A rapid and sensitive separation of retinol and retinyl palmitate using a small, disposable bonded-phase column: kinetic applications

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Summary A method utilizing small disposable C18 bonded-phase columns has been developed to separate retinol and retinyl palmitate mixtures into their individual components in high yield and purity. Up to ten mixtures can be processed in 1 hr and the columns are reusable after suitable washing. Although the method was developed with standard retinoid mixtures, it was shown that it is also applicable to the assay of the kinetics of both a bile salt-stimulated human milk lipase-catalyzed hydrolysis reaction and an acyl transfer reaction. This rapid, accurate, and inexpensive method is complementary to other chromatographic techniques, especially in kinetic investigations, and enables one to detect these fluorescent retinoids in quantities as small as 2 picomoles. —**O'Connor, C. J., and B. Yaghi.** A rapid and sensitive separation of retinol and retinyl palmitate using a small, disposable bonded-phase column: kinetic applications. *J. Lipid Res.* 1988. **29:** 1693–1697.

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The procedures most commonly used for determining the activity of lipases that hydrolyze retinyl esters fall into two general categories: *a*) conversion of free fatty acid released during the hydrolysis into the sodium salt and separation, using a method similar to that described by Belfrage and Vaughan (1), followed by radiochemical assay of the fatty acid (2); *b*) extraction of the fatty acid released into an ethanolic solution and titration with NaOH (3, 4).

Although these methods produce satisfactory results, methods in category "*a*" require purchase of expensive radiochemicals and instrumentation, while those in category "*b*" require at least 10 μmoles of retinyl ester per assay. Methods based on separation of retinol from the retinyl ester have been rarely used in lipolysis studies. This is not surprising, since separation and analysis of retinoids are difficult, due to similarity in polarity among the major retinyl esters and susceptibility of all vitamin A molecules to oxidation, especially during thin-layer procedures. Moreover, equimolar concentrations of the various retinyl esters and of retinol have very similar absorbances in their UV spectra (5) and similar excitation and emission fluorescent spectra in a variety of solvents (6). Even current reversed-phase high performance liquid chromatography (HPLC) separation methods of retinyl esters generally show long retention times, broad peaks, and low sensitivity (7).

In 1976, de Ruyter and de Leenheer (8) found that they could identify as little as 50 $\mu g \cdot l^{-1}$ of retinol in serum or plasma. Only 100 μl of fluid was required and fast separation (< 12 min) was achieved by a simple high-speed liquid chromatographic assay coupled to on-line UV detection at 328 nm. Later, Ross (5) found that, by using an octyl-substituted reverse-phase column and mixtures of acetonitrile with water, she could resolve completely both retinyl esters and more polar neutral retinoids, such as retinol. The limits of detection were 2 and 75 pmol and the retention volumes were 8 and 120 ml for retinol and retinyl palmitate, respectively. Recently van Kuijk, Handelman, and Dratz (9) improved both the speed (within 12 min) and sensitivity (to a detection limit of 1 ng) of separation of retinol from retinyl esters by using a C18 column and an HPLC step-gradient solvent system of methanol and isopropanol.

Cooper and Olson (10) have recently described a new sensitive method (detection limit 4 pmol at 5:1 signal to noise ratio) for retinyl ester hydrolase activity. Radioactive substrate was not required because the formation of retinol could be adequately quantitated by reversed-phase HPLC. Enzyme activity was measured against a number of retinyl esters. The reaction mixture (10 ml) containing 500 μM CHAPS was incubated with 770 μg liver homogenate extract. After 1 hr the reaction was quenched by the addition of 0.5 ml ethanol. Retinol was extracted twice with 2 ml hexane, dried under a gentle stream of N_2, redissolved in 500 μl isopropanol, and eluted through a 4.6 × 250 mm Partisil 10/25 ODS-2 column with methanol–water 9:1 in 7.2 min. Absorbance was monitored at 325 nm. This method has also been used in another laboratory (11) and is very similar in principle to the assay of enzymatic activity presented in this paper. Moreover, the two methods have comparable time requirements and limits of detection.

