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Determination of Total Cholesterol in Serum by Gas Chromatography-Mass Spectrometry

KAOQI LIAN[1], PINGPING ZHANG[2], WEI WANG[1], TINGTING DAI[1] and LEI LI[3,*]

[1]The School of Public Health, Hebei Medical University, Shijiazhuang 050017, P.R. China
[2]Institute of Basic Medicine, Hebei Medical University, Shijiazhuang 050017, P.R. China
[3]Department of Hygienic Analysis and Detection, School of Public Health, Nanjing Medical University, Nanjing 211166, P.R. China

*Corresponding author: E-mail: drleili@hotmail.com

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In this study, a reliable protocol for determination of total cholesterol in serum using gas chromatography-mass spectrometry was established. The total free cholesterol was extracted from the serum with chloroform and derivatized with *bis*(trimethylsilyl)trifluoroacetamide, analyzed without the saponification step commonly used. Linear calibration curves of cholesterol were obtained from the concentration 0.1 to 15 mmol L^{-1}. The limit of detection was 0.04 mmol L^{-1}. Finally, the proposed method was to explore the influence of high cholesterol diet on total cholesterol in serum of rats.

Keywords: Cholesterol, Gas chromatography-mass spectrometry, Serum, High cholesterol diet.

INTRODUCTION

Cholesterol is one of the major constituents of cellular membranes. It is the precursor of bile acids, steroid hormones and provitamin D_3 and is found in foods of animal origin, such as eggs, meat, fish and dairy products. Substantial amounts of total body cholesterol are obtained from the diet even though a major portion of the cholesterol needed for normal body functions is synthesized endogenously. A high-cholesterol diet is a major environmental contributor to an unbalanced lipoprotein metabolism. It is associated with an increased prevalence of atherosclerosis which is the major source of morbidity and mortality in the developed world and it claims more lives than all types of cancer combined[1]. Previous reports have clearly indicated a positive correlation between serum cholesterol level and the risk of cardiovascular disease[2]. Despite the fact that there are drugs available clinically for treating hypercholesterolemia, the consumption of functional foods/dietary supplements in lowering/controlling serum cholesterol levels and risk of cardiovascular diseases has gained enormous global acceptance over the years by the general public. Thus, cholesterol measurement is one of the most common laboratory tests performed in clinical laboratories[3-4].

Numerous biochemical methods for assaying cholesterol have been published, such as non-enzymatic method[5] and enzymatic method[6]. The nonenzymatic methods are very time consuming and require the use of corrosive chemicals. Consequently, the nonenzymatic methods are not suitable for the rapid and direct automated analysis of serum cholesterol using a limited amount of sample. The enzymatic method will result in false positives in any method measuring consumption of oxygen unless steps are taken to remove it. The oxygen will be consumed by other substances, which are present in clinical samples, such as ascorbic acid[7].

The determination of cholesterol has been done using chromatography techniques, including gas chromatography (GC)[8], high-performance liquid chromatography(HPLC)[9,10] and capillary electrophoresis (CE)[11]. All these methods share the same procedure for sample preparation: a saponification extraction step of total cholesterol and a multistage solvent extraction followed by purification, concentration and may need derivatization. It is worth to note that there are many conflicting points of view and results in relation to the analytical methods for cholesterol determination and in relation to the results obtained.

In this study, we report a sensitive and reliable technique to monitor the total cholesterol in serum of rats. The cholesterol of samples was extracted, followed by purification and derivatization for GC analysis. The extraction and derivatization have been mainly researched. Finally, the proposed method was to explore the influence of high cholesterol diet on total cholesterol in serum of rats.

EXPERIMENTAL

Cholesterol and standard 5α-cholestane, were obtained from Sigma (St. Louis, MO, USA). The derivatizing agent

99 % *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1 % trimethylchlorosilane (TMCS) and amino acid standard solution and 10 % boron trifluoride methanol solution (BF_3/MeOH solution) were also purchased from Sigma-Aldrich (St Louis, MO, USA). Potassium hydroxide (Guanghua Chemical Agent Com., China). The solvents used in this study were liquid chromatographic grade and purchased from Tianjin Chemical Agent Com., China). All chemicals used in this study were analytical-reagent grade.

The experimental protocols for this study were approved by the Institutional Animal Care of Hebei 16 male Sprague-Dawley rats were purchased (Shijiazhuan, China) and housed individually in stainless steel cages in a room maintained at 18-25 °C with a 12:12-h light-dark cycle. After one week of acclimatization, the rats were divided randomly into a control group (CON) and a high cholesterol diet group (CHO) for 3 months. The control group fed a diet containing 1 % cholesterol and 0.5 % cholic acid, *i.e.* cholesterol diet group fed the high cholesterol. Cholic acid was added to diets to improve cholesterol absorption by the intestine. The composition of high cholesterol diets was detailed in Table-1. Diets and tap water were freely available.

