% methanol and hexane was found to be effective in extracting more than 90% of the polar metabolites and the fatty acyl esters of retinol. The 99% methanol and hexane extracts were evaporated

separately and the residues were combined and redissolved in chloroform—methanol (1:1). An aliquot was counted and used for HPLC.

High-performance liquid chromatography

HPLC was carried out on a Beckman Model 322 MP programmable liquid chromatographic system. The UV spectrophotometer was a Hitachi Model 100-40 equipped with a wavelength variable between 195 and 850 nm (maximal sensitivity, 0.01 a.u.f.s.). The HPLC column, Partisil 10 ODS-1 (25 cm × 4.6 mm I.D.) was obtained from Whatman (Clifton, NJ, U.S.A.). Ultrasphere ODS (5-μm particle size, 25 cm × 4.6 mm I.D.) was purchased from Beckman Instruments (Toronto, Canada). The Ultrasphere column was found to be the column of choice for the separation of RFAE. In a typical separation the column was eluted with methanol—water (98:2) at a flow-rate of 1.5 ml/min for the first 62 min to elute retinyl laurate, myristate, palmitoleate, linoleate, pentadecanoate, palmitate and oleate, and 2.0 ml for the following 28 min to elute retinyl heptadecanoate and stearate.

Determination of extinction coefficient ($\epsilon^{1\%}_{1\,cm}$ in ethanol) of standard RFAE

$\epsilon^{1\%}_{1\,cm}$ of various RFAE were measured at 325 nm in ethanol and the following values were obtained: retinyl laurate 1052, myristate 990, pentadecanoate 970, palmitoleate 944, palmitate 948, linoleate 900, oleate 890, heptadecanoate 916 and stearate 870. These values are in close agreement with the theoretical values calculated from the reported $\epsilon^{1\%}_{1\,cm}$ of retinyl palmitate, which is 940 at 325 nm [9].

Quantitation of RFAE

The procedure is standardized by adding known amounts of a mixture of standard RFAE to 1 g of liver tissue from a vitamin A deficient rat and taking these through the entire procedure of lyophilization, extraction and separation by HPLC. Peak areas of different RFAE were measured at 325 nm by triangulation [10] and plotted against the amounts. The losses (usually 5—10%) due to extraction and evaporation were corrected for the exact amount. The amounts of RFAE in the unknown samples were determined by measurement of the peak area and by use of the standard curve.

Hydrolysis of RFAE

Either the UV or radioactive RFAE peaks were collected in large amounts by repeated injections and were saponified at 55°C for 90 min in 200 μl of 0.4 *M* ethanolic potassium hydroxide under nitrogen. The mixture was then diluted with 500 μl of water and extracted 3—4 times with 2 ml of petroleum ether in order to remove retinol. The petroleum ether fraction was then concentrated and directly injected onto an ODS-1 column to detect retinol [8]. The hydrolysis and recovery of retinol from RFAE, based on radioactivity, was greater than 90%.

Determination of radioactivity

Samples from the column were collected at 0.5- or 1.0-min intervals and were counted after adding 7.5 ml of phase combining system (Amersham).

Samples were counted in a Beckman Model LS-230 liquid scintillation counter. The efficiency of counting for ^{3}H was 31%.

RESULTS

Separation of RFAE by HPLC

Fig. 1 illustrates the chromatography profile from a reversed-phase HPLC system that separates standard fatty acyl esters of retinol. In this system, an Ultrasphere ODS column (5 μm) was used and developed with a solvent system of methanol—water (98:2). An effective separation of known naturally occurring RFAE was achieved, including the separation of retinyl myristate and palmitoleate and of retinyl palmitate and oleate. Peak areas of various RFAE were measured at 0.01 a.u.f.s. setting as described in Materials and methods. A linear relationship between the peak areas of different RFAE and their amounts (40—120 pmol) was observed.

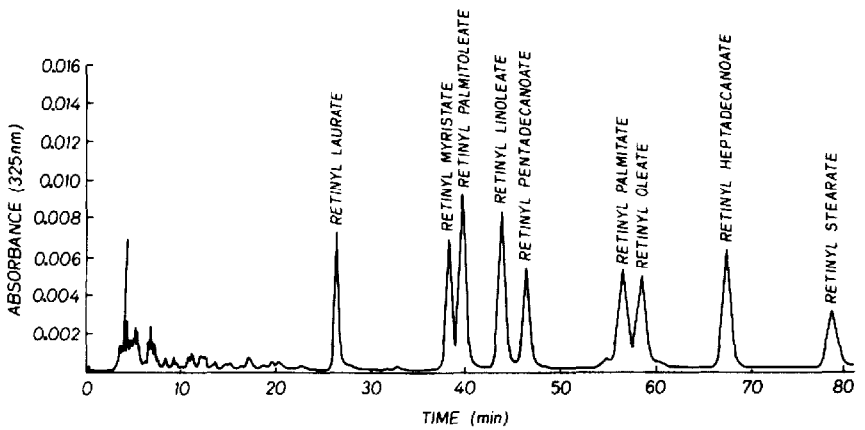

Fig. 1. Separation of a mixture of nine standard RFAE on an Ultrasphere ODS (5 μm) column. Conditions of elution are given in Materials and methods. The quantity of each standard RFAE was 150—200 ng.

