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Simultaneous quantification of 18 saturated and unsaturated fatty acids and 7 sterols as their *tert*-butyldimethylsilyl derivatives in human saliva using gas chromatography-tandem mass spectrometry

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ABSTRACT

The profiling of fatty acids (FAs) or sterols has been applied in clinical studies, but still needs to be improved to enable their simultaneous quantification. Moreover, little progress has been made in determining the levels of FAs and sterols in human saliva in a single run. In this study, gas chromatography-tandem mass spectrometry (GC–MS/MS) using one-step *tert*-butyldimethylsilyl (TBDMS) derivatization was developed for comprehensive profiling of 18 FAs (eight saturated, five monounsaturated, and five polyunsaturated FAs) and 7 sterols (cholesterol and its precursors). The TBDMS derivatization process was also optimized in terms of reaction solvent, catalyst, temperature, and reaction time. The optimized conditions resulted in better derivatization efficiency with good chromatographic separation through a high-temperature column within 23 min. The present method provided good linearity ($r > 0.993$), precision (coefficient of variation, 2.7% to 10.4%), and accuracy (91.5% to 103.4%). The overall recovery ranged from 73.8% to 114.3% for the 18 FAs, and from 68.9% to 79.8% for the 7 sterols. The validated method was applied to characterize FAs and sterols in human saliva samples. This is the first report of a GC–MS/MS method for the simultaneous determination of various FAs and sterols from a small volume (100 μL) of saliva. This approach can be used as a primary screening tool to examine the levels of both FAs and sterols in saliva, providing detailed information about their homeostasis for diagnostic and prognostic purposes.

1. Introduction

Fatty acids (FAs) and cholesterol play important structural, energetic, and signaling roles in essential biological processes in all mammalian cells [1]. Evidence has shown the interconnection of these two lipid classes, which was underlined by the finding that the important transcription factors, sterol regulatory element-binding proteins (SREBPs) control both FAs and cholesterol biosynthesis (Fig. 1) [2]. Their homoeostasis can be disturbed under acute or chronic physiological conditions [3], which could alter the biosynthesis of FAs or cholesterol within cells, as reflected in their concentration in the blood. This can subsequently progress to diseases such as cancer [4], Huntington's disease [5], or cardiovascular disease [6].

Saliva specimens have recently been suggested to be an appropriate substitute for blood samples, in that saliva sampling is simple and non-invasive; moreover, disease-signaling biomarkers in saliva have been suggested to be suitable as indicators of health status in humans [7–9]. Indeed, several studies have reported the potential of salivary biomarkers in the diagnosis and prognosis of diseases, including dental diseases, diabetes, cardiovascular diseases, and cancer [7, 8]. However, little progress has been made in determining the levels of various FAs and sterols in human saliva using a single run of an experiment. Therefore, a comprehensive analytical method is needed for the simultaneous measurement of FAs (saturated, monounsaturated, and polyunsaturated) and sterols (cholesterol and its precursors) in saliva samples.

Many analytical methods have been developed for the determination of FAs or sterols using fluorometric enzyme immunoassays [10], and gas or liquid chromatography coupled to mass spectrometry (GC- or LC-MS) [9, 11–18]. Immunoassays have limited applicability in this context due to their overestimation of the analyzed levels as a result of cross-reactions between similar structures, which may lead to misinterpretation of the clinical results [19]; in contrast, MS-based profiling is a proven technique for this purpose, exhibiting high sensitivity

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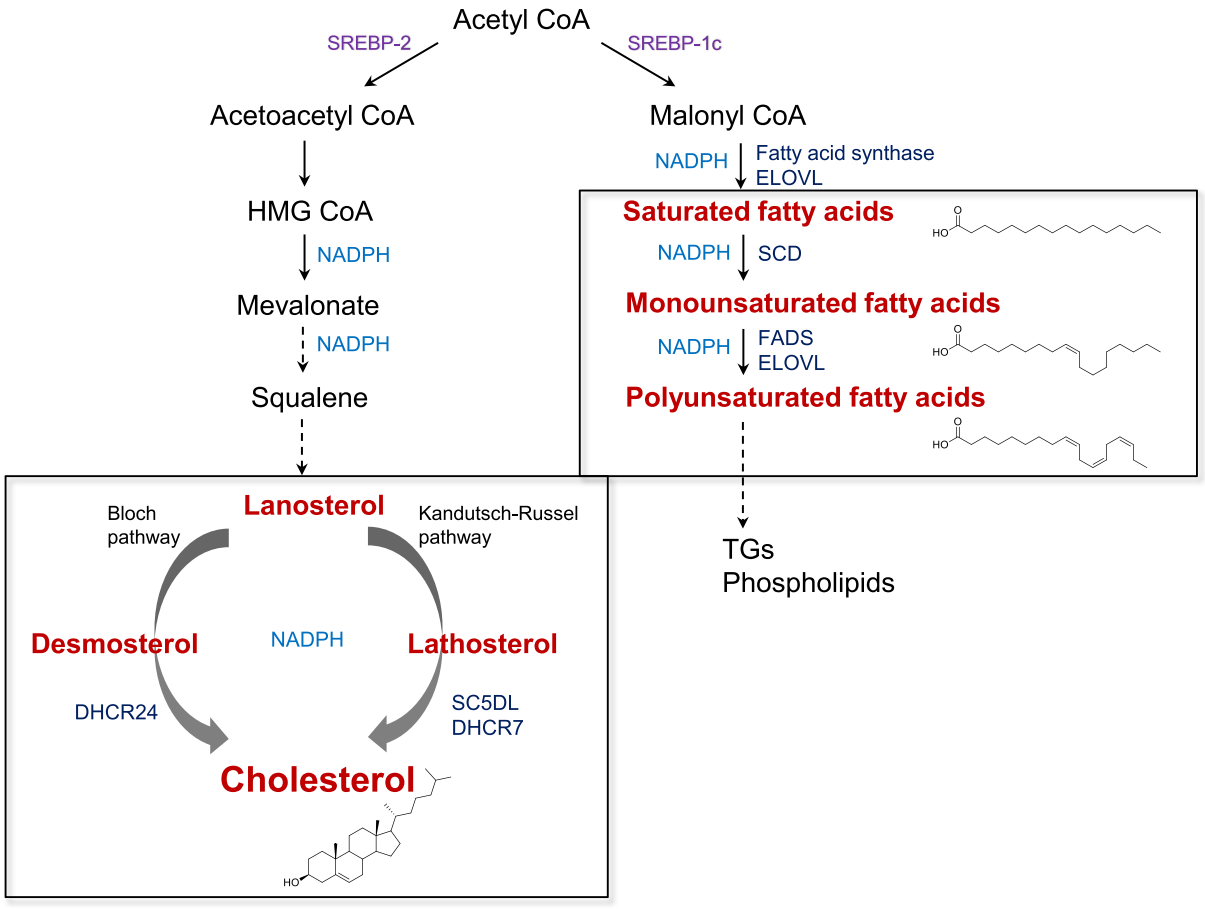

