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Note

Cholesteryl ester quantitation: non-equivalent rates of transesterification of molecular species in the presence of silica gel

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Numerous methods exist for the quantitation of cholesteryl esters from tissues and fluids. Most commonly, cholesteryl ester in lipid extracts can be determined by subtracting free cholesterol values from those obtained for total cholesterol of the extract after saponification of the total lipid mixture in methanolic base. However, for studies involving diet- or disease-induced aberrations in lipid metabolism, it is often necessary to determine individual molecular species of cholesteryl esters since this can reflect a general alteration in lipid metabolism. This may be accomplished by various transesterification procedures or conversion of the released fatty acids to their methyl esters after saponification of the cholesteryl ester. Commonly used methods include acid methanolysis [1—3], sodium methoxide methanolysis [4, 5], methanolysis in the presence of a Lewis acid such as boron trifluoride [6], and saponification followed by conversion of the relased fatty acids to their methyl esters [7].

In addition, recent advances have allowed direct quantitation of cholesteryl esters without prior derivatization by the use of on-column injection capillary gas chromatography (GC) [8]. In general, quantitation of cholesteryl esters by molecular species determination requires the use of a suitable cholesteryl ester containing an odd-chain fatty acid added as an internal standard to the extract (e.g. cholesteryl heptadecanoate). Prior to derivatization and analysis, it is necessary to isolate the pure cholesteryl ester fraction from the extract, free of other contaminating lipids; this is accomplished by column procedures, high-performance liquid chromatography (HPLC) or preparative thin-layer chromatography (TLC) in a non-polar solvent. In the case of preparative TLC, it has been convenient to derivatize the silica gel/cholesteryl ester complex after

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scraping, without elution of the compound from the gel. We, however, were unable to achieve consistent quantitative results using this method, and investigated the effects of silica gel on various derivatization procedures. When an internal standard is used, it is necessary that the derivatization reaction goes to 100% for all molecular species, or that the individual species be hydrolyzed at rates identical to the internal standard. We present evidence, after surveying various derivatization procedures, that the presence of silica gel in the reaction mixture has a profound effect on the rates of hydrolysis of cholesteryl ester molecular species. The results indicate that the use of saponification followed by fatty acid methyl ester formation, or extended methanolysis in the presence of Lewis acid, are superior to other derivatization procedures for the analysis of cholesteryl esters when silica gel is present in the reaction mixture.

EXPERIMENTAL

Cholesteryl esters (99% purity) of varying fatty acyl chain length and degree of unsaturation (palmitoyl, 16:0; stearoyl, 18:0; heptadecanoyl, 17:0; linoleyl, 18:2; and arachidonyl, 20:4), and standard fatty acid methyl esters were obtained from Nu Chek Prep (Elysian, MN, U.S.A.). TLC plates (250 μm, silica gel H) were purchased from Analtech (Newark, DE, U.S.A.). Boron trifluoride (14% in methanol) was obtained in sealed ampules from Applied Sciences (State College, PA, U.S.A.) and trimethyl (α,α,α-trifluorotolyl) ammonium hydroxide (TMTFTH) was purchased from Regis Chemical (Morton Grove, IL, U.S.A.). Chemicals were reagent grade and purchased from Fisher (Pittsburgh, PA, U.S.A.) and solvents were glass-distilled and supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Chromatographic procedures

Standard cholesteryl ester mixtures were prepared in hexane. Aliquots of the mixture were taken directly for derivatization and analysis, or spotted versus a known standard using an AIS multispotter (Analytical Instrument Specialties, Libertyville, IL, U.S.A.) and developed in a solvent system of hexane—diethyl ether—glacial acetic acid (70:30:1). A first survey of the derivatization procedures utilized cholesteryl heptadecanoate as an internal standard. The lane containing the spotting standard was sprayed with 0.2% 2,5-dichlorofluorescein and visualized under ultraviolet light. Plate areas corresponding to cholesteryl ester were scraped into tubes containing methyl-nonadecanoate as an internal standard to monitor recoveries, and derivatization procedures were carried out as follows.

