Determination of Cholesterol in Milk and Dairy Products by High-Performance Liquid Chromatography

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ABSTRACT : A sensitive high-performance liquid chromatographic method was developed to determine the content of cholesterol in milk and dairy products. To optimize separation of cholesterol, mobile phases including acetonitrile:2-propanol (8:1, v/v), acetonitrile:methanol (3:1, v/v), and acetonitrile:methanol:2-propanol (7:3:1, v/v/v) were compared. Acetonitrile/methanol/2-propanol was superior to the other mobile phase systems for separating cholesterol. Liquid-liquid extraction (LLE) of cholesterol was simplified using a non-polar solvent, hexane, to remove interfering compounds, and had an excellent recovery (100±1.0%) of cholesterol. A solid phase extraction (SPE) method using Sep-pak C_{18} was developed and compared with LLE. The SPE method was rapid and highly reproducible. Both extraction methods were useful when used in combination with saponification of esterified cholesterol to facilitate total cholesterol determination. The detection limit of cholesterol was 0.01 μg. The newly developed HPLC method was rapid, simple, and accurate, and has advantages over the many methods commonly used. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 10 : 1465-1469)*

Key Words : Cholesterol, Milk, Dairy Products, High-Performance Liquid Chromatography

INTRODUCTION

Cholesterol is a major component of all mammalian plasma membranes. Although it is vital for cell growth and survival, there is a statistically significant correlation between elevated serum cholesterol levels and cardiovascular diseases in general and atherosclerosis in particular. Increased consumption of milk fat might be linked to the incidence of coronary arterial diseases. Thus, a rapid, simple, and precise method for determining cholesterol in milk and dairy products should be developed (McGill, 1979; Gurr, 1992).

Punwar (1975) developed a widely used approach for determining cholesterol in foods. The method involves lipid extraction, saponification and silyl derivatization, and detection by gas chromatography (GC). Although the lipid extraction and saponification steps are cumbersome, the derivatizing reagents are unstable, and cholesterol is thermally decomposed in the GC column, the method is based on sound scientific principles and is still relied upon.

The determination of cholesterol by HPLC has received less attention than GC approaches. Since the weak absorbance of cholesterol at low wavelengths presents problems in spectral detection, the application of HPLC with UV detection for determining cholesterol is limited in a complex sample matrix (Hamill and Soliman, 1994).

The objectives of this study were to compare various extraction methods and various mobile phases for separating cholesterol in HPLC in order to develop a simple and accurate method for determining cholesterol in milk and dairy products.

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MATERIALS AND METHODS

Materials

Milk (commercial milk containing 2.6, 3.6, and 4.6% milk fat) and dairy products (whipping cream, ice cream, drinking yogurt, cheddar processed cheese, and butter) were purchased from local retail stores. Standard cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

HPLC conditions for cholesterol determination

HPLC was performed using a multisolvent delivery pump (Waters, Model 600 pump, Milford, MA, USA) equipped with a 20-μL injection loop and a Nova-pak C_{18} column (Waters Assoc. Milford, MA, USA; 3.9 mm I.D. × 300 mm, 10 μm). Elution of cholesterol was monitored at 205 nm using a Waters 486 UV detector. To optimize the separation of cholesterol, isocratic mobile phases, including acetonitrile:2-propanol (8:1, v/v), acetonitrile:methanol (3:1, v/v), and acetonitrile:methanol:2-propanol (7:3:1, v/v/v) were compared with a flow rate of 1.6 mL/min. The peak areas obtained using injected samples and standard were compared.

Extraction of cholesterol

Method A : One mL of milk was accurately weighed and transferred into a test tube fitted with a Teflon-lined screw cap. Direct saponification was achieved with 1 mL of 10% KOH in ethanol (w/v) for 30 min at 70°C. The unsaponifiable fraction was extracted using 5 mL of diethyl ether and 2 mL of distilled water. Diethyl ether extraction was repeated 3 times and the sample was rinsed thoroughly.

An aliquot of the diethyl ether extract was transferred into a 50-mL round-bottomed, glass-stoppered flask and evaporated to dryness on a rotary vacuum evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 50°C, then redissolved in 1 mL methanol. An aliquot (20 μL) was injected directly into the HPLC.

