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Research Article

Microwave-Assisted Derivatization of Bile Acids for Gas Chromatography/Mass Spectrometry Determination

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Bile acids derived from cholesterol are produced in the liver, and their analysis is difficult due to their complex natures and their low concentrations in biological fluids. Mixtures of various derivatives, created via conventional heating, are used for such analyses. Microwave radiation is proposed to accelerate the derivatization process. This paper presents a mass fragmentation study and microwave-assisted derivatization (MAD) for the silylation of bile acids (cholic and ursodesoxycholic) prior to gas chromatography and mass spectrometry analysis. The derivatization was performed using the two-step process of methoximation and silylation. The reaction time, power, and quantity of N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TCMS) were optimized to improve the derivatization. The optimized derivatization conditions required 210 W for 3 min. The MAD method exhibited linearity with respect to cholic acid between 0.78 and 20.0 μg mL^{-1} with an LOQ of 0.23 μg mL^{-1} and a precision ranging from 1.08% to 9.32% CV. This optimized derivatization method is valid for the analysis of bile acids in different matrices.

1. Introduction

Bile acids (BAs) are steroidal compounds synthesized in the liver during cholesterol metabolism, and their major structural components include a steroid nucleus with a side chain and carboxyl groups [1–3]. BAs are predominantly present in biological fluids in their ionized form. The composition of BA in serum and urine varies with different physicochemical properties and the rate of intestinal absorption by the liver [2, 4]. Its hepatic and intestinal metabolism can also be influenced by liver and gastrointestinal diseases. Therefore, modifications to the hepatic synthesis, intracellular metabolism, hepatic uptake, and biliary excretion can result in a disturbance in the metabolism of bile acids [1, 4, 5]. An increase in blood bile salts appears to result from changes in their hepatocellular uptake that are induced by chemical substances and can serve as a biological marker for the detection of liver damage [1, 4–6].

Bile acids are present in serum and urine at millimolar levels, and due to large differences in their chemical properties, such as lipophilicity and polarity, their separation and identification require accurate and sensitive methods. Several analytical methods based on chromatography techniques have been reported for detecting bile acids in biological fluids [1, 7–9]. Liquid chromatography (LC) coupled with evaporative light-scattering detection (ELSD) or conventional UV-Vis detection have limited sensitivity [1]. Iida et al. [8] proposed a gas chromatography method using flame ionization detection (GC/FID) to analyze derivatized BA.

In analytical procedures using gas chromatography, derivatization is utilized to generate compounds with better volatility and thermal stability. For GC analysis, molecules such as aldehydes, carboxylic acids, esters, and phenols are of concern due to their ability to form hydrogen bonds between compounds, which can lead to low volatility, insufficient thermal stability, or an interaction with the solid column packing [10–13].

The most common reactions used in GC analysis are silylation, acylation, alkylation, and condensation [14]. The derivatization of the –COOH, –OH, and oxo-functional groups present in bile acid molecules can modify a compound to increase its volatility and improve its thermal stability,

but this procedure increases the total time required for the analysis [1, 3, 15–18].

Silylation is the most established derivatization technique, with common reagents including N-methyl-(trimethylsilyl) trifluoroacetamide (MSTFA), N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), trimethylchlorosilane (TMCS), and trimethylsilylimidazole (TMSI) used in the analysis of compounds such as estrogen steroids [19, 20], bile acid in aqueous environments [16, 21], and metabolomics [10, 22].