Base hydrolysis. Methanolic base (0.3 *M* potassium hydroxide in 90% methanol, 1 ml) was added to each tube, the tube was tightly sealed, and saponification carried out for 0—4 h in an 80°C sand bath. At the end of each specified hydrolysis time, 0.1 ml of 5 *M* hydrochloric acid was added to neutralize, followed by 2 ml hexane—diethyl ether (2:1) and 1 ml water to extract the released fatty acids. The extracts were taken to dryness under nitrogen in 5-ml conical centrifuge tubes. Hexane (5 ml) and TMTFTH (0.05 ml) were added, the tubes vigorously handshaken for 3 min, and centrifuged at 1000 *g* for 5 min [5]. Aliquots of the lower TMTFTH phase were then analyzed by capillary GC [10].

Boron trifluoride methanolysis. Tubes containing the cholesteryl ester standard mixture with or without silica gel, and with chloroform (0.5 ml) and boron trifluoride—methanol (0.5 ml) were heated in a boiling water bath for specified times. Then 2 ml of hexane—diethyl ether (2:1) and 0.5 ml water were added to extract fatty acid methyl ester. The upper hexane layer was removed, dried under nitrogen, and the residue redissolved in a small volume of hexane. The fatty acid methyl esters were then purified by preparative TLC on silica gel H plates and developed in benzene versus a known standard. After spraying the standard lane with 2,7-dichlorofluorescein as discussed earlier, areas corresponding to fatty acid methyl esters were scraped into tubes, and 1 ml water and 2 ml hexane—diethyl ether (2:1) added to extract fatty acid methyl esters. The hexane—diethyl ether extract was then dried under nitrogen, redissolved in a small amount of hexane and analyzed by GC.

Acid methanolysis. A 2-ml volume of 0.5% hydrochloric acid in methanol was added to the tubes containing the cholesteryl ester standard mixture with or without silica gel. The samples were then heated for 3.5 h in an 80°C sand bath. After cooling to room temperature, 1 ml water and 2 ml hexane—diethyl ether (2:1) were added, and fatty acid methyl esters extracted. The extracted methyl esters were purified by preparative TLC as described earlier.

Sodium methoxide methanolysis. Cholesteryl esters from the stock solution or scraped from a TLC plate were added to tubes containing methyl nonadecanoate and 2 ml of 1 *M* sodium methoxide in methanol. The tubes were heated in sand bath at 80°C for 1 h. Then, 2 ml of 0.7 *M* sulfuric acid in methanol was added, and the tubes heated at 80°C for an additional 1 h. After cooling to room temperature, 1 ml of water was added and methyl esters were extracted with 2 ml hexane—diethyl ether (2:1). The extracted methyl esters were then purified by TLC as described earlier.

Gas chromatography. GC analysis of fatty acid methyl esters derived from the cholesteryl ester mixture hydrolyzed and derivatized by the various methods, was performed on either a Hewlett-Packard 5700A or a Varian 6000 instrument with flame-ionization detection. Separations were achieved on either a DB-1 column (30 m × 0.242 mm I.D., 0.1 mm film thickness) or a DB-5 column (15 m × 0.245 mm I.D., 0.1 mm film thickness) obtained from J & W Scientific (Rancho Cordoba, CA, U.S.A.). The instrument conditions for the two columns are given below.

DB-5: injector temperature 240°C; detector temperature 300°C; initial column temperature 125°C, no hold; final temperature 220°C with a program rise of 8°C/min; final hold, 5 min; column head pressure 206.84 mPa; hydrogen flow-rate 40 ml/min; splitter ratio 100:1; air flow-rate 300 ml/min; auxiliary nitrogen flow-rate 40 ml/min.

DB-1: injector temperature 350°C; initial temperature 140°C, held 2 min; final temperature 250°C, 8°C program rise; final hold, 5 min; column head pressure 55.16 mPa; hydrogen flow-rate 36 ml/min; splitter ratio 40:1; air flow-rate 280 ml/min; auxiliary nitrogen flow-rate 40 ml/min.