Method B : Diethyl ether extract from Method A was passed through a silica Sep-pak (Waters Associates, 0.18 cm dia. and 1.3 cm long). The combined extracts were evaporated to dryness, redissolved in 1 mL methanol and analyzed using HPLC.

Method C : One mL of milk was saponified as described in Method A and the unsaponifiables were extracted with three portions of hexane (5 mL each) instead of diethyl ether. The remaining steps were the same as in Method A.

Method D : Two mL of 15% acetic acid were added to the saponified sample as in Method A and this mixture was centrifuged at 5000×g for 10 min in an HMR-220IV centrifuge (Hanil Industrial Co., Seoul, Korea). After centrifugation, 5 mL of ethanol and 2 mL of 15% acetic acid were added to the upper phase, which was centrifuged again. Then, the upper phase was directly transferred into an activated Sep-pak C_{18} cartridge (Waters Associates, 0.18 cm dia. and 1.3 cm long) as an SPE tube. Afterwards the Sep-pak C_{18} cartridge was washed with the following reagents in order: 2 mL of distilled water, 2 mL of acetic acid, and 0.5 mL of 50% methanol. Then, the Sep-pak C_{18} cartridge was dried with nitrogen gas. The cholesterol in the Sep-pak C_{18} cartridge was eluted three times with 0.5 mL of ethyl acetate:hexane (20:80, v/v). The collected eluent was evaporated to dryness under nitrogen; the residue was dissolved in 1 mL methanol, and 20 μL was injected into the HPLC system.

Recovery test

A recovery test was performed by adding standard cholesterol solutions (1.0, 2.0, and 3.0 mg/mL ethanol) to a milk sample before extraction.

Cholesterol determination in milk and dairy products

Cholesterol in milk (1 mL), cream (0.3 g), ice cream (0.5 g), yogurt (1.0 g), cheese (0.3 g), and butter (0.2 g) was extracted as described in Method C. Then, the extracted cholesterol was analyzed by HPLC using acetonitrile:methanol:2-propanol (7:3:1, v/v/v) as the mobile phase.

RESULTS AND DISCUSSION

Mobile phase selection for cholesterol determination

In preliminary experiments, different stationary phases based on C_{18} (Nova-Pak C_{18} and Bondapak C_{18} columns) were tested for separating cholesterol. The Nova-Pak C_{18} column was better at separating cholesterol in milk and dairy products, while the cholesterol peak overlapped that of other milk components with the Bondapak C_{18} column. Any overlapping of peaks influences the analytical results, as it is difficult to determine the peak area of specific compounds accurately. Therefore, we used Nova-Pak C_{18} columns for cholesterol separation in subsequent experiments. For a given set of analytical columns in HPLC separation, the mobile phase composition is the principal factor that affects resolution, whereas the flow rate and temperature play secondary roles (Razzazi-Fazeli et al., 2000). To optimize separation of cholesterol, we compared isocratic mobile phases consisting of acetonitrile:2-propanol (8:1, v/v), acetonitrile:methanol (3:1, v/v), and acetonitrile:methanol:2-propanol (7:3:1, v/v/v) at a flow rate of 1.6 mL/min. Fig. 1 shows HPLC chromatograms of cholesterol obtained using the different mobile phases. When 2-propanol was used as the mobile phase, increasing the 2-propanol volume decreased the retention time of cholesterol in the column, although the column and pump were affected by the high pressure caused by the high viscosity of 2-propanol with increasing 2-propanol volume (fig. 1A). With acetonitrile and methanol as the mobile phase, the best solvent ratio for cholesterol separation was 3:1 of acetonitrile:methanol (fig. 1B) and increasing methanol or acetonitrile volume decreased the separation effect. Adding 2-propanol to the mobile phase of acetonitrile:methanol decreased the residence time of cholesterol in the column (fig. 1C). In this case, however, if the volume ratio of 2-propanol was increased, cholesterol was incompletely separated due to overlapping peaks and the resulting column pressure was also increased. Consequently, acetonitrile: methanol:2-propanol (7:3:1, v/v/v) was superior to and more rapid than the other mobile phase systems for separating cholesterol. With this mobile phase system, the retention time was approximately 8 min and the detection limit of cholesterol at 205 nm was 0.01 μg.