The work detailed in this report outlines a procedure for the use of small, disposable C18 bonded phase columns, to isolate retinol from retinyl palmitate. Up to ten mixtures of these retinoids can be processed in 1 hr.

Our specific interest in this project was to find an efficient and rapid method for analyzing the products of bile salt-stimulated human milk lipase-catalyzed hydrolysis of retinyl palmitate and we have not tested it against other long-chain fatty acids of retinol. In the course of our studies we found that this enzyme also catalyzes the acyl transfer reaction between retinol and palmitic acid. Data utilizing both standard retinoid mixtures and mixtures arising from these bile salt-stimulated human milk lipase-catalyzed reactions are presented.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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MATERIALS AND METHODS

Materials

Retinol (all *trans,* type D) and retinyl palmitate (all *trans,* type IV) were obtained from Sigma and Serva, respectively. Bile salt-stimulated lipase was isolated from fresh human milk using the method of Bläckberg and Hernell (12). The specific activity of the enzyme was 54.9 μmol · $min^{-1} \cdot mg^{-1}$ (1 mM *p*-nitrophenyl acetate, 2 mM sodium taurocholate, pH 7.5, 37°C).

Methanol and hexane were reagent grade. The hexane was stored over sodium. Cyclohexane was BDH AnalaR.

Disposable columns containing C18 (octadecyl bonded silica gel) (100 ± 0.5 mg) were constructed from the barrels of 1-ml plastic syringes (4.9 × 73 mm) using bonded phase packing obtained from Analytichem International, Harbor City, CA.

It was essential to remove every trace of the silicone plunger lubricant from the barrel to prevent contamination of the packing. Six layers of Whatman GF/C glass-fiber filter paper (punched into 6.35-mm diameter circles) were pressed firmly into the bottom of the barrel, two layers at a time, using a 4-mm diameter square-ended glass rod. The Sepralyte (40 μm C18 silica) was weighed directly into the column, tapped firmly to make it settle, and then held in place by a further six layers of GF/C. A cross-sectional diagram of the column construction is shown in **Fig. 1.**

Analysis of retinyl palmitate and retinol mixtures

Stock solutions of retinol and retinyl palmitate, both 2.3 mM, were prepared in ethanol under a stream of N_2, and from these solutions standard mixtures (mole percent retinol:retinyl palmitate = 100:0 to 0:100) were made up in steps of 10 mole percent ratio in the mixture. The same C18 column was used to establish a set of calibration curves. Forty μl from each mixture (92 nmol) was loaded onto the column and eluted first with 1.5 ml methanol-water 93:7 (v/v) (eluant A which removes only the retinol), followed by 1.5 ml hexane (eluant B which removes retinyl palmitate). After each separation, the column was regenerated by washing with 2 ml hexane, followed by 2 ml methanol, and finally 1 ml methanol-water 93:7 (v/v).

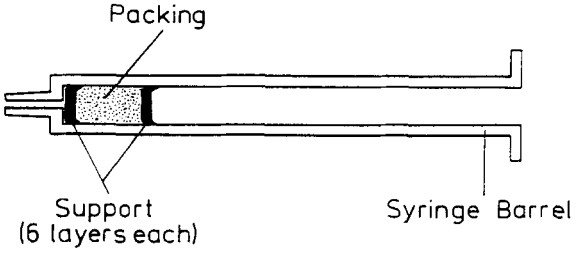

Fig. 1. Cross section of disposable bonded-phase column showing details of construction.

Mixtures of retinol and retinyl palmitate were also prepared in the range of mole percent ratio of the minor component from 0:100 to 10:90. Samples differing by as little as 1 mole percent ratio were quite clearly differentiated.

Fluorescence analyses were made on a Shimadzu RF540 spectrofluorophotometer, using an excitation wavelength of 325 nm and an emission wavelength of 475 nm. For the more concentrated samples, typically 150 μl of eluant A was diluted with 3 ml methanol and 150 μl eluant B was diluted with 3 ml cyclohexane before determination of the relative fluorescent intensity. (The eluted hexane was collected in a 1.5-ml Eppendorf tube and was contaminated with traces of aqueous solvent which was immiscible with both hexane and cyclohexane. The contaminant was therefore first removed by centrifuging the tube and contents for 5 min at room temperature at ca. 750 *g*.) For the samples containing only 1–10 mole percent ratio of the minor component in the mixture, 500 μl of the respective eluants was diluted with 2.5 ml of solvent before analysis. These quantities were measured on scale 2 of the detector, but one to two orders of magnitude less compound could also be measured on a more sensitive scale.