This derivatization reaction should be performed using controlled temperature and time to prevent the formation of additional unexpected derivatives (artifacts). Imprecise control over the derivatization parameters might significantly enhance the interference of the compound's concentration. Otherwise, hydroxyl groups are likely silylated at room temperature [23]. The strength of donor silyl groups represents another possibility for improving the derivatization using reagents such as TMSI, BSTFA, and MSTFA, the first of which are the strongest donors of a silyl group. Recently, Zhou et al. [20] reported that the addition of 0.5% TMSI in BSTFA or MSTFA effectively prevents the formation of multiple trimethylsilyl derivatives in the derivatization of ethinylestradiol and enhances the derivatization efficiency. The silylation reaction is a nucleophilic substitution on the silicon atom of the silyl donor. The TMSI activates the hydroxyl groups and serves as an acid scavenger by removing the acidic products [20]. In a conventional derivatization, the time is fundamental to reaction completion, but conventional derivatization is not as rapid as microwave heating. As with conventional derivatization, microwave-assisted derivatization requires the power and time to be optimized for complete derivatization and to avoid the formation of artifacts. Kumar et al. [24] used four derivatization mixtures: MSTFA + 1% TMCS, BSTFS + 1% TCMS, MSTFA : NH_4I : DTE, MSTFA : TMSI : TMCS, and BSTFA : pyridine [24].

Various methods for the derivatization of bile acids are described in the literature using gas chromatography with conventional derivatization [7, 16, 21, 25]. The method proposed by Tyagi et al. [25] silylated the bile acids with the addition of BSTFA/pyridine (1 : 1, v/v) at 60°C for 30 min. In another method proposed by Casas-Catalán et al. [26], the bile acids were identified using a derivatization method that combines the formation of ethyl esters from the carboxylic group using ethyl chloroformate (ECF) with the trimethylsilyl ethers from the hydroxyl group using trimethylsilylimidazole (TMSI) at 40°C for 20 min. To optimize the sensitivity, many derivatization mixtures were tested by Kumar et al. [24], including MSTFA/NH_4I/DTE (500 : 4 : 2, v/w/w, 40 μL at 60°C for 30 min), MSTFA/TMSI/TMCS (100 : 2 : 5, v/v/v, 40 μL at 60°C for 30 min), MSTFA/TMCS (100 : 1, v/v,40 μL at 60°C for 30 min), and BSTFA/TMCS (100 : 1, v/v, 40 μL at 60°C for 30 min). These authors concluded that the MSTFA/NH_4I/DTE mixture exhibited improved stability and MS fragment abundance [24, 25]; however, the time required for the derivatization was 10-fold longer than that required for microwave-assisted derivatization. Several reagent mixtures have been tested for the derivatization (silylation) of bile acids and have been found to improve the sensitivity and efficiency without reducing the derivatization time [24]. Recently, Ranz et al. [27] and Kouremenos et al. [10] evaluated the potential of microwave-assisted derivatization for different analytes, such as herbicides and metabolomes; however, they did not examine microwave irradiation for the derivatization of the bile acids. Microwave-assisted derivatization (MAD) has the additional advantage of reducing the analysis time [28, 29]. The microwave energy does not directly induce chemical reactions or degradation, but it efficiently heats the reagent mixtures via "microwave dielectric heating." This reaction is attributed to the rapid transfer of energy and the ability of the material to absorb the microwaves. In addition, this process reduces the energy demand, increases the ease of use, and eliminates the need to heat the reaction vial [30]. Kouremenos et al. [10] studied the derivatization of many classes of compounds (sugars and keto acids) and demonstrated that the amino acids present in a mixture exhibit incomplete derivatization and require additional time to react. The conventional derivatization methods for bile acid analysis may require an extended time (50 to 120 minutes), but the use of microwaves can decrease this time to a few minutes and provide a safer heating method. The present study describes an analytical procedure for determining bile acids via gas chromatography/mass spectrometry using microwave-assisted derivatization.

2. Materials and Methods

2.1. Bile Acid Standards. The following bile acids were included in this study: cholic acid (CA; 98%) and ursodesoxycholic acid (UDCA; 99%) purchased from Sigma-Aldrich Vo. (St. Louis, MO, USA). Methanol (reagent grade) and dichloromethane (chromatographic grade) were obtained from Merck KGaA (Darmstadt, Germany). The 99.9% w/w MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide), BSTFA (N,O-bis (trimethylsilyl)-trifluoroacetamide) + 1% v/v TMCS and 99.8% w/w pyridine (anhydrous-grade solvents) were purchased from Sigma-Aldrich Vo. (St. Louis, MO, USA), and the methoxyamine PA was obtained from Fluka (St. Louis, MO, USA).