Selection of cholesterol extraction method and recovery study

To develop and evaluate a simple, rapid, and precise method for the determination of cholesterol in milk and dairy products, preferably while minimizing the use of expensive, dangerous, and environmentally objectionable solvents and reagents, various methods of extracting cholesterol, including liquid-liquid extraction (LLE) and solid phase extraction (SPE), were assessed.

Cholesterol forms complexes with other biological molecules, primarily phospholipids and proteins, and this changes their overall physical properties, resulting in inefficient extraction of cholesterol. This explains the poor

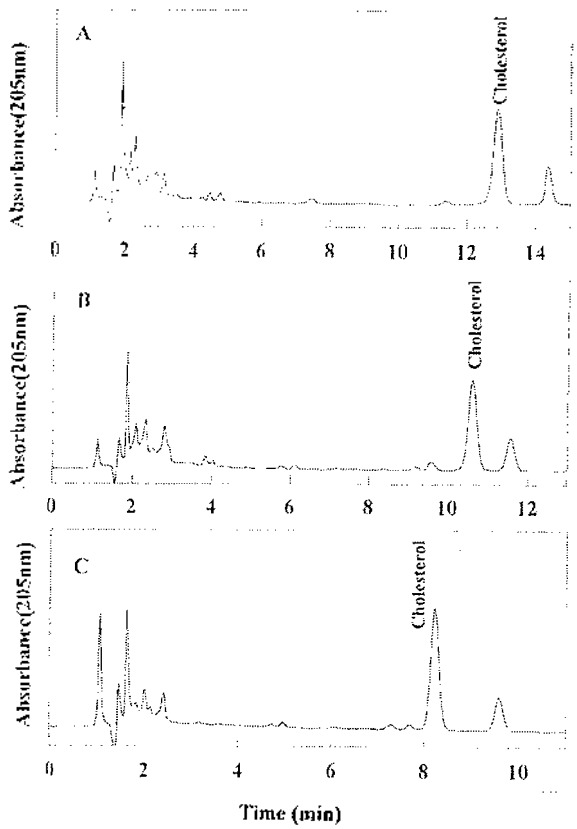

Figure 1. HPLC chromatograms of milk cholesterol.
A : Acetonitrile : 2-propanol (8:1)
B : Acetonitrole : methanol (3:1)
C : Acetonitrile : methanol : 2-propanol (7:3:1)

recovery of cholesterol during what is considered one of the most critical steps in lipid analysis, fat extraction (Kovacs et al., 1979). On the other hand, the direct saponification procedure appears to be a rapid method that has excellent recovery of cholesterol and requires fewer solvents. Ulbreth and Reich (1992) compared direct saponification of a food sample with fat extraction followed by saponification, and concluded that the results of fat extraction were no better than those of direct saponification. Therefore, we used direct saponification with all methods for cholesterol extraction, without lipid extraction, to avoid cholesterol loss.

Figure 2 shows the effects of various cholesterol extraction methods on cholesterol separation from other components. Method A is similar to the AOAC method, which is widely used for cholesterol extraction. Diethyl ether extracted free fatty acids, glycerol, and polar materials, as well as cholesterol, from the saponified sample in Method A. These materials interfered with either the separation or quantification of cholesterol (fig. 2A) and decreased the lifetime of the column. Moreover, diethyl ether is not only extremely flammable, but is also a health hazard; thus Method A is not appropriate for cholesterol