The maximum amount of sample that could be loaded onto this column without overload was 26 μmol retinol and 29 μmol retinyl palmitate, whether as a mixture or as a single component.

The limit of detection wsa 4 pmol retinol and 2 pmol retinyl palmitate, at 10:1 signal:noise ratio, for the coupled chromatographic and fluorescence determination steps. Even using these small amounts of substrate, reproducibility of the fluorescence assay, before and after recovery from the column, was ± 1%.

Bile salt-stimulated human milk lipase-catalyzed hydrolysis of retinyl palmitate

A mixture of 60 μl retinyl palmitate solution (0.0236 g ml^{-1}) and 0.54 ml Tris buffer (0.1 M, pH 7.5, containing 10 mM sodium taurocholate) was sonicated under nitrogen, in ice, for 45 sec, and to this emulsion was added 30 μl of a solution of bovine serum albumin (4% w/v in the same Tris buffer). Aliquots (100 μl) of this stock were allowed to equilibrate to 37°C, 5 μl of enzyme solution (5 mg ml^{-1}) was added, and the reaction was allowed to proceed for preset times before being quenched by the addition of 1.20 ml methanol. After vortexing and centrifugation to separate the precipitated sodium taurocholate, 280 μl of the supernatant was loaded onto a bonded phase column and eluted first with 1.22 ml methanol-water 93:7 (v/v) and secondly with 1.5 ml hexane. One hundred fifty μl of each eluant was diluted with 3 ml solvent and the relative fluorescence intensity was determined, as described above.

This experiment was repeated, but with an additional amount of retinol (30 μl of a 0.045 M solution) added to

the substrate mixture before sonication. This quantity corresponded to that which would have resulted from 50% hydrolysis of the retinyl palmitate.

Bile salt-stimulated human milk lipase-catalyzed synthesis of retinyl palmitate

A sonicated mixture of 60 μl retinol solution (0.045 M), 60 μl palmitic acid solution (0.045 M), 540 μl Tris buffer (0.1 M, pH 7.5, containing 10 mM sodium taurocholate) was prepared and 60 μl of bovine serum albumin solution (4% w/v in the Tris buffer) was added. Aliquots (100 μl) of this emulsion were incubated with 5 μl of the enzyme solution for preset times at 37°C, and the quenching and assay procedures were carried out as before.

RESULTS

Fig. 2 (panel A) shows the calibration curves obtained for determination of 0–100 mole percent retinol or retinyl palmitate after separation of these components from mixtures containing both compounds in the range of molar ratios 0:100 to 100:0.

Panel B of Fig. 2 shows the calibration curves obtained for determination of 0–10 mole percent retinol or retinyl palmitate after separation of these minor components from mixtures prepared in the range of mole percent ratios 0:100 to 10:90.

Fig. 3 (panel A) shows the time course assay for the increase in the concentration of retinol and the decrease in the concentration of retinyl palmitate during the enzyme-catalyzed hydrolysis of the ester. The experiment was carried out under two sets of conditions, differing in the initial concentration of retinol.

Panel B of Fig. 3 shows the time course assay for the increase in the concentration of retinyl palmitate and the decrease in the concentration of retinol during the enzyme-catalyzed acyl transfer reaction between retinol and palmitic acid.

DISCUSSION

In the retention behavior of a solute in a reversed-phase system, only the role of the hydrophobic interactions is clearly understood. Retention is not due to favorable interaction with the stationary phase but to the effect of the mobile phase forcing the compound to the hydrocarbon stationary phase.