The recovery of all standard RFAE from the column based on UV spectrophotometry was greater than 95%. Known amounts of individual RFAE were mixed with 1 g of liver tissue from a vitamin A deficient rat, extracted and estimated as described in Materials and methods. The recoveries of various esters of retinol varied between 85 and 95%.

Tissue distribution of radioactivity present in retinol and its metabolites 24 h after the injection of [11-^{3}H] retinyl acetate

The distribution of radioactivity (dpm/g or dpm/ml) as well as the percentage of injected radioactivity in different tissues of a normal rat is presented in Table I. The highest concentration of radioactivity was observed in liver. This was followed in descending order by kidneys, adrenals, lungs, intestine, trachea, testis, blood, heart and spleen tissues. The radioactivity, expressed as the percentage of injected dose was greater in liver (29.2%) followed by kidneys, intestine and lungs.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF A NORMAL RAT, INJECTED WITH [11-^{3}H]RETINYL ACETATE

Normal rat was injected intraperitoneally with 25 μCi (2.83 μg) of [11-^{3}H]retinyl acetate and sacrificed after 24 h. Retinol and its metabolites were extracted from the tissues as described in Materials and methods.

Tissue	^{3}H (dpm/g or dpm/ml • 10^{-6})	Percentage of injected dose
Liver	2.033600	29.20
Kidney	1.056971	3.16
Adrenals	0.435429	0.04
Lungs	0.274784	0.74
Intestine	0.187210	1.06
Trachea	0.154280	0.01
Testis	0.057220	0.24
Blood	0.056686	0.51
Heart	0.054026	0.09
Spleen	0.044505	0.04

Separation of RFAE in normal rat tissues

Fig. 2A shows the chromatograms of the lipid extract of normal rat liver tissue. Several peaks (4–11) were positively identified as fatty acyl esters of retinol by UV monitoring at 325 nm and also by co-chromatography with standard RFAE. Furthermore the hydrolysis of each of these fractions yielded retinol as the only material absorbing at 325 nm. The labelling of RFAE by [11-^{3}H]retinyl acetate is shown in Fig. 2B. Hydrolysis of each of the radioactive peaks (3–11) yielded [^{3}H]retinol. The polar metabolites of retinol, such as retinyl phosphate, mannosyl retinyl phosphate, retinyl glucuronide, retinoic acid, are eluted in the area of radioactive peak 1 and hence constitute a mixture of polar metabolites of retinol. The radioactive peak 2 in Fig. 2B was identified as being [^{3}H]retinol, as judged by its retention time with standard retinol when separated on an ODS-1 column with acetonitrile—water mixtures [8]. Radioactive peak 3 appears to be an ester of retinol, but was not identified further and was not found to be present in other tissues.

Since retinyl palmitate was present in large quantities in liver tissue, a broad peak of retinyl palmitate can be seen in Fig. 2A as peak 9. Retinyl oleate which elutes after retinyl palmitate, therefore was not resolved properly. For this reason, the lipid extract was further diluted 20-fold and injected into the chromatograph to resolve retinyl oleate from palmitate. Fig. 3A illustrates such a chromatogram which indicates that retinyl oleate is not present as one of the major RFAE in the liver tissue. This was further confirmed in the labelling of the RFAE (Fig. 3B).

Quantitation of RFAE in normal rat tissues

The amounts of endogenous RFAE expressed as nmol per g or per ml in various tissues are presented in Table II. Retinyl palmitate and stearate were

