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Simultaneous determination of retinyl esters and retinol in human livers by reversed-phase high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of retinol and retinyl esters in human liver samples is presented. The free retinol and the prevalent retinyl esters (retinyl palmitate, oleate and stearate) are resolved within less than 30 min, using an octasilyl (C_8)-substituted column and an isocratic elution with methanol–water as mobile phase. This method allows to determine in duplicate all retinyl ester concentrations in small liver samples (3–10 mg of fresh tissue). The results obtained from thirteen patients without liver disease are described.

1. Introduction

Vitamin A (retinol) is a fat-soluble vitamin which is essential for vision and reproduction and which regulates differentiation and growth of many cell types in animals and humans. Over 90% of the total body reserve of vitamin A is stored in the liver of well nourished individuals. Stellate cells (Ito cells or fat-storing cells) are the main storage site of vitamin A derivates (75–85% of liver vitamin A), mostly esterified (98%) with long-chain fatty acids. Retinyl palmitate is the major ester with retinyl oleate and stearate the next most prevalent esters (for review see Ref. [1]). During liver injury, especially in chronic alcoholics, a significant depletion in hepatic vitamin A was observed [2,3] and the retinyl ester composition of the liver was altered: retinyl oleate was increased with a corresponding decrease in retinyl palmitate, and an increase in the relative amounts of unesterified retinol was also observed [4–6].

Normal levels of plasma retinol are maintained over a wide range of hepatic concentrations. Plasma levels of vitamin A correlate poorly with depletion of hepatic retinol concentrations [2,7]. Thus, analysis of liver vitamin A concentrations should generally provide the best estimate of body stores and it may be of importance to know the amounts of the main retinyl esters.

Different reversed-phase high-performance liquid chromatographic (HPLC) methods have been described. Some of them are time-consuming [9–11], or did not achieve a sufficient separation between palmitate and oleate [8,12–14].

We developed a sensitive micromethod to

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simultaneously determine retinyl esters and retinol with good resolution of retinyl palmitate and oleate. This method was applied to human liver samples.

2. Experimental

2.1. Instrumentation

The HPLC analysis was performed using a Waters Model 600E Multisolvent Delivery system and a Waters 715 UltraWisp autosampler (Waters Chromatography Division, Millipore, Milford, MA, USA). The analytical column used was a 5-μm Supelcosil LC-8 250 mm × 4.6 mm I.D. (Supelco, Bellefonte, PA, USA) preceded by a RP-8 New Brownlee guard cartridge system (7-μm particles; 15 × 3.2 mm I.D.; Applied Biosystems, San José, CA, USA). Detection was carried out with a Waters Model 940 Programmable Photodiode Array detector. Acquisition, integration and processing were performed by Baseline Work Station (Waters) using a NEC APC III Microcomputer (NEC Information System, Boxborough, MA, USA).

2.2. Reagents

Absolute ethanol and methanol were analytical-grade reagents purchased from Carlo Erba (Milan, Italy); *n*-hexane from Merck (Darmstadt, Germany). Pyrogallol and all-*trans*-retinol were purchased from Fluka Chemika (Buchs, Switzerland), and all-*trans*-retinyl acetate, all-*trans*-retinyl palmitate type IV and oleoyl chloride from Sigma (St. Louis, MO, USA). Retinyl stearate was a gracious gift from Hoffmann-La Roche Division (France). Retinyl oleate was synthesized by reaction of retinol with the corresponding acyl chloride as described by Huang and Goodman [15].

2.3. Standard curves

All solid components (all-*trans*-retinol, all-*trans*-retinyl acetate, all-*trans*-retinyl palmitate, retinyl stearate) were kept at −20°C. Ethanolic working standard solutions of all-*trans*-retinol (2 mmol/l) and all-*trans*-retinyl acetate (0.3 mmol/l) were also kept at −20°C for one month. The accurate concentration of vitamin A solution was determined spectrophotometrically at 325 nm (Spectrophotometer Uvikon 930, Kontron Instruments, Zurich, Switzerland), using $\epsilon = 52600$ mol^{-1}. A retinol calibration curve was generated by injecting 50 μl of methanolic solutions of retinol from 0.15 to 15 μmol/l with 30 μmol/l of retinyl acetate as the external standard. The standard curve was established using retinol/retinyl acetate peak-area ratios versus retinol concentrations. Retinyl ester concentrations were calculated with a retinol calibration curve and expressed as retinol equivalent.

2.4. Preparation of liver samples

The whole procedure was performed under lighting as weak as practicable.