Many hydrophobic substrates do not completely elute from a reversed-phase liquid chromatographic column when using semiaqueous mobile phases containing methanol or acetonitrile, due to their low solubility in water and methanol (13). Incomplete elution of sample components significantly contributes to the steady deterioration of the column. Therefore, in our separation, we have used a combination of aqueous methanol to elute the relatively polar retinol, followed by hexane, a solvent in which the ester of low polarity has appreciable solubility. This technique offers several advantages. First, sample solubility is no longer a problem and column life is extended; second-

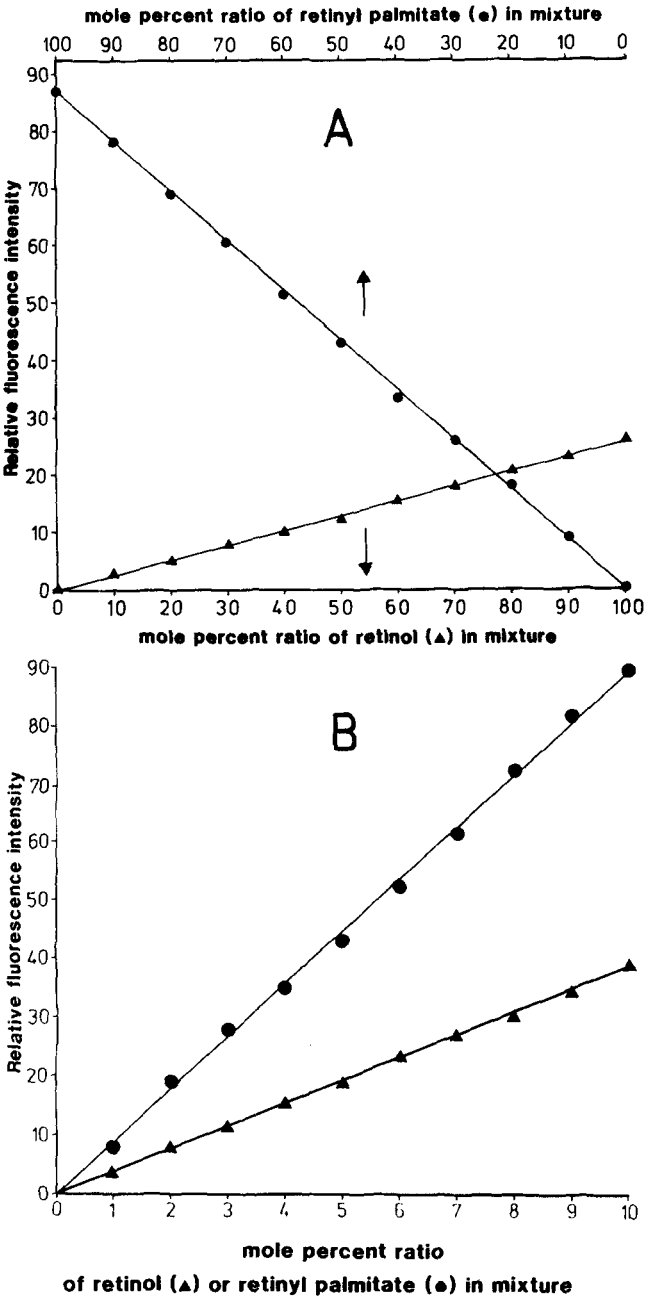

Fig. 2. Standard curves for retinyl palmitate and retinol. Mixtures of 0–100 mol percent retinyl palmitate (●) in the presence of 100–0 mol percent retinol (▲) (panel A) and of 0–10% retinol (▲) or retinyl palmitate, ●) in the presence of 100–90% retinyl palmitate (or retinol) (panel B) were separated on C18 bonded phase columns and assayed as described in Materials and Methods. Each point represents the mean of three to six replicates. The reproducibility of the assay was ± 1% and lies within the size of the symbols used in the figure.