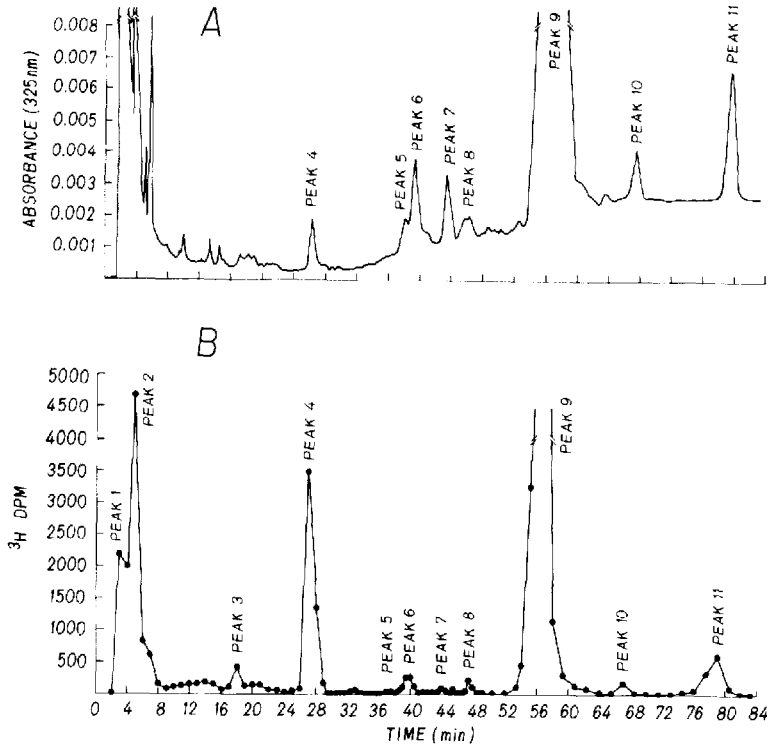

Fig. 2. HPLC of lipid extract of normal rat liver. One rat was injected with 25 μCi of [11-^{3}H]retinyl acetate intraperitoneally and sacrificed after 24 h. An aliquot of the lipid extract (20 μl) was injected in chloroform—methanol (1:1) onto an Ultrasphere column and eluted with methanol—water mixtures as described in Materials and methods. The UV was monitored at 325 nm to detect RFAE (A) and 0.5- or 1.0-min fractions were collected and counted (B) as described in Materials and methods. Peaks: 4 = retinyl laurate, 5 = myristate, 6 = palmitoleate, 7 = linoleate, 8 = pentadecanoate, 9 = palmitate, 10 = heptadecanoate and 11 = stearate, identified by co-chromatography with standard RFAE. Radioactive peaks: 3 = an unidentified ester of retinol, 2 = retinol and 1 = a mixture of polar metabolites of retinol.

present in all the tissues examined. Retinyl oleate was present in small amounts in intestine and kidney and could not be detected in liver tissue.

In liver tissue, retinyl palmitate constituted 79% of the total RFAE, followed by stearate (7%), palmitoleate (3.7%), linoleate (2.9%), heptadecanoate (2.2%), myristate (2.1%), pentadecanoate (1.6%), and laurate (1.2%). However, the labelling of these esters by [11-^{3}H]retinyl acetate indicated an entirely different pattern (Table II). Retinyl laurate was found to be highly labelled and its specific radioactivity (S.A.) (dpm/nmol) was 66-fold greater than that of retinyl palmitate. In addition to retinyl laurate, the S.A. of several other RFAE were also found to be greater than that of retinyl palmitate, retinyl pentadecanoate (5-fold), and palmitoleate (4-fold).

DISCUSSION

The separation of naturally occurring RFAE by HPLC has been previously

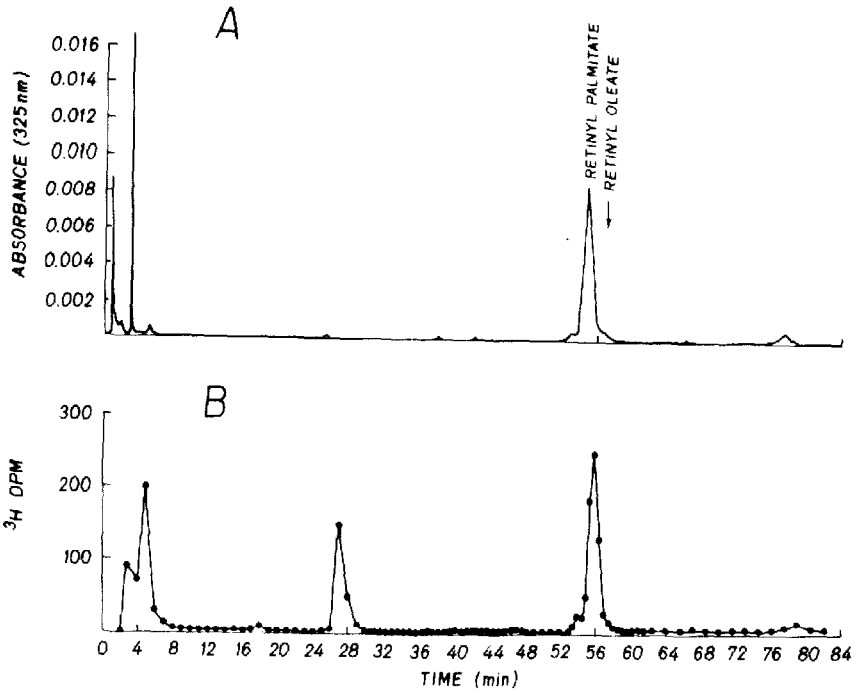

Fig. 3. HPLC of lipid extract of normal rat liver. The lipid extracts were prepared from liver tissue as described in Materials and methods and diluted 20-fold to resolve retinyl oleate from retinyl palmitate. (A) represents the detection by UV and (B) is the tracing of radioactive peaks. The conditions of elution were as described in Materials and methods.