TABLE-1
COMPOSITION OF EXPERIMENTAL DIETS (g/kg diet)

Ingredient	Casein	Sucrose	Corn starch	Cholesterol	Cholic acid
	200	50	500	10	5

Body weight and food intake were monitored in all rats throughout the experimental period. On the final day of 1st month, 2nd month and 3rd months, blood was taken from tail vein and allowed to coagulate on ice, serum was recovered by centrifugation. The serum samples were frozen and stored at -80 °C until analysis.

Extraction and derivatization procedure: 200 µL serum sample was transferred to a 1.5 mL centrifuge tube and submerge in 1 mL mixture solution of chloroform followed by addition of 100 µL 5α-cholestane as internal standard. The mixture was then sonicated for 60 min to extract cholesterol and precipitate proteins. The precipitated proteins were separated out by centrifugation at 12,000 rpm for 10 min, with other components remaining in the solution. Then 600 µL supernatant was collected from each sample into a vial with PTFE-lined screw cap and evaporated under nitrogen gas at 50 °C to dryness. After the sample evaporated to dryness for derivatization, 100 µL of BSTFA with 1 % TMCS was added into vial and the derivatization reaction was carried out under 70 °C for 40 min. After derivatization and cooling to room temperature, 1 µL derivative was injected in the GC-MS for analysis.

GC-MS analysis: GC-MS analysis were performed on an Agilent (Little Falls, DE, USA) gas chromatograph 7890 equipped with an electronically controlled split/splitless injection port, an inert 5975C mass selective detector with electron impact (EI) ionization chamber and a 7683B Series injector/autosampler. The GC separation was conducted with an HP-5MS 30 m × 0.25 mm I.D., 0.25 µm film thickness column (Agilent, CA, USA). Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. A splitless injection of 1 µL was made at 260 °C. The GC oven was operated with the following temperature program: initial temperature 120 °C held for 1 min, ramped at 10 °C min^{-1} to 250 °C and held for 5 min. Total run time was 19 min. The MS was operated in electronic impact (EI) mode and selected ion monitoring (SIM) mode was used for the identification and quantification of cholesterol. The MS transfer line temperature was held at 280 °C. Mass spectrometric parameters were set as follows: electron impact ionization with 70 eV energy; ion source temperature, 230 °C; MS quadrupole temperature, 150 °C and solvent delay 3 min.

RESULTS AND DISCUSSION

Optimization of sample preparation procedure: Standard addition of 5 mmol L^{-1} cholesterol were pre-treated with three methods including silanization, methylation and saponification followed by GC-MS analysis for determining the peak areas.

BSTFA silanization: The BSTFA silanization procedure described in section extraction and derivatization procedure.

BF_3 methylation: Pre-treatment with BF_3 methylation was adopted by the method described by literature[12]. 200 µL cholesterol solution was transferred to a 1.5 mL centrifuge tube and then 4 mL 0.5 n KOH/CH_3OH solution was added to initiate reaction. The sample was then refluxed at 90-100 °C for 13 min, followed by supplementing with 3 mL BF_3/CH_3OH solution and continuing to reflux for 2 min. Finally, 2 mL ether was added to the mixture and further refluxed for another 1 min. The reaction was terminated by adding 10 mL saturated NaCl solution and cooled down to room temperature. The ether layer was carefully collected and subjected to GC analysis.

Saponification: Pre-treatment with saponification was adopted by the method described by literature[13]. 200 µL cholesterol solution was transferred to a 1.5 mL centrifuge tube, then 1 mL 1 mol L^{-1} KOH/MeOH were added to initiate saponification reaction. The sample was then refluxed at 50 °C for 60 min, Finally, 1 mL *n*-hexane was added to the mixture and further refluxed for another 1 min. the *n*-hexane layer was carefully collected and subjected to GC analysis.

Using three different pre-treatments, the determination of total cholesterol in serum samples were also compared. As shown in Fig. 1, the cholesterol peak areas were higher in those obtained from the pre-treatment with BSTFA silanization than those from BF_3 methylation or saponification. Therefore, BSTFA silanization was selected in the experiments. The total ion current chromatogram of the cholesterol and 5α-cholestane derivatives utilized BSTFA were shown in Fig. 2. Mass spectra of the cholesterol derivative utilized BSTFA were shown in Fig. 3.

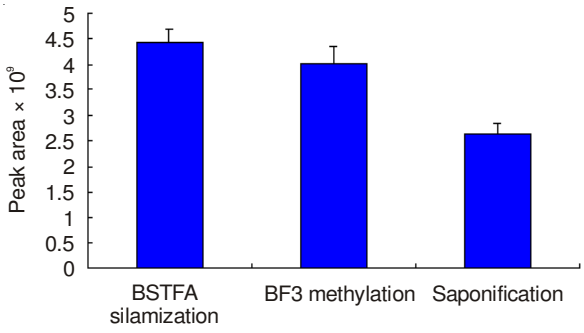

Fig. 1. Peak areas of cholesterol using different pre-treatments

TABLE-3
COMPOSITION OF CHOLESTEROL IN SERUM OF THE CHO AND CON RATS (n = 6, $\bar{x} \pm s$)