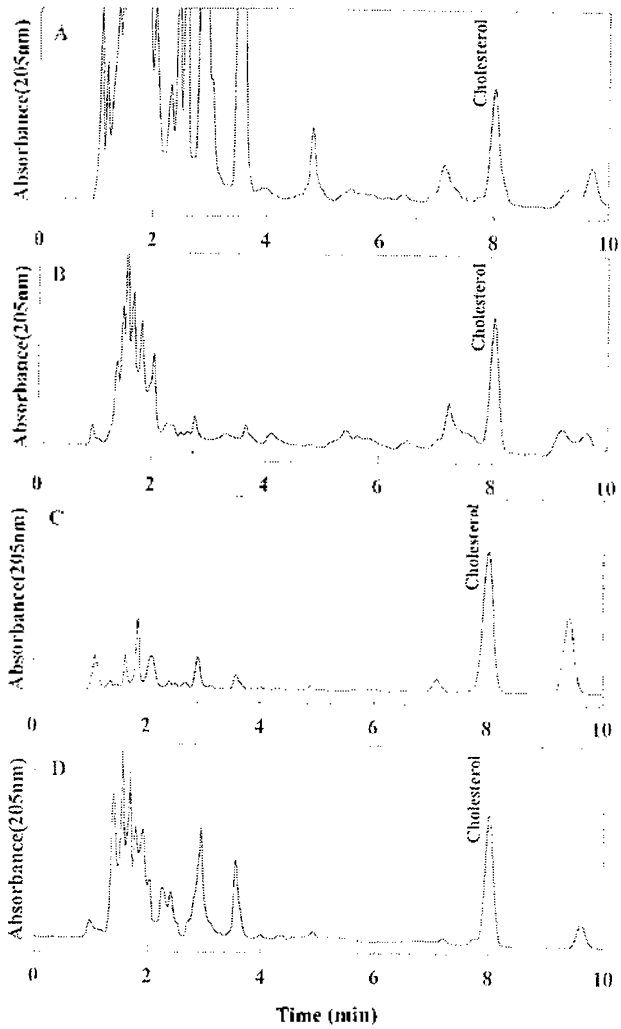

Figure 2. HPLC chromatograms of milk cholesterol.
A : Extraction Method A, B : Extraction Method B
C : Extraction Method C, D : Extraction Method D

extraction. In Method B, the extracted diethyl ether from Method A was passed through a silica Sep-pak. As shown in fig. 2B, some components were removed in comparison with Method A, but the baseline indicated that other materials still remained in the sample extracted using Method B. Accordingly, Methods A and B were not significantly different at separating cholesterol from milk. Hexane extracts fewer polar materials, such as free fatty acids, than diethyl ether, so the chromatogram of Method C (fig. 2C), which used hexane as the extraction solvent, had an excellent baseline and no interference was detected. Recent work has shown that hexane can be substituted for diethyl ether (Al-Hasani et al., 1990). Since solid phase extraction (SPE) can prepare many samples in a short time, it is widely used for the pre-treatment of samples. The chromatogram of cholesterol extracted by SPE using a Sep-pak C_{18} cartridge is shown in fig. 2D. In Method D, the pH

was adjusted to 2-5 using 2 mL of 15% acetic acid to decrease interference by free fatty acids. Consequently, it had an excellent baseline and good selectivity for separating cholesterol from a complex matrix, such as milk and dairy products.

A cholesterol recovery study using extraction Methods A, B, C, and D was carried out by adding 1.0, 2.0, and 3.0 mg of cholesterol to milk samples; the results are presented in table 1. The mean recoveries (%) of Methods A, B, C, and D were 99.3-100.2, 98.8-99.3, 100.1-100.3, and 99.7-100.7%, respectively. The differences were not significant. In Methods A, C, and D, good recoveries were observed and these methods were highly reproducible.

The comparison of chromatograms and the recovery study indicated that Methods C and D were more suitable for cholesterol extraction from milk and dairy products than Methods A and B. Although the SPE method is relatively expensive, hexane extraction and the SPE method are useful when used in combination with saponification of esterified cholesterol to facilitate total cholesterol determination.

Our results are similar to those of Al-Hasani et al. (1990), who reported that when direct saponification was used with hexane as the extraction solvent, recoveries ranged from 98.0 to 104% with a mean of 100.2% and a standard deviation of 2.86. Moreover, Beyer and Jensen (1989) used direct saponification of specific products to speed up the cholesterol assay, and Kovacs et al. (1979) also developed a method for the microdetermination of cholesterol and some plant sterols in fishery-based products. Tsui (1989) designed an extraction system using SPE and capillary GC to rapidly determine cholesterol from milk. Oles et al. (1990) optimized Punwar's (1975) lipid extraction and saponification step, combining it with an NH_2 solid phase extraction cleanup cartridge and capillary GC to achieve excellent reproducibility.