The 1.0 mg mL^{-1} bile acid standard solutions were prepared in methanol and stored at −20°C. To optimize the MAD, the standard solutions were diluted to generate individual working standards in dichloromethane/methanol (2 : 1, v/v) at a final concentration of 1.0 μg mL^{-1} and stored at 4°C.

2.2. Instrumentation and GC/MS Analysis. The GC/MS analysis was conducted using a Thermo Electron Trace gas chromatographic system from Thermo Scientific Inc. (West Palm Beach, FL, USA) coupled with an POLARIS Q model ion trap spectrometer and equipped with a capillary column (30 m × 0.25 mm id × 0.25 μm film) containing 5% diphenyl and 95% dimethylpolysiloxane HP-5 ms (Agilent Technology Inc.). The initial oven temperature was 120°C, which was maintained for 1 min. The temperature was increased at a rate of 15°C min^{-1} to 250°C; then, it was increased to 300°C at 5°C min^{-1} and held for 20 min.

The helium flow was 1.0 mL min^{-1}. The injector was operated at 280°C in the splitless mode for 3 min, followed by a 1 : 20 split ratio (RD). The mass spectrometer was operated in electron impact mode (EI) at 70 eV. The temperature of the ion source was 200°C and that of the GC/MS interface was 250°C. The analysis was performed in full scan mode (range 50–90 *m/z*) with quantification in selected-ion monitoring (SIM) mode using the *m/z* 426 (cholic acid) and 518 (ursodesoxycholic acid).

A domestic microwave from Philco (model P.R.C, power 10–700 Watts) was used for the MAD derivatization. Xcalibur software from Thermo Finnigan Inc. (West Palm Beach, FL, USA) was used to acquire the signal and process the data.

2.3. Derivatization Procedure. A 10.0 μL working standard solution with a concentration of 1.0 μg mL^{-1} was transferred to a glass insert (flat bottomed) and dried at room temperature. Then, 10.0 μL of methoxyamine in pyridine (20 mg mL^{-1}) was added, followed by 50.0 μL of BSTFA with 1% TCMS. The glass insert was introduced into a GC vial, and the mixture was subjected to heating via microwave irradiation. Initially, the power (160, 240, 320, 480, and 640 W) and time (1, 2, 3, 4, and 5 minutes) were varied to evaluate the response, reproducibility, and speed of the analysis using microwave derivatization. The yield of the microwave derivatization was compared with that of conventional derivatization.

3. Results and Discussion

3.1. Fragmentation Patterns of the Bile Acids. The fragmentation patterns of the bile acids were studied to identify the different compounds. The *m/z* of the molecular ion, the molecular ions without methyl or trimethylsilyl groups, and other fragmentation products in the steroid nucleus were found in greater abundances. The experimental results for the bile acid ions (*m/z*) are presented in Table 1.

In the study of cholic acid, the molecular ion without a methyl group produced an *m/z* 681 fragment, which was used to identify this compound. However, this fragment presented a lower intensity (15.76%), as shown in Table 1. In contrast, the *m/z* 253 ion (100% abundance) could not be used to identify or quantify the cholic acid because it is an artifact of the derivatization. The quantity of the *m/z* 516 ion is related to the loss of 2 TMSOHs. The quantity of the *m/z* 426 ion is dependent on the loss of 3 TMSOHs (47.94% abundance). This ion was used to identify the cholic acid due to its higher relative intensity. The *m/z* 428 ion, which corresponds to the compound that lost 2 TMSOHs and $Si(CH_3)_4$, presents a low intensity, and its total ion chromatogram (TIC) presents many artifacts. The chromatogram and mass spectrum found in Figure 1 demonstrate the efficiency of the cholic acid derivatization.