RESULTS

Different rates of conversion were obtained for individual molecular species

of cholesteryl esters using various derivatization procedures. The presence of silica gel in the reaction mixture greatly inhibited conversion of unsaturated species to the corresponding fatty acid methyl esters (Table I). We therefore undertook experiments using the most promising methods (base hydrolysis and boron trifluoride methanolysis) to determine optimum conditions for the complete conversion of all molecular species in the presence of silica gel.

By using an odd-chain fatty acid methyl ester as an internal standard, we were able to determine the relative rates of hydrolysis of different molecular species of cholesteryl esters from a standard mixture in the presence of silica gel by the two methods mentioned previously. Cholesteryl heptadecanoate was included in the standard mixture since it is commonly used as an internal standard. As shown in Fig. 1 and 2, different rates of hydrolysis for cholesteryl ester species occurred using both derivatization procedures. Base hydrolysis followed by TMTFTH methylation resulted in complete hydrolysis of all species only after 4 h at 100°C (Fig. 1), although conversion of saturated

TABLE I

COMPARISON OF PUBLISHED HYDROLYSIS AND DERIVATIZATION METHODS FOR CHOLESTERYL ESTERS: EFFECT OF SILICA GEL

Cholesteryl ester standard mixtures (plus or minus silica gel) were derivatized by the various methods as described in the text. The values in the table represent percentage recovery of each cholesteryl ester species using cholesteryl heptadecanoate as an internal standard.

Method	Percentage hydrolysis			
	16:0	18:0	18:2	20:4
Boron trifluoride—methanol	100	100	100	100
Boron trifluoride—methanol+TLC	95.1	100.4	69.5	54.4
Potassium hydroxide—methanol+TMTFTH	100	100	70.6	75
Potassium hydroxide—methanol—TMTFTH+TLC	100	100	76.2	80.1
Hydrochloric acid—methanol	100	100	61.7	21.1
Hydrochloric acid—methanol+TLC	93.1	69.6	44.9	24
Sodium methoxide—methanol	100	100	59.7	52.9

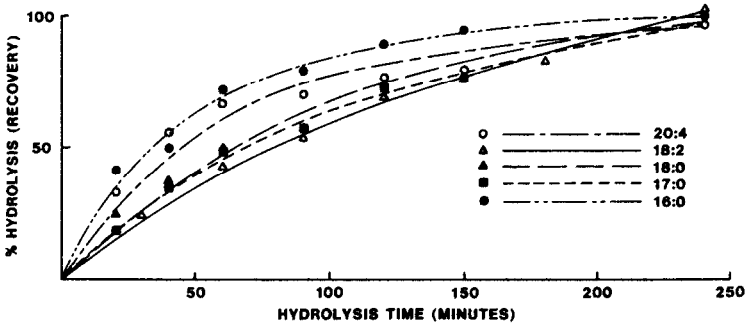

Fig. 1. Time course for derivatization of cholesteryl ester species in the presence of silica gel. A standard mixture of cholesteryl esters was saponified in base and individual species quantitated by methylation of the released fatty acids by TMTFTH as described under Experimental. Numerical abbreviations on the figure (e.g. 20:4) refer to carbon chain length and double bonds of the esterified fatty acids.

