Rapid Synthesis and Purification of Vitamin A Esters

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Various esters of retinol (vitamin A) have been synthesized in high yields from fatty acids in one step, at room temperature in the presence of 4-dimethylaminopyridine and dicyclohexylcarbodiimide. Under anhydrous conditions, this technique avoids the occurrence of undesirable substances. The esters are separated quickly and efficiently from unreacted starting materials by flash chromatography on a silica gel column with a hexane/ether mixture as elution solvent.

KEY WORDS: Esterification, dicyclohexylcarbodiimide, 4-dimethylaminopyridine, flash chromatography, retinol.

In animal liver, retinyl esters are the storage form of vitamin A (retinol) and are involved in several steps of its metabolism. Retinyl palmitate is the main ester but, depending on the dietary conditions, numerous other esters also occur (1,2), which are typically not commercially available. Because they are required in a large number of experiments to investigate vitamin A metabolism and functions, one has to synthesize these retinyl esters. Several different procedures have been used (3-5). All involve multiple steps for synthesis and purification and may become tedious without leading to high isolated yields.

Neises and Steglich described in 1978 a simple method for esterification of carboxylic acids (6) with 4-dimethylaminopyridine (DMAP) as catalyst and dicyclohexylcarbodiimide (DCC) as reagent. The formation of side-products is thus suppressed, and high yields are reported. Separately, flash chromatography purification was first developed by Still *et al.* (7) and adapted to lipids by Jensen *et al.* (8). This technique is simple and allows fast and efficient separations.

We adapted both these techniques to retinol esterification with several fatty acids and describe a two-step method for synthesis and purification of retinyl esters, which is fast and easy to perform and which leads to high yields of isolated products.

EXPERIMENTAL PROCEDURES

Dryness precautions. Avoiding the presence of traces of water in the solvent and the reaction vessel is crucial for the synthesis. Glassware is carefully dried in an oven at 100°C, the solvents are distilled over calcium hydride just before the synthesis, and the reaction glassware is flushed with high-grade nitrogen when introducing compounds.

Synthesis. Under red light, 2.25 mmoles of retinol (Fluka, Buchs, Switzerland, 99% pure), 2 mmoles of fatty acid (Sigma, St. Louis, MO) and 0.2 mmoles of DMAP (Sigma) are dissolved in anhydrous dichloromethane (Prolabo, Paris, France, analytical grade) by gentle stirring and cooled in an ice bath. DCC (Aldrich, Gillingham, U.K.) (3 mmoles) is separately dissolved in dichloromethane and slowly added to the previous compounds, still in the ice bath. The reaction mixture is allowed to stand 3 h in the dark at room temperature. The urea precipitate is then filtered, and the filtrate is washed in a decantation funnel three times with water, then with 0.5 M HCl and finally with a saturated solution of $NaHCO_3$. After drying over $MgSO_4$, the organic phase is evaporated under nitrogen and dissolved in a small volume of hexane containing 50 mg/L of 3,5-di*tert*-butyl-L-hydroxytoluene (BHT) as antioxidant. It can either be kept at −20°C under nitrogen for up to one week or preferably, immediately processed for purification.

Purification. The glass column used for flash chromatography (i.d. = 2.5 cm, l = 15 cm) is filled with Silica gel (0.4 ± 0.063 mm, Merck, Darmstadt, Germany, ref. 9385). Some diethyl ether is added to the solution in hexane to reach a solvent composition of 98:2 (hexane/ether, vol/vol). The sample is then poured in the top of the column and forced into the silica gel with a stream of nitrogen. The elution solvent (hexane/ether, 98:2) is then forced in the same way, at approximately 2 cm/min. Thirty-five to 40 fractions of 5 mL each are collected, and the purification can be achieved in less than 25 min. The yellow color of the retinol and its ester allows one to follow their respective elutions and, if needed, to accelerate the elution of retinol by increasing the ether proportion in the solvent mixture.

Identification procedures. An aliquot of the mixture before purification and of each elution fraction is chromatographed on reverse-phase high-performance chromatography (HPLC) [Column, Nucleosil 5 μm 250 × 39 mm; solvent, methanol 100% at 2 mL/min; detection, ultraviolet (UV) 325 nm]. When available, pure standards are co-chromatographed. Peak area ratios, molecular weights and optical density of the mixture allow calculation of synthesis yield.

RESULTS AND DISCUSSION

Synthesis yields are in the same range of those obtained by previously described methods (3,4), but it is noticeable (Table 1) that, in most cases, the purification step is performed with little loss of the newly synthesized ester. This allows good final isolated yields. Careful optimization of the experimental conditions of the synthesis should lead to increased synthesis yields, but our purpose was mainly to provide a simple (two steps) and fast (one day) method.

TABLE 1

Yields of Synthesis and Purification of Retinyl Esters

Fatty acid esterified	Synthesis yield (%)	Isolated yield (%)
Lauric acid (C12:0)	96.5	77.4
Myristic acid (C14:0)	53.2	51.8
Pentadecanoic acid (C15:0)	52.1	52.0
Palmitic acid (C16:0)	86.0	75.3
Palmitoleic acid (C16:1 n-7)	80.0	78.5
Stearic acid (C18:0)	68.0	66.0
Oleic acid (C18:1 n-9)	54.0	49.5
Linoleic acid (C18:2 n-6)	70.2	53.7
Linolenic acid (C18:3 n-3)	70.0	69.8

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We noticed that synthesis yields are much decreased when the initial quantities of products are diminished. One can assume that the amount of water remaining in the reaction mixture is the same whatever the quantities of compounds are and that its effect is greater when the reaction involves small amounts of products.

The reaction is gentle and occurs in the dark at room temperature for a limited duration. This makes unlikely the formation of *cis*-isomers, which are generally photo-induced. Cyclicized or isomerized compounds have been observed during acid catalysis of retinyl esters (9), but our experimental conditions are different and no side-products were detected during HPLC, even before purification of the synthesis mixture. For polarity reasons, it is reasonable to assume that free fatty acids are tightly retained on the column during flash chromatography.

Separation of short-chain retinyl esters (*e.g.,* retinyl laurate) from retinol is easier to achieve with a slightly decreased proportion of ether in the elution solvent (*e.g.,* 98.5:1.5, hexane/ether). Length and unsaturation level of the fatty acid do not seem to significantly affect the synthesis and purification of the corresponding ester.

We used this technique for synthesis of radioactive retinyl palmitate from tritiated retinol (New England Nuclear, Boston, MA) and obtained a 56% final yield with a specific radioactivity of 2.9×10^9 cpm/mmol, starting from 0.02 mmoles of palmitic acid.

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