Retinyl esters extraction

After being accurately weighed, each biopsy sample was extracted. The liver sample (2 to 10 mg) was homogenized, using a 7-ml Tenbroeck grinder (Bioblock Scientific, Illkirch, France) with 1.5 ml absolute ethanol containing 1% of pyrogallol (0.158 mmol/l). After shaking 30 min at + 4°C, distilled water (100 μl) was added and the mixture was extracted twice with 3 ml of *n*-hexane. After centrifugation (15 min, 3000 *g*, + 4°C), the two *n*-hexane layers obtained were treated separately, all drawn off, and evaporated quickly to dryness in a 50°C water bath under a gentle stream of nitrogen. The evaporation must be as quick as possible to avoid hydrolysis of the retinyl esters. The residues were dissolved in 200 μl of methanol and spiked with retinyl acetate (30 μmol/l).

Total retinol determination

Weighed liver samples were homogenized with 1.5 ml of 10% potassium hydroxide in 95% ethanol and submitted to hydrolysis. The sam-

ples were saponified for 30 min at 60°C, cooled in an ice bath and then extracted twice with 800 μl of distilled water and 1.9 ml of *n*-hexane (as described in Ref. [16]). The *n*-hexane layers were then treated as above without retinyl acetate spiking.

2.5. Chromatographic analysis

A 50-μl volume of each extract was injected onto the column. Separations were achieved by isocratic elution using methanol–water (94:6, v/v) as mobile phase at a flow-rate of 1.5 ml/min and the effluent was monitored at 325 nm during 30 min. All runs were performed at ambient temperature. Retinol, retinyl palmitate, oleate and stearate were identified by comparison of retention times with those of standards injected separately onto the same column, and also by spiking liver extracts with the different retinyl esters standards. In addition to chromatographic analysis monitored at 325 nm, some extracts were monitored at 292 nm to facilitate recognition of non-retinoid peaks. The photodiode array spectrophotometric detector was also used to confirm the characteristic absorption spectrum and purity of retinyl ester peaks. Each retinol or retinyl ester/retinyl acetate peak-area ratio was compared to the calibration curve. The results obtained for the two hexanic extractions of the same sample were summed to give the final result. All data were expressed in nmol equivalent retinol per gram of liver weight.

2.6. Subjects

Human liver samples were obtained during abdominal surgery for diagnosis purposes from 13 hospitalized patients, 2 women and 11 men, 29 to 73 years old. None of the patients had alcoholic liver disease or overt malnutrition or fat-soluble vitamins supplementation. Each specimen was placed in a hermetically closed vial, immediately frozen and stored in the dark at −80°C until used.

3. Results

3.1. Chromatographic separation

The separation of a mixture of retinol and four retinyl esters is presented in Fig. 1A. This chromatogram, obtained by injecting about 7.5 nmol retinol equivalent, allowed ester peak identification and retention time determination. Fig. 1B shows a chromatogram obtained from a liver sample. Retention times for retinol and the three main retinyl esters (retinyl palmitate, oleate and stearate) were 3, 17, 18 and 23 min, respectively. The retention time for retinyl acetate was 3.7 min. However, for some very lipidic samples, little variations of palmitate, oleate and stearate retention times were observed. In these cases, the retinyl esters were identified by spiking the evaporated residues with retinyl ester standards. Retinyl esters clearly predominate in liver, but retinol may also be quantitatively measured.

3.2. Recovery

Recoveries of 81 to 92% of retinyl palmitate, 80 to 100% of retinyl oleate and stearate were obtained after a single *n*-hexane extraction. A second extraction with *n*-hexane improved the extraction to 100% of palmitate. But this procedure recovered at best only 50% of retinol and retinyl acetate. This explains why inclusion of retinyl acetate as an internal standard failed to correct differences in the efficiency of the extraction. So the samples were spiked with retinyl acetate just before injection.

After basic hydrolysis of two samples containing 1220 nmol/g and 1950 nmol/g of total retinyl esters, the recovery of total retinol was 102% and 96%, respectively.