The conventional derivatization of cholic acid (1 h at 100°C) demonstrated results similar to those obtained using microwave heating (3 min), as shown in Table 1. Furthermore, the same derivative structure was obtained in each step of the procedure using the MAD method to derivatize the bile acids. The trimethylsilyl (oxime) ether/esters of the cholic and ursodesoxycholic acid derivatives were produced at the same power and time as the carboxylic acids and the phenol groups. Therefore, the use of microwaves for heating during the derivatization reduced the reaction time from 60 to 3 minutes. After derivatization, the ursodesoxycholic acid had a molecular ion of *m/z* 608.5. The *m/z* 413 ion is produced by the loss of 2 TMSOH + CH_3 and presents a lower intensity (32% abundance). These two ions present more artifacts in the total ion chromatogram (TIC). Another fragment ion is *m/z* 518, which is formed by the loss of TMSOH (Figure 2).

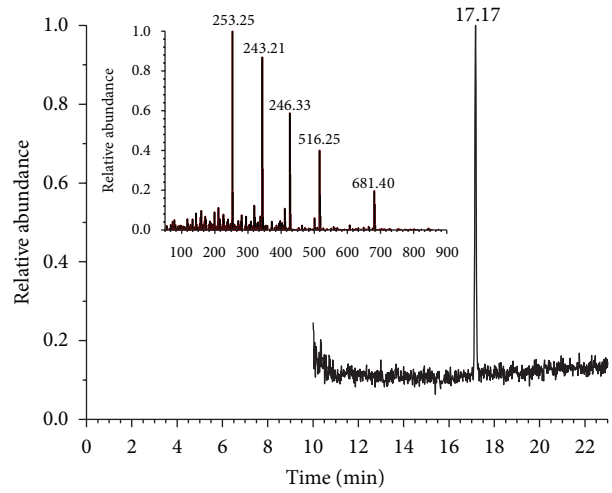

Figure 1: Chromatogram and mass spectra of the trimethylsilyl (oxime) ether/ester of cholic acid obtained using microwave radiation for 3 min at 240 W in the positive mode; *m/z* 426 (1.0 μg mL^{-1}).

This fragment has the highest relative intensity (100% abundance) but also corresponds to chenodeoxycholic acid, as demonstrated by Sebok et al. [21]. Thus, the identification and quantification of the ursodesoxycholic after the microwave-assisted derivatization were performed using the *m/z* 428 ions, as shown in Table 1.

3.2. Optimization of the Microwave-Assisted Derivatization. The order by which derivatization was optimized is as follows: the quantity of the methoximation and silylation reagents, followed by the power and time of the microwave irradiation. The silylation was performed on the BSTFA + 1% v/v and TCMS in volumes of 10.0 μL, 20.0 μL, 40.0 μL, 50.0 μL, 60.0 μL, and 100.0 μL. A volume of 40 μL was chosen because it presented the highest peak area. The smaller volumes were insufficient for complete derivatization, and volumes above 40.0 μL resulted in increased artifacts, such as new peaks in the TIC.

The microwave time and power can potentially affect the responses, including the peak area. Therefore, after establishing the reagent quantities, the power (watts) and time (min) were optimized. The analysis held one factor constant while varying the other. The powers studied were 160, 240, 320, 480, and 640 W at a constant time of 1, 2, 3, 4, or 5 minutes. Each condition was tested three times. The optimization results for the derivatization of the bile acids at the various powers

Table 1: Fragmentation patterns of the bile acid TMS (oximes) derivatives obtained using gas chromatography and mass spectrometry (GC/MS) and the common ions in the fragmentation patterns of cholic acid (conventional and microwave-assisted derivatization) and ursodesoxycholic acid (MAD) presented as relative intensities.

Bile acid	t_r (min)	Selected fragment ions for identification and quantification		Frequent ion in fragmentation			
		$[M]^{+\bullet}$	$[M-15]^+$	Further abundant fragment ion, m/z	m/z	Relative intensity (%) microwave	Relative intensity (%) conventional
Cholic	17.19	696.5	681	$[M-2\text{TMSOH}]^+ = 516$	253	100	100
				$[M-(2\text{TMSOH}+\text{SiCH}_4)]^+ = 428$	426	47.94	56.74
				$[M-3\text{TMSOH}]^+ = 426$	516	32.23	39.61
					681	15.76	14.87
Ursodesoxycholic	17.59	608.5	593	$[M-2\text{TMSOH}]^+ = 428$	413	32	
				$[M-(15+2\text{TMSOH})]^+ = 413$	428	56	
					518	100	

