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Gas chromatography analysis of serum cholesterol synthesis and absorption markers used to predict the efficacy of simvastatin in patients with coronary heart disease

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ABSTRACT

Objectives: We investigated the changes in cholesterol absorption and synthesis markers before and after simvastatin therapy in Chinese patients with coronary heart disease.

Design and method: We developed a gas chromatography method to identify cholesterol synthesis and absorption markers and measured them in patients with coronary heart disease. We then tested their use in predicting the efficacy of simvastatin in lowering cholesterol. Serum samples from 45 patients and 38 healthy humans (controls) were analyzed in a gas chromatography–flame ionization detector.

Results: Squalene and five non-cholesterol sterols—desmosterol and lathosterol (synthesis markers) and campesterol, stigmasterol, and sitosterol (absorption markers)—were detected. The recovery rates of the markers were 95–102%. After simvastatin treatment for four weeks, the total cholesterol and low-density lipoprotein cholesterol levels had significantly decreased from the baseline values ($p < 0.05$). The baseline lathosterol level was significantly higher in good responders than in poor responders ($p < 0.05$), and the stigmasterol level was significantly lower in good responders than in poor responders ($p < 0.05$).

Conclusions: This method should be suitable for the detection of serum squalene and non-cholesterol markers and can be used to predict the efficacy of simvastatin in patients with coronary heart disease.

Introduction

Currently, clinicians in China usually rely on the total cholesterol (TC)[4], low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and apolipoprotein levels to reflect a patient's cholesterol status. However, these indices are only indicative of the cholesterol concentration and not the state of cholesterol metabolism in the body. Blood cholesterol is mainly the result of both liver synthesis and intestinal absorption — processes that regulate the cholesterol level [1]. It is important to know the efficiency of cholesterol absorption and synthesis in order to identify individual differences in cholesterol metabolism, which can then be used as guidance for choosing appropriate medications.

Traditionally, oral radioisotope tracers [2] are used to determine cholesterol metabolism, which can be measured in terms of the isotope-labeled cholesterol concentrations in the blood at timed intervals after the tracers are administered. The measured radioactivity in the serum and feces reflects the efficiency of cholesterol transference [3]. With the development of chromatographic techniques in recent years, traces of cholesterol precursors and plant sterols have been found in human serum. Their structures are similar to those of cholesterol and have been used as markers of cholesterol synthesis and absorption to reflect the state of cholesterol metabolism in the body [4,5].

Statins are cholesterol-lowering drugs that are widely used for primary [6–9] and secondary [10] prevention of coronary heart disease (CHD). Although statins can decrease the cholesterol levels by inhibiting cholesterol synthesis [11], thereby reducing the risk of cardiovascular disease, they benefit some patients more than others [12–14]. Recently, studies have applied gas chromatography to study the changes in cholesterol absorption and synthesis markers before and after statin treatment [15–17]. However, no studies have investigated the changes in cholesterol absorption and synthesis markers before and after simvastatin therapy in Chinese patients

Abbreviations: TC, total cholesterol; RSD, Relative standard deviations; LDL-C, low density lipoproteins cholesterol; TG, triglyceride; HDL-C, high density lipoproteins cholesterol; CHD, coronary heart disease; Lp, lipoprotein; BMI, body mass index; ApoA, apolipoprotein A; ApoB, apolipoprotein B; GC, gas chromatography; HMDS, hexamethyldisiloxane; TMCS, tri methyl chloro Silane; BST-FA, bis-(trimethylsilyl)trifluoroacetamide; TMSI, trimethylsilyl imidazole; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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with CHD. Therefore, we developed a method for the simultaneous identification of squalene and five non-cholesterol sterols—desmosterol, lathosterol, campesterol, stigmasterol, and sitosterol—in the serum using gas chromatography with a flame ionization detector. Individual differences in cholesterol metabolism in patients with CHD were also examined to obtain an insight into the better selection of lipid-lowering drugs.

Methods

Methods for detecting cholesterol synthesis and absorption markers

Main reagents

The reagents included cholestane, cholesterol, squalene, desmosterol, lathosterol, campesterol, stigmasterol, and sitosterol (Sigma, St. Louis, USA); potassium hydroxide (analytically pure); ethanol (chromatographically pure); *n*-hexane (chromatographically pure) (Merck, Darmstadt, GER); derivatization reagent hexamethyldisiloxane (HMDS) +trimethylchlorosilane (TMCS) + pyridine (3:1:9; Supelco, Bellefonte, USA); and ultra-pure water (Millipore, Billerica, USA).

Preparation of standard solutions and the internal standard solution

Serial dilutions of squalene, cholesterol, desmosterol, lathosterol, campesterol, stigmasterol, and sitosterol were prepared in differently labeled solutions (Supplementary Table 1). They were then sealed in ampoules numbered from 1 to 5 and stored at −20 °C. The internal standard solution was 5α-cholestane (0.01301 g), which was diluted to 25 mL with hexane.