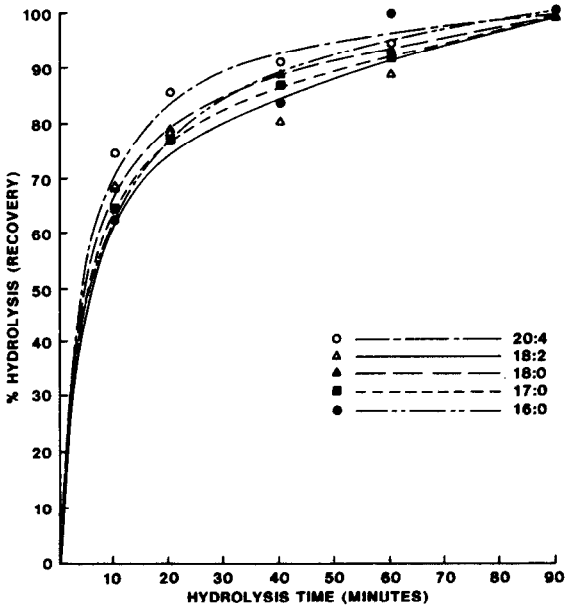

Fig. 2. Time course for derivatization of cholesteryl ester species by boron trifluoride—methanol. A standard mixture of cholesteryl esters was heated with boron trifluoride—methanol for specified times and quantitation achieved as described under Experimental.

species occurred more rapidly. Since all cholesteryl ester species were hydrolyzed at different rates, it is essential for quantitation that this derivatization reaction be allowed to proceed for 4 h when silica gel is present. Similar results were obtained using boron trifluoride—methanol as the derivatization mixture (Fig. 2), although a much shorter (90 min) reaction time was required for complete conversion. Inter-assay variation (n = 4) was $\leqslant$ 5% and intra-assay variation $\leqslant$ 6% for all time points and molecular species. These variations may have resulted from the inability to have identical quantities of silica gel in all reactions. Since 100°C was the temperature used in a variety of other published cholesteryl ester derivatization procedures, we adhered to this temperature for both methods we further evaluated.

DISCUSSION

Many analytical methods for cholesteryl ester determination have appeared in the literature. These have utilized either an odd-chain cholesteryl ester or cholestane as an internal standard. The use of an odd-chain cholesteryl ester as an internal standard, of course, presumes that it is extracted, hydrolyzed and derivatized with the same efficiency (at a given time point) as normally occurring species. The data presented in this paper, however, demonstrate that this is not always the case. Acid methanolysis of cholesteryl esters and sodium methoxide-catalyzed transesterification proved to be incomplete when silica gel was present. The effect of silica gel on cholesteryl ester analysis by derivatization has not been previously reported. Many times this fraction is isolated from a lipid extract by preparative TLC and the cholesteryl ester not eluted from the gel but rather added directly to the derivatization reagent. The data we present

here suggest that caution must be exercised when choosing a method and reaction time for derivatization.

Cholesteryl palmitate was previously shown to be essentially 100% converted to free cholesterol and methyl palmitate after 60 min reaction with sodium methoxide reagent at 27°C [5]. This compares favorably with our results (Table I). However, we found incomplete conversion of unsaturated species using this procedure (Table I) and further studies using this method were abandoned. It should be noted, however, that this method has more recently been modified to eliminate the hydrolysis step [9]. Unfortunately, only cholesteryl palmitate transmethylation was tested in this report, and it cannot be certain that unsaturated cholesteryl esters of different fatty acyl chain length and unsaturation are similarly derivatized, even in the absence of a hydrolysis step.

The base hydrolysis/on-column pyrolytic methylation procedure described by Ishikawa et al. [10] again used cholesteryl heptadecanoate as a standard to assess conversion efficiency of methyl palmitate. Unfortunately this paper also does not adequately address hydrolysis rates of different molecular species of cholesteryl esters. It is common practice in many laboratories to isolate lipid classes from mixtures by preparative TLC and to analyze their fatty acids by scraping the appropriate areas of the TLC plate directly into the derivatization mixtures, without prior elution. The work presented here demonstrates that longer hydrolysis/derivatization times are required when silica gel is present. In addition, not all published procedures for conversion of lipid-esterified fatty acids are adaptable to cholesteryl esters, especially unsaturated species. Alternatively, HPLC procedures such as that of Carroll and Rudel [11] can be applied for the quantitation of cholesteryl esters from lipid extracts of biological samples. The data presented here, however, suggest that cholesteryl esters can be accurately quantitated using potassium hydroxide—TMTFTH or boron trifluoride—methanol even when silica gel is present, providing that longer hydrolysis times are used.

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