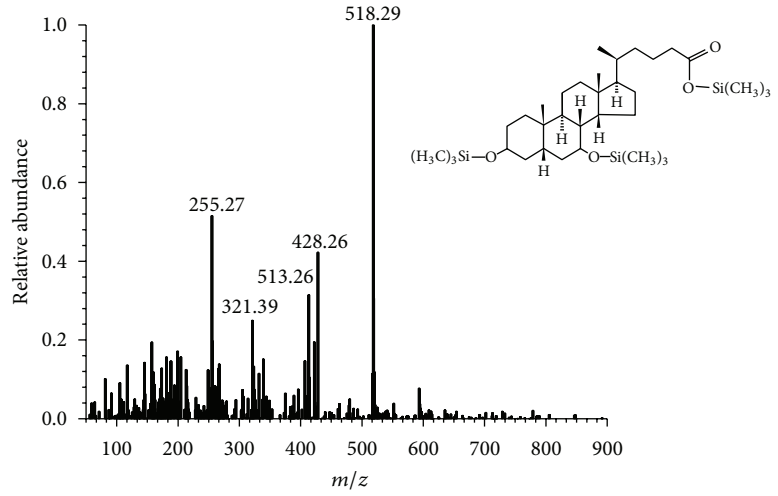

Figure 2: Mass spectra of the ursodesoxycholic acid standard trimethylsilyl (oxime) derivative at a concentration of 1.0 μg mL^{-1} and an m/z of 518, corresponding to the loss of TMSOH and the presence of a fragmented compound.

and times are presented in Figure 3. Increasing the power and time did not result in improvement in the peak area. Likewise, a higher power at 3 minutes decreased the relative peak areas of the cholic and ursodesoxycholic acids. The same decrease in the peak area can be observed at 1.5 min. The relative responses of the analyte peaks indicate that 240 W at 3 min provided the best sensitivity. In addition, this condition also improved the reproducibility.

The presence of artifacts in the silylation is a common problem with functional groups such carboxylic acids and phenols. The bile acids were silylated at these groups. However, optimizing the derivatization reagents can decrease this interference. The formation of artifacts was also observed by Kouremenos et al. [10] and Zhou et al. [20]. The artifacts have not been specifically identified. In addition, the heating and reaction times for conventional derivatization and microwave-assisted derivatization changed as expected.

The highest resolution was achieved with a mixture containing 10.0 μL of bile acid standard, 20.0 μL of methoxyamine, and 40.0 μL of silylation reagent. Figure 4 provides the peak areas obtained using different quantities of silylant. The derivatization results enabled the correct identification and quantification of the bile acids.

Acceptable results were obtained for the derivatization using microwaves as a heating source because the fragmentation patterns are comparable to those obtained by Sebok et al. [21] using conventional derivatization at 100°C for 60 min to determine bile acids via gas chromatography/mass spectrometry of trimethylsilyl (oxime) ether/ester derivatives.

A 3-fold decrease in the formation of TMS (oxime) ester/ether derivatives was observed when the power was increased from 150 to 300 W, leading to an increase in the irradiation time. The shortest reaction time was 30 min, which was a 10-fold increase in time over that required for microwave

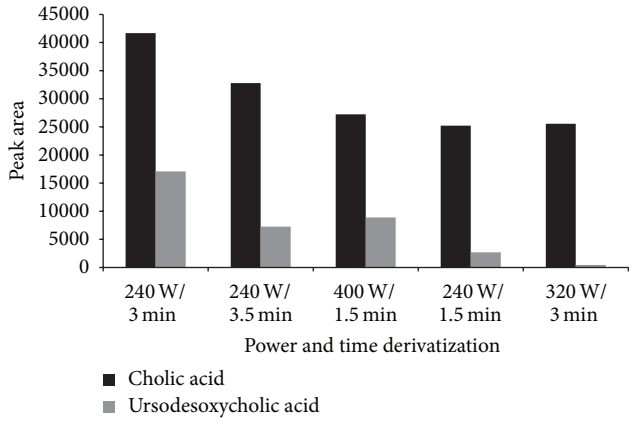

Figure 3: Effect of power and time on the yield of the derivatization of bile acids.