Sample pretreatment

Serum (100 μL) was added to a 5 mL graduated test tube, followed by 100 μL of the internal standard solution, 1 mL of absolute ethyl alcohol, and 960 μL of potassium hydroxide solution (8.90 mol/L). The mixture was vortexed for 15 s and then saponified for 1 h at 67 °C. Water (1 mL) and hexane (2 mL) were added for extraction. The upper layer containing the non-saponifiable materials was transferred into clean glass tubes, and the extraction procedure was repeated twice. Then, the combined extracted hexane was washed with water to neutralize any acid therein, and the hexane was separated and dried with nitrogen. Thereafter, 200 μL of silylating reagent was added to the tube and incubated for 1 h at 67 °C, and the residual silylating reagent was dried under a steady stream of nitrogen. The resulting substances were solubilized in 300 μL of hexane for gas chromatography analysis (Fig. 1).

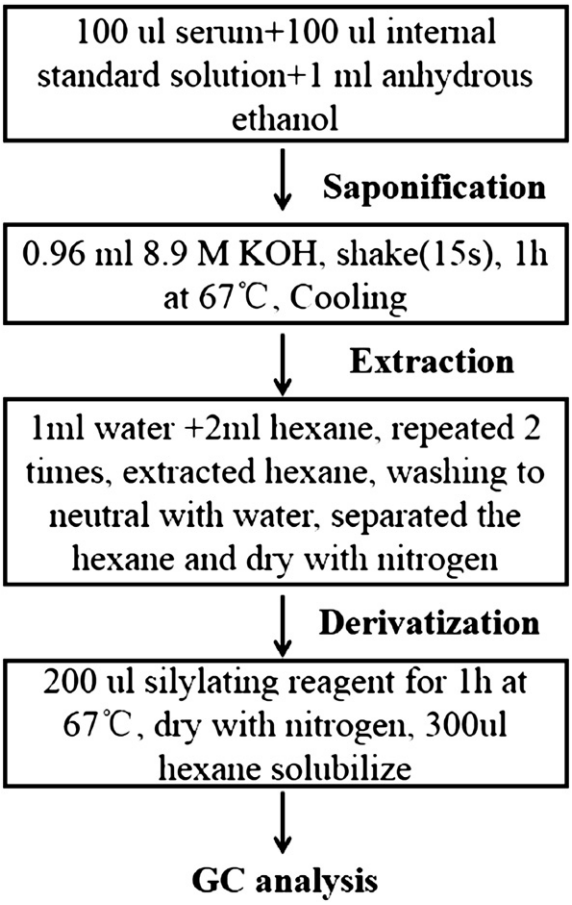

Fig. 1. Experimental flow chart.

Gas chromatography conditions

Gas chromatography was carried out in an HP-5 quartz capillary column (5% phenyl-methyl silicone; Agilent Technologies, Santa Clara, USA) (30 m × 0.32 mm × 0.25 μm) under the following conditions: temperature at the starting point, 150 °C; retention, 3 min; temperature-programmed rate, 30 °C/min to 250 °C and 5 °C/min to 280 °C; retention, 30 min; temperature of the flame ionization detector, 290 °C; sample injection temperature, 290 °C; sample introduction pressure, 15 psi; and splitless mode sampling, 1 μL. High-purity nitrogen (99.99%) was used as the carrier gas.

Cholesterol synthesis and absorption markers

An Agilent 7890 gas chromatograph was used for image acquisition. The markers were determined by the single internal standard curve method.

Measurement of cholesterol synthesis and absorption markers

Study population

Male and female patients from 40 to 70 years of age who had been diagnosed with CHD but had not been treated with lipid-lowering drugs over the last four weeks were recruited to this study. The study population comprised patients who visited Beijing Anzhen Hospital, China, between June 2008 and July 2009. The study protocol was reviewed and approved by the Ethics and Research Committee of Beijing Anzhen Hospital which follows the Declaration of Helsinki protocol and all participants provided their written informed consent. During the experimental period, patients were fed a low cholesterol diet. Patients who met any of the following criteria were excluded from the study: allergic to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor or ezetimibe; had active inflammatory myopathy (creatine kinase more than three times the upper limit of normal); had chronic viral hepatitis or abnormal liver function (alanine aminotransferase > 1.5 times the upper limit of normal); had renal dysfunction (serum creatinine level > 2 mg/dL) or nephrotic syndrome; had hematological disease; or had a malignant tumor. Healthy controls were subjects with no other diseases and with normal liver and kidney functions.

Criteria for the diagnosis of CHD

Patients who met the following criteria were diagnosed with CHD: the typical symptoms of angina pectoris; the dynamic change of electrocardiogram; echocardiography showed ventricular wall motion abnormalities; the clear old or acute myocardial infarction history; markers of myocardial damage abnormalities; and coronary angiography showed at least one main coronary vessel or its collateral vessel stenosis more than 50%.

Measurement of blood lipid and cholesterol synthesis and absorption markers

Venous blood samples were collected after overnight fasting at baseline and after four-week simvastatin therapy. TC, TG, HDL-C, LDL-C, apolipoprotein A (apoA), apolipoprotein B (apoB), and lipoprotein (a) were