Time (month)	0	1	2	3
CON (mmol L^{-1})	2.05 ± 0.08	2.06 ± 0.11	2.02 ± 0.13	2.06 ± 0.08
CHO (mmol L^{-1})	2.04 ± 0.06	2.10 ± 0.12	2.35 ± 0.10 [a,b]	2.67 ± 0.11 [a,b]

[a]Contrasted with CON, $p < 0.05$. [b]Contrasted with previous month, $p < 0.05$; CON = Control group; CHO = Cholesterol diet group

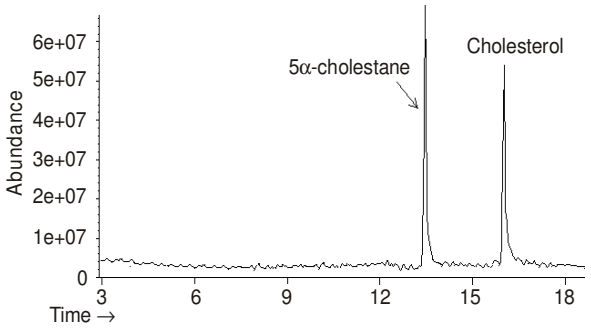

Fig. 2. Total ion current chromatogram of the cholesterol and 5α-cholestane derivatives by BSTFA

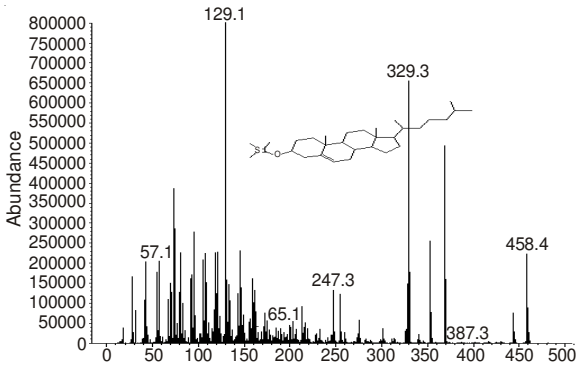

Fig. 3. Mass spectra of the cholesterol derivative by BSTFA

Method validation: Linearity and detection limits. For cholesterol analysis in serum, different standard solutions were prepared to contain different concentrations cholesterol along with 5.0 mmol L^{-1} 5α-cholestane as internal standard. Linear calibration curves of cholesterol were obtained from the concentration 0.1 to 15 mmol L^{-1}. The correlation coefficient was 0.9994. The limit of detection (LOD, based on S/N = 3) was 0.04 mmol L^{-1}.

Precision and recovery: Different spiked concentrations in the serum (0.2, 1.0, 5.0 mmol L^{-1}) were adopted to examine the recovery and precision of the method (Table-2). The data indicate that the intra-day precision (%, RSD) in serum for cholesterol at the low concentration level was 3.8 %, at medium and high concentration levels were ≤ 1.7 %. The aver age recoveries of different concentrations ranged from 94.9 % to 98.5 %. The inter-day recovery and precision were determined at the same three concentration levels over a period of 3 days (n = 6). The inter-day precision (%, RSD) at the low concentration level was 5.2 %, at medium and high concentration levels ≤ 2.8 %. The assay accuracy ranged from 92.8 % to 95.5 %. The results showed the method had good precision and recovery even at the low concentrations.

Application to real samples: The proposed method was to explore the influence of high cholesterol diet on total cholesterol in serum of rats. After getting through the above described analytical procedure, the relevant data were displayed in Table-3. Fig. 4 shows a typical chromatogram of serum. Analysis showed that total cholesterol levels in serum of rats significantly increased ($p < 0.05$) and positively correlated with time in the condition of high cholesterol diet.

TABLE-2
RECOVERIES AND PRECISIONS OF CHOLESTEROL IN SERUM

	Intra-day (n = 6)			Inter-day (n = 6)		
Nominal concn. (mmol L^{-1})	0.2	1.0	5.0	0.2	1.0	5.0
Recovery (%)	96.6	98.5	94.9	95.5	92.8	94.4
Precision (% RSD)	3.8	1.4	1.7	5.2	2.8	2.5

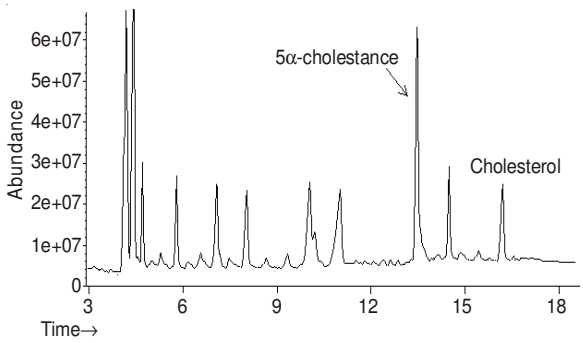

Fig. 4. A typical chromatogram of serum example

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