Fig. 1. The major metabolic pathway of fatty acid and cholesterol synthesis. In general, SREBP-2 preferentially activates genes related to cholesterol metabolism, whereas SREBP-1c preferentially activates genes related to fatty acid metabolism. (A) Cholesterol biosynthesis starts from acetyl-CoA, which leads to the formation of lanosterol, the first cholesterol precursor with a steroidal skeleton. This is further metabolized via two alternate pathways, including lathosterol or desmosterol as cholesterol precursors. (B) For fatty acid biosynthesis, acetyl-CoA is converted into malonyl-CoA, and subsequent elongation and further desaturation produce saturated, unsaturated, and polyunsaturated fatty acids with different carbon numbers. SREBP, sterol regulatory element-binding proteins; DHCR, dehydrocholesterol reductase; SC5DL, sterol-C5-desaturase; ELOVL, fatty acid elongase; SCD, stearoyl-CoA desaturase; FADS, fatty acid desaturase.

and selectivity in biological and clinical applications [20]. Although chemical derivatization is necessary to increase the volatility and sensitivity of FAs or sterols, the GC–MS method is still an excellent platform with good chromatographic resolution and isomer separation for both FAs and sterols [20]. Two-step derivatization strategies have been used to measure both FAs and cholesterol using different derivatization reagents as follows: i) acid-catalyzed methanolysis and subsequent silylation with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) [17] and ii) two-step silylation with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) followed by BSTFA (1% TMCS) [18]. These methods are capable of quantifying FAs and cholesterol at low levels, but they are time-consuming because of the need for additional derivatization steps. As an alternative, we designed a one-step derivatization method using MTBSTFA for both FAs and sterols. This reagent has advantages in terms of the formation of intense characteristic ions and highly stable *tert*-butyldimethylsilyl (TBDMS) esters [18], but for sterols, the TBDMS reaction tends to be incomplete when MTBSTFA alone is used [21]. Therefore, we optimized the TBDMS derivatization procedure to ensure a complete reaction for both classes.

Here, the GC–MS/MS method in combination with one-step TBDMS derivatization was developed to allow the simultaneous quantification of 18 FAs and 7 sterols. These included eight saturated FAs (C12:0, C14:0, C16:0, C18:0, C20:0, C22:0, C24:0, and C26:0), five monounsaturated FAs (C14:1, C16:1, C18:1, C22:1, and C24:1), five polyunsaturated FAs (C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:4 n-6, and C20:5 n-3), cholesterol, three cholesterol precursors as cholesterol synthesis markers (desmosterol, lathosterol, and lanosterol), and three phytosterols as cholesterol absorption markers (campesterol, stigmasterol, and β-sitosterol) (Table 1) in human saliva. The present method was successfully applied to the quantification of several FAs and sterols in human saliva samples. This method would be useful to evaluate the levels of both FAs and sterols in biological fluids, such as urine, blood, amniotic fluid, or cerebrospinal fluid, as well as saliva.

2. Experimental

2.1. Chemicals

The 18 FAs and seven sterols examined in this study (Table 1) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Steraloids (Newport, RI, USA), respectively. The internal standards (ISs), namely, d_3-myristic acid (d_3-C14:0) and d_{31}-palmitic acid (d_{31}-C16:0) for the 18 FAs, and 2,2,3,4,4,6-d_6-cholesterol for the seven sterols, were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA) and C/D/N isotopes (Pointe-Claire, Quebec, Canada). Silylating agents such as MTBSTFA and *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH_4I), pyridine (≥99.9%), sodium acetate (reagent grade), human serum albumin (HSA) (lyophilized powder, fatty acid-free, ≥99%), and acetic acid (glacial, ≥99.99%)