3.3. Precision

The within-day and between-day assays were done by analysing human liver samples of ca. 5

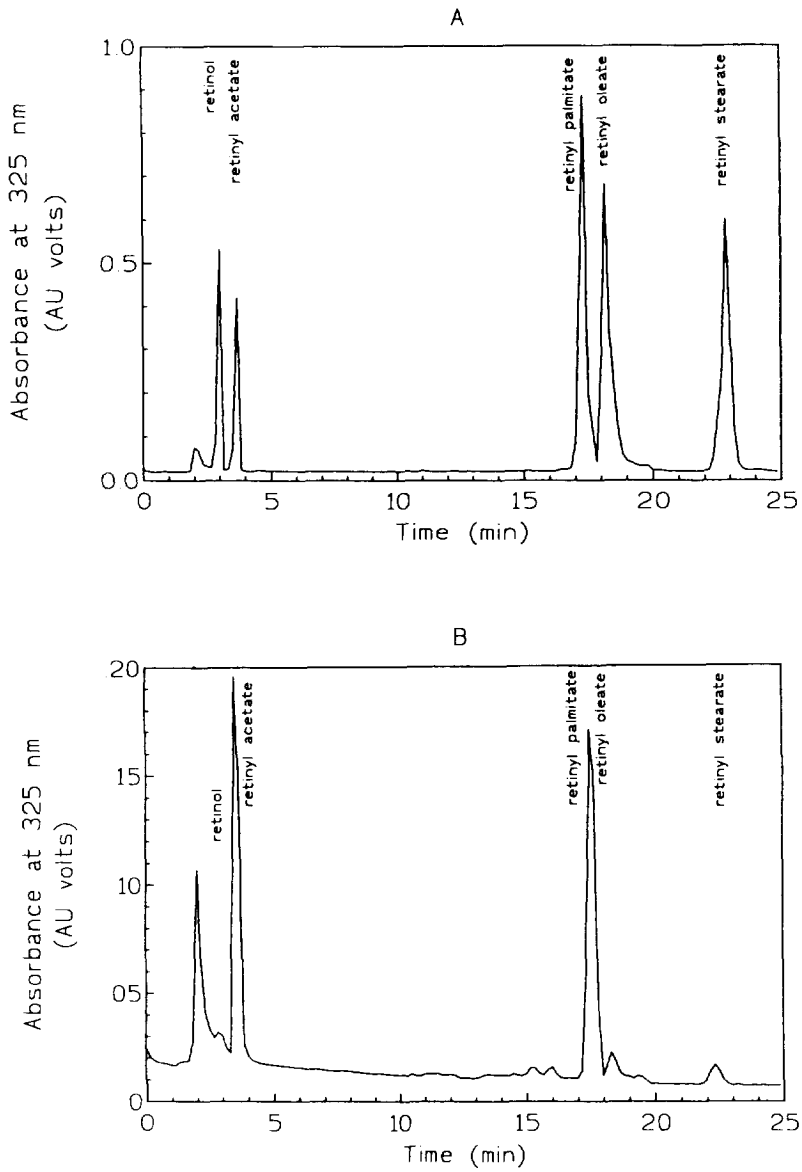

Fig. 1. Chromatographic separation: (A) mixture of retinol, retinyl acetate, palmitate, oleate and stearate containing ca. 7.5 nmol retinol equivalent injected; (B) human liver sample containing 692 nmol/g of total vitamin A.

mg weight. The results obtained are shown in Table 1.

The C.V. did not exceed 11% for the within-day assay and 15% for the between-day assay, except for retinol. This could be explained by the lowest concentrations of retinol found in human liver samples.

3.4. Linearity and detection limit

Assay linearity and quantification limit were examined by analysing methanolic solutions of retinol and retinyl palmitate because of endogenous concentrations of retinol and retinyl esters in the biological samples.

Table 1
Precision: within-day and between-day assays

Compound	Within-day				Between-day	
	Sample A ($n = 4$)		Sample B ($n = 3$)		Sample A ($n = 5$)	
	Mean ± S.D. (nmol/g)	C.V. (%)	Mean ± S.D. (nmol/g)	C.V. (%)	Mean ± S.D. (nmol/g)	C.V. (%)
Total vitamin A	1933 ± 177	9.1	567 ± 59	10.4	1920 ± 280	14.6
Free retinol	20 ± 6	30	nd[a]	–	16 ± 4	25
Retinyl palmitate	1665 ± 152	9.1	506 ± 56	11	1668 ± 240	14.4
Retinyl oleate	104 ± 8	7.7	33 ± 3	9	112 ± 14	12
Retinyl stearate	145 ± 11	7.6	28 ± 0.6	2	143 ± 19	13

[a] nd = Not detected.

The linearity was tested by injecting increasing concentrations of retinol over a range from 0.15 to 15 μmol/l. The analytical method was linear up to 15 μmol/l. Specimens with retinyl ester concentrations outside of the linear range were diluted with methanol and reanalyzed.