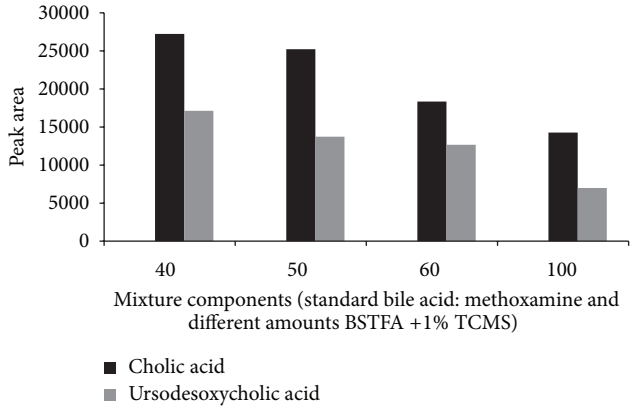

Figure 4: Comparison of various derivatization reagent mixtures and bile acids using microwave derivatization.

derivatization. The MSTFA and BSTFA were used for the silylation of the bile acids; however, neither was required to achieve complete derivatization.

3.3. Method Validation. The calibration curve for the cholic and ursodesoxycholic bile acids was constructed at six concentration levels with three replicates for each level. The curve obtained for the cholic acid ($y = 4849.8x - 764.15$) presented good linearity in the range of 0.78 ± 0.01 to $20.0 \pm 0.02\,\mu g\,mL^{-1}$ with $R^2 = 0.999$ (P value < 0.0001). This range represented 0.129 to 3.30 ng of cholic acid, a quantity smaller than that found by Sebok et al. [21] (0.432 to 3.46 ng). For the ursodesoxycholic acid, the obtained curve ($y = 4849.8x - 764.15$) exhibited good linearity in the range of 0.44 ± 0.01 to $20.0 \pm 0.02\,\mu g\,mL^{-1}$ with $R^2 = 0.999$ (P value < 0.0001).

The limits of detection (LOD) and quantification (LOQ) were calculated according to the recommendations of the EURACHEM Guide [31] through ten consecutive measurements of the blank. The LOD values were $0.23\,\mu g\,mL^{-1}$ and $0.13\,\mu g\,mL^{-1}$ for cholic acid and ursodesoxycholic acid, respectively. The LOQ values were $0.78\,\mu g\,mL^{-1}$ for cholic acid and $0.44\,\mu g\,mL^{-1}$ for ursodesoxycholic acid and were lower than those obtained in other studies [21, 24]. To assess the intra-assay precision (repeatability), ten replicates with concentrations of 1.0, 10.0, and $20.0\,\mu g\,mL^{-1}$ were analyzed on the same day. For cholic acid, the coefficients of variation were 9.32%, 3.90%, and 1.08%. The ursodesoxycholic acid presented coefficients of variation of 11.71%, 13.02%, and 1.38%. The precisions obtained using this derivatization are similar to those obtained by Damm et al. [28] for the microwave-assisted derivatization of morphine.

4. Conclusion

This study presented an alternative method for the gas chromatography/mass spectrometry identification and quantification of bile acids using microwave-assisted derivatization. The fragmentation patterns were evaluated in this study by varying the MAD heating protocol. This procedure was validated and demonstrated precision and linearity. A study of the variables used in the derivatization was performed, and a complete derivatization was obtained only after the addition of methoxyamine. The microwave irradiation did not directly induce the chemical reactions and degradation, but it was an efficient method of heating the reagent mixtures and reduced the sample preparation time required to obtain the derivatized products and properly identify and quantify the bile acids. This derivatization method can be used to analyze bile acids in different matrices, such as urine, serum, or bile. However, the complexity of these biological matrices will require selectivity studies to complement the validation of the proposed method.

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