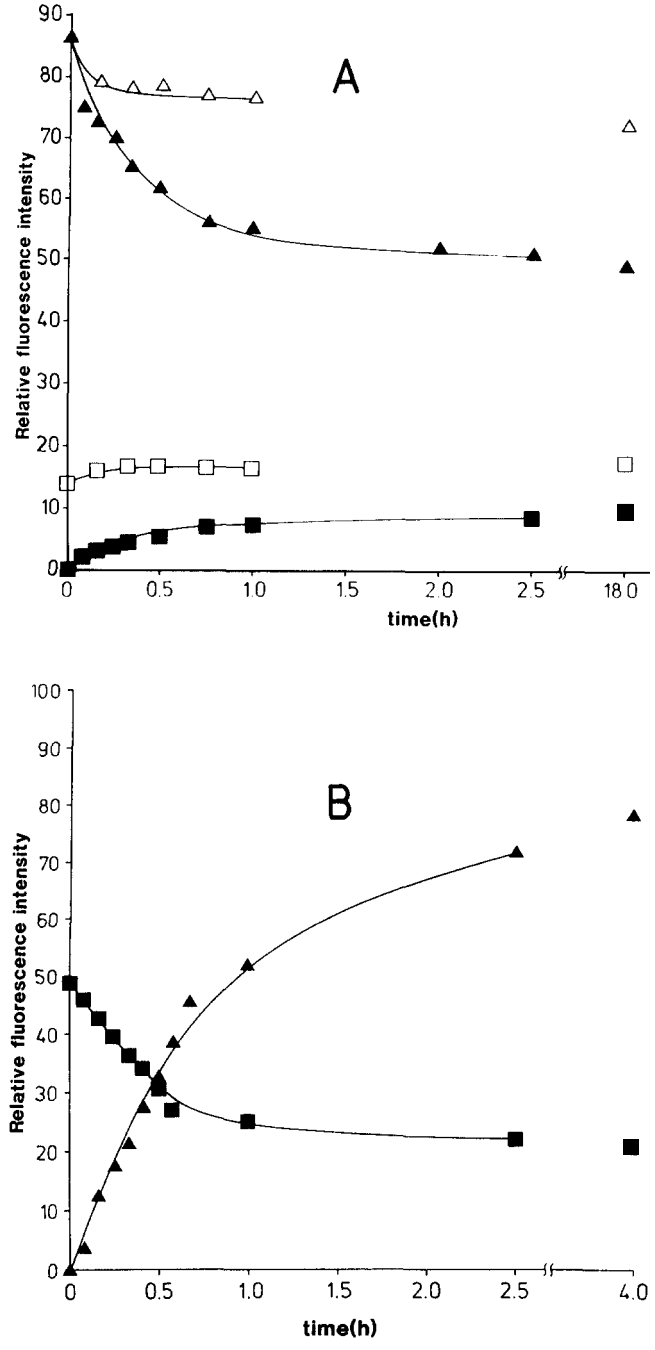

Fig. 3. Time course assay for bile salt-stimulated human milk lipase-catalyzed reactivity. The ordinate is a measure of the rate of attainment of the equilibrium between retinol and retinyl palmitate. Panel A: hydrolysis of retinyl palmitate. Retinyl palmitate was incubated with 10 mM sodium taurocholate and the enzyme at 37°C and the retinol released (■ and □) and ester remaining (▲ and △) were assayed as described in Materials and Methods. Closed symbols: initial concentration of retinol 0 mM; open symbols: initial concentration of retinol 2.07 mM. Panel B: synthesis of retinyl palmitate. Retinol and palmitic acid were incubated with 10 mM sodium taurocholate and the enzyme at 37°C and the retinyl palmitate formed (▲) and retinol remaining (■) were assayed. Each point represents the mean of three to six replicates. The reproducibility of the assay was ± 2% and lies within the size of the symbols used in the figure.

ly, due to the higher solubility, a larger sample can be loaded and, thus, an improvement of the detection limit can be seen.

We have developed a rapid economical method for the preparative scale isolation and separation of retinol and retinyl palmitate. Since the columns can be regenerated by washing in reverse-elution order, the method is more convenient than thin-layer procedures and up to ten retinoid mixtures can be processed in 1 hr using just 100 mg of C18 in one column.