reported by other workers [6, 7, 11]. De Ruyter and De Leenheer [7] obtained reasonably good separation of RFAE using silver ions in the mobile phase. However, in their study, no attention has been given to the effect of silver ions on the retention of polar metabolites of retinol. Moreover, the applicability of the method for the separation of complex RFAE present in tissues like liver has not been reported. In our method, we have shown that polar metabolites of retinol are clearly separated from RFAE. It can be seen from Fig. 1, that the effective separation of a variety of esters of retinol was achieved by an Ultrasphere ODS column with methanol—water as the eluting solvent. Using this solvent system the polar metabolites of retinol are eluted in the void volume of the column.

Futterman and Andrews [3] reported the presence of at least eight RFAE in rat liver: retinyl palmitate, stearate and oleate being the major components. In our studies, retinyl oleate could not be detected in liver tissue. This discrepancy could be due to the method used by Futterman and Andrews [3] for the separation and identification of RFAE. They separated RFAE by thin layers of silica gel and identified them by determining the fatty acids using gas—liquid chromatography. Although they were able to separate various RFAE by thin-layer chromatography, it is not certain that they have separated RFAE from other lipids present in liver tissue. Therefore separation and identification of RFAE by these techniques might have given misleading results. Since retinyl palmitate and oleate are eluted closely in our system, it is possible

TABLE II

COMPOSITION OF RETINYL FATTY ACYL ESTERS IN TISSUES OF A NORMAL RAT

RFAE	Liver		Kidneys		Lungs		Intestine		Testis	
	nmol/g	S.A.*	nmol/g	S.A.	nmol/g	S.A.	nmol/g	S.A.	nmol/g	S.A.
Retinyl laurate	9.9	12952	N.D.**		N.D.		N.D.		N.D.	
Retinyl myristate	17.4	302	N.D.		N.D.		N.D.		N.D.	
Retinyl palmitoleate	30.7	740	0.4	3242	N.D.		N.D.		0.069	9244
Retinyl linoleate	24.1	234	0.3	8500	N.D.		3.50	600	0.034	10900
Retinyl pentadecanoate	13.1	1038	N.D.		N.D.		N.D.		N.D.	
Retinyl palmitate	651	195	38.5	606	15.5	498	7.43	248	20.1	478
Retinyl oleate	N.D.		N.D.		N.D.		2.3	416	N.D.	
Retinyl heptadecanoate	18.4	454	N.D.		N.D.		N.D.		N.D.	
Retinyl stearate	57.10	529	1.57	3931	5.99	508	N.D.		0.14	2753

*S.A. = specific radioactivity, expressed as dpm/nmol.
**N.D. = not detected endogenously.

that small amounts of retinyl oleate, if present, might have been masked by the presence of large amounts of retinyl palmitate. Nevertheless, the results indicate that retinyl oleate is not one of the major RFAE present in the liver tissue as claimed by other workers [3, 12].

At 24 h after the injection of [11-^{3}H]retinyl acetate to the normal rat, the highest radioactivity was observed in liver and kidney tissues. This confirms the previous observations of Goodman et al. [12] who reported similar radioactivity distribution in liver and kidney tissues after intravenous administration of [15-^{14}C]retinol in chylomicrons. In addition, we also observed a considerably high concentration of radioactivity in adrenals. This is in general agreement with previous studies by Sundaresan and Sundaresan [13] and Willmer and Laughland [14].

The study of the labelling of various esters of retinol in liver tissue yielded interesting results. The specific radioactivity (dpm/nmol) of some RFAE such as retinyl laurate, pentadecanoate and palmitoleate was found to be higher than that of retinyl palmitate 24 h after the injection of the label. According to the reports of Reitz et al. [15] and Varma and Beaton [16] it is unlikely that newly administered labelled vitamin A mixes uniformly with the endogenous pool of RFAE within 24 h after injection. However, recent reports [17, 18] indicate that a portion of newly absorbed vitamin A is rapidly metabolized in the liver possibly in a separate compartment (pool 1) and that the excess would go for storage (pool 2). Labelling of RFAE at 24 h may represent the metabolism of retinol in pool 1 of liver. This is supported by the finding that the specific radioactivity of certain RFAE increase rapidly, possibly due to the rapid turnover of these esters in pool 1.

In conclusion, it is believed that the HPLC technique described in this paper will provide an excellent tool to study further the distribution, turnover and metabolism of natural RFAE in tissues.

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