The quantification limit tested for retinol and retinyl palmitate, assuming that the signal-to-noise ratio should be at least 3, was 0.15 μmol/l (7.5 pmol injected) and 0.5 μmol/l (25 pmol injected), respectively. With 5 mg hepatic sample, the quantification limit of retinol and retinyl palmitate was 6 nmol/g and 20 nmol/g, respectively, in the biopsy.

The linearity of the extraction was tested over a range from 3 to 10 mg of liver weight. Satisfactory analyses were possible with biopsy samples as small as 3 mg and linear up to 10 mg with reliable chromatographic resolution of retinyl esters.

3.5. Human liver samples

The results obtained from human liver samples are detailed in Table 2. The median value, the first and third quartiles are shown for total vitamin A, free retinol and retinyl esters. Free retinol and retinyl esters are also expressed as percentage of total retinol.

4. Discussion

In the last few years, the reversed-phase HPLC was used to achieve the separation of several long-chain retinyl esters. De Ruyter and

Table 2
Hepatic concentrations, percentage of free retinol and of the main retinyl esters in thirteen human liver samples

Compound	Median (Q_1, Q_3)[a] (nmol/g)	Percent of total retinol (mean ± S.D.)
Total vitamin A	422 (362, 1252)	–
Free retinol	10 (–[b], 27)	2.9 ± 3.2
Retinyl palmitate	398 (311, 1015)	85.8 ± 5.1
Retinyl oleate	24 (17, 94)	5.4 ± 2.5
Retinyl stearate	29 (17, 85)	5.8 ± 2.4

[a] Q_1 = first quartile, Q_3 = third quartile.
[b] For three patients, the free retinol was under the detection limit.

De Leenheer [8,14] used an octadecylsilane column with methanol–water eluents and later with an argentation system to obtain a good separation of retinyl palmitate and oleate. Cullum and Zile [10] have employed a three-step gradient reversed-phase HPLC but failed to separate retinyl palmitate and oleate. Furr et al. [12] have also described a gradient reversed-phase HPLC method using an octadecylsilane column. They could analyse small amounts of liver tissue obtained by needle biopsy in 20-min runs but they did not resolve retinyl palmitate from oleate. Furr et al. [13] reported another HPLC method using an octadecylsilane column and acetonitrile–dichloromethane eluents, but the resolution of retinyl palmitate and oleate peaks was not completely achieved. As reported by Ross [17], the use of a column with a less hydrophobic substituent, an octyl or phenyl group, allowed good resolution of retinyl palmitate and oleate. So we chose a C_8 column with a simple isocratic elution with methanol–water as described by Rasmussen et al. [18]. The modification of the extraction procedure (less water and more *n*-hexane with a second extraction) gave good results for total recovery of the three main retinyl esters. We obtained good resolution of the "critical pair" palmitate–oleate in 30 min. With this procedure, retinol could also be quantified in some liver samples, for example in cases of alcoholic liver disease for which decreased amounts of total liver vitamin A with increasing amounts of free retinol has been described [18]. However, we observed that hydrolysis of retinyl esters to retinol may have occurred during the *n*-hexane layer evaporation step. So we evaporated separately and quickly the two hexane extracts. In addition, the solvent mixtures used for sample extraction failed to achieve a complete recovery of retinol and long-chain retinyl esters simultaneously because they differ quite considerably in polarity and we chose to obtain the best recovery of retinyl esters. These two points and the low hepatic concentrations of free retinol may explain the great variations in retinol values both in the within-day and the day-to-day assays and in human liver samples. However, a C.V. of 20% on retinol concentrations involved a small variation of the retinol absolute values in the biopsy.

Because liver biopsy is conventionally obtained for histological examination related to clinical diagnosis, vitamin A analysis might readily be performed on the remainder of the sample. Indeed, the technique described here allowed quantification in samples as little as 3 mg of liver tissue. So the determination in duplicate of retinyl esters in biopsy samples was possible and it is of interest to reduce variations due to the analytical method.

The human liver retinyl ester concentrations and their composition, obtained from thirteen patients, were similar to those already reported in the literature [19–25]

In conclusion, we described here a simple procedure for simultaneous determination of retinyl esters and retinol in small human liver samples by reversed-phase high-performance liquid chromatography with an isocratic elution. Good resolution of retinyl esters and retinol was achieved. This technique allowed quantification of small amounts of retinyl esters in liver biopsy samples.

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