The separation and recovery that were obtained by this bonded-phase procedure were a function not only of retinoid polarity, but also of solvent polarity. The methanol–water 93:7 mixture was found to be the most efficient for complete elution of retinol but retention of retinyl palmitate. Pure methanol carries some retinyl palmitate with it and therefore an intermediate wash with this solvent, to remove traces of water before the addition of the hexane, was not possible. However, centrifugation satisfactorily separated this contaminant from the hydrocarbon phase. Tests showed that the volumes of eluant used, 1.5 ml, were sufficient to completely remove both the retinol and retinyl palmitate from the column. The data in Fig. 2 show that the calibration curves were linear over a loading range on the column from 0.92 to 92 nmol for both retinol and retinyl palmitate, each present in a mixture of them both with a total retinoid loading of 92 nmol. The total retinoid concentration could be increased up to 50 μmol without overloading the column. At the other end of the scale, the sensitivity of the fluorescence technique is such that the method will detect as little as 2–4 pmol of retinoid. It must be reemphasized that the composition of the eluants should not be changed, since the physical environment of the column may be altered such that separations, recoveries, and degrees of purity become less than optimal. Providing that the bonded phase columns are carefully packed, the standard curves can be used for calculating the concentration of retinol and retinyl palmitate, eluted as described above. Analysis of mixtures subjected to chromatography through ten separate columns agreed within a concentration range of ± 1%.

The fluorescence yield of retinyl palmitate in cyclohexane is much greater than that of retinol in methanol. This difference is due to the nature of the solvents. Kahan (6) notes that the ratio of fluorescence intensity in cyclohexane and ethanol is 15.6:1 for both retinol and retinol acetate. The difference in polarity of the eluants, a necessity for efficient separation, unfortunately did not allow for equalizing the fluorescence yield by diluting the column eluants into the same solvent. Thus, it was not possible to use a single standard curve for both retinol and retinyl palmitate.

The analytical techniques can now be readily used in monitoring the course of an enzyme-catalyzed reaction.

None of the other substances present in the reaction medium, i.e., Tris buffer, the cofactor (sodium taurocholate), the enzyme (bile salt-stimulated human milk lipase) or the fatty acid inhibitor (bovine serum albumin), affect the efficiency of the separation or the subsequent spectrofluorimetric analysis of the retinoids. Moreover, it is now possible to monitor both the loss of retinoid-substrate and the synthesis of retinoid-product, whereas the previously available techniques of radiochemical assay and titration could only be used to monitor the concentration of fatty acid. We have, therefore, been able to identify for the first time that bile salt-stimulated human milk lipase does not promote complete hydrolysis of retinyl palmitate, but rather catalyzes the rate of attainment of an equilibrium mixture of retinol and its palmitate ester. Moreover, although it takes ca. 18 hr to establish the equilibrium position when retinyl palmitate is the initial substrate, equilibrium is attained in ca. 4 hr, when one starts with retinol and palmitic acid under the same experimental conditions. The equilibrium constant for the reaction

$$\text{retinyl palmitate} \underset{k_{\text{acyl transfer}}}{\overset{k_{\text{hydrolysis}}}{\rightleftharpoons}} \text{retinol} + \text{palmitic acid}$$

can be calculated from these data and the values obtained are: 2.50 mM (from equilibrium concentrations after hydrolysis of retinyl palmitate (initial retinol concentration 0 mM, Fig. 3, panel A); 1.72 mM (from equilibrium concentrations after the acyl transfer reaction between retinol and palmitic acid, Fig. 3, panel B); and 2.82 mM (from the ratio of the rate constants for the forward and reverse reactions calculated after monitoring the loss of substrate for the enzyme catalyzed hydrolysis and acyl transfer reactions). This last value has been corrected for the small contribution of blank reactions carried out under conditions identical to those described above, but in the absence of the enzyme. The comparability of these results is a pleasing feature of the separation and subsequent analysis.

Bile salt-stimulated human milk lipase is known to be a very nonspecific enzyme, since it catalyzes the hydrolysis of a very large range of mono-, di-, and triglycerides as well as cholesteryl, aryl, and alkyl esters (14). There is also a little evidence that an acyl transfer role can be attributed to this enzyme since it catalyzes the synthesis of cholesteryl oleate (15) and [^{3}H]oleic acid is incorporated into mono-, di-, and trioleoylglycerol during trioleoylglycerol hydrolysis (16). However, this is the first evidence that it is this role that is significant in the enzyme-vitamin A interactions. This finding reveals new perspectives in the contribution of bile salt-stimulated human milk lipase to infant nutrition. We are currently investigating details of this acyl-transfer reaction.

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