Table 1. Recovery of cholesterol as extracted by different methods

Extraction method	Recovery [a]		
	1 mg	2 mg	3 mg
Method A	100.2±0.2	99.3±0.2	99.5±0.5
Method B	99.1±0.4	99.3±0.5	98.8±0.5
Method C	100.3±0.2	100.2±0.2	100.1±0.4
Method D	99.7±0.3	100.5±0.3	100.7±0.5

[a] Mean value of six replicates
Method A: Saponification and extraction with dietyl ether
Method B: Extracted sample from Method A passed through silica Sep-pak
Method C : Saponification and extraction with hexane
Method D : Solid phase extraction (SPE) (Unit : %)

Table 2. Cholesterol content in milk and milk products [a]

Sample	Cholesterol [b]	Literature value
2.6% [c] milk	10.1±0.3	9.52±0.22 [d]
3.6% [c] milk	12.5±0.2	13.14±0.22 [d]
4.6% [c] milk	17.7±0.2	16.83±0.30 [d]
Cream	111.4±0.5	123.40±1.93 [d]
Yogurt	12.7±0.3	7.33-7.82 [e]
Ice cream	26.5±0.5	30.6-44.7 [f]
Processed cheese	84.6±0.6	78.8-97.9 [f]
Butter	253.5±1.4	227.3-307.0 [f]

[a] Extracted by Method C, [b] Mean value of six replicates.
[c] Milk fat content(%), [d] Oles et al., 1990, [e] Tsui, 1989.
[f] Sweeney and Weihrauch, 1976. (Unit : mg/100 mL or 100 g)

Cholesterol determination of milk and various dairy products

To establish and test the performance of the extraction (Method C) and HPLC method developed for cholesterol, the amounts of cholesterol in milk and various dairy foods (whipping cream, ice cream, drinking yogurt, processed cheese, and butter) were measured. Cholesterol was detected in all these products, indicating that the detection limit is sufficient for testing cholesterol in different matrices. The results of cholesterol analyses in the various dairy products were compared with literature values and are shown in table 2. The cholesterol concentrations in commercial milk containing 2.6, 3.6, and 4.6% milk fat were 10.1±0.3, 12.5±0.2, and 17.7±0.2 mg/100 mL, respectively. As expected, the cholesterol concentration increased with the milk fat percentage. The concentration of cholesterol in milk and cream determined by HPLC in this study was in close agreement with values of Lee et al. (1997) determined by the GC method. In addition, milk containing 2.8% milk fat, and cheddar processed cheese, contained 9.6-9.7 and 78.8-97.9 mg cholesterol/100 g, respectively (Sweeney and Weihrauch, 1976), which are very similar to our values, determined by the HPLC method. Literature values of cholesterol for butter determined by GLC (227.3 mg/100 g) and gravimetric procedure (307.0 mg/100 g) were somewhat different from our value (253.5±1.4 mg/100 g). The cholesterol values in yogurt and ice cream in this study were significantly different from those extracted by Roese-Gottlieb's method (Lacroix et al., 1972) and determined by GC in other studies (Sweeney and Weihrauch, 1976; Ulbreth and Reich, 1992). The difference of cholesterol content in some dairy products between literature and our values is probably due to various factors, including milk fat content, sample variation, extraction method, and determination method used.

CONCLUSION

Acetonitrile:methanol:2-propanol was superior to other mobile phase systems in separating cholesterol using HPLC.

SPE had a high recovery and a shorter extraction time than LLE. Moreover, the SPE method is rapid and highly reproducible. Extraction with hexane followed by SPE removed interfering compounds so that HPLC with UV detection could be applied for determining cholesterol in a complicated sample matrix. The newly developed extraction and HPLC method for cholesterol analysis is rapid, simple, and accurate, and has advantages over the many methods commonly used.

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