PRACA ORYGINALNA

Application of the pyrolytic methylation to GC/MS profiling of short-chain fatty acids in selected pharmaceutical preparations

Zastosowanie pirolitycznej metylacji w oznaczaniu profilu krótkołańcuchowych kwasów tłuszczowych w wybranych preparatach farmaceutycznych techniką GC/MS

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ABSTRACT

Lactic Acid Bacteria (LAB) belong to the normal flora of human alimentary tract and vaginal epithelium. The main products of LAB metabolism are short chain fatty acids (SCFA), which are known to have significant influence on human health. The aim of this study was to examine the suitability of direct pyrolytic methylation in GC/MS profiling of SCFA in the selected pharmaceutical preparations of LAB. For method optimization, standard SCFA samples consisting of acetic, propionic, butyric and lactic acids were pyrolyzed under various temperature conditions in the presence of methanolic solution of tetramethylammonium hydroxide (TMAH) as a derivatizing reagent. It was demonstrated that pyrolytic derivatization of standard SCFA to methyl esters was the most efficient with the use 10% TMAH and the pyrolytic filaments with Curie temperature of 480°C, when the pyrolysis cell was kept at 150°C. It was shown that the method is suitable for GC/MS qualitative analysis of SCFA profile in the pharmaceutical preparations that contain LAB in lyophilized form.

KEY WORDS
pyrolysis, GC/MS, TMAH derivatization, SCFA, LAB

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STRESZCZENIE

Bakterie kwasu mlekowego (*Lactic Acid Bacteria* – LAB) należą do naturalnej flory bakteryjnej zasiedlającej przewód pokarmowy oraz nabłonek pochwy człowieka. Głównym produktem metabolizmu LAB są krótkołańcuchowe kwasy tłuszczowe (*short-chain fatty acids* – SCFA) wywierające istotny wpływ na zdrowie człowieka. W pracy poddano ocenie przydat-

ność techniki bezpośredniej metylacji pirolitycznej w oznaczaniu profilu SCFA w wybranych preparatach farmaceutycznych LAB techniką GC/MS. W celu optymalizacji metody, wzorcowe próbki SCFA zawierające kwas octowy, propionowy, masłowy i mlekowy poddano pirolizie w obecności metanolowego roztworu wodorotlenku tetrametyloamoniowego (TMAH) w różnych warunkach temperaturowych. Stwierdzono, że derywatyzacja analizowanych kwasów tłuszczowych do estrów metylowych zachodziła z największą wydajnością pod wpływem 10% TMAH, gdy zastosowano druty pirolityczne o temperaturze Curie 480°C, a komorę pirolizera utrzymywano w temperaturze 150°C. Wykazano, że zoptymalizowana metoda jest przydatna w prowadzonej techniką GC/MS analizie jakościowej profilu SCFA w wybranych preparatach farmaceutycznych zawierających LAB w formie liofilizatu.

SŁOWA KLUCZOWE
piroliza, GC/MS, derywatyzacja, TMAH, krótkołańcuchowe kwasy tłuszczowe, bakterie kwasu mlekowego

INTRODUCTION

Lactic Acid Bacteria (LAB) are one of the constituents of normal flora of the oral cavity, the intestinal tract, and the vaginal epithelium in humans. The microorganisms obtain energy only from carbohydrate fermentation, and the main products of their metabolism are short chain fatty acids (SCFA). Since these compounds are known to have significant influence on human health, their determination in samples of various types is an important problem. The chromatographic methods that are commonly used for higher fatty acids analysis are unsuitable for SCFA, mainly due to unsatisfactory yields resulting from high volatility of SCFA as well as their strong affinity to aqueous phase. To overcome this problem, some derivatization methods have been developed that are based on various chemical modifications of fatty acid functional groups leading to the formation of derivatives of desirable chromatographic properties. Esterification of carboxylic groups prior to GC analysis is one of the commonly used methods of fatty acids derivatization. Long-chain fatty acids are usually derivatized to more volatile methyl esters [1,2]. Preparation of less polar propyl or butyl esters is the method of choice in GC analysis of short-chain fatty acids [3], although an alternative methods based on the silylation [4] of carboxylic group or preparation of pentafluorobenzyl bromide derivatives [5] were also described.

Several problems can be encountered, however, when SCFA are derivatized by conventional methods. In general, these methods require prolonged heating of the analyzed fatty acids with appropriate reagents. Under such conditions, short-chain α-hydroxy acids are easily transformed to cyclic lactides or they form polyesters [6]. Especially in trace analysis, Teflon® test tubes or silylated glassware should be used since adsorption on the surface of the glass may lead to decreased recoveries or even total loss of the determined small hydroxy acids [4]. SCFA are readily water-soluble, which make their quantitative transfer to organic phase difficult. Such transfer is essential, because derivatization efficiency decreased dramatically in the presence of water.

Dworzański et al. [7,8] developed a new technique of pyrolytic on-line derivatization coupled with GC/MS analysis of bacterial long-chain fatty acids. Whole bacterial cells were pyrolytically methylated using tetramethylammonium hydroxide (TMAH), and the fatty acid methyl esters formed were thermally extracted directly to the stream of the carrier gas (helium). An alternative method of bacterial fatty acids profiling by direct pyrolytic derivatization to picolinyl esters was developed by Kurkiewicz et al. [9]. Pyrolytic chromatography coupled with mass spectrometry was also successfully applied by Urakami et al. [10] for compositional analysis of copoly (DL-lactic/glycolic acid).

In this study, the method of direct pyrolytic methylation was applied to GC/MS analysis of short-chain fatty acids (SCFA) in the selected pharmaceutical preparations of LAB. For the method optimization, standard SCFA samples were pyrolyzed under various conditions, using methanolic solution of TMAH as a derivatizing reagent.

MATERIAL AND METHODS

In all experiments, the glassware silanized according to the procedure of Landeghem et al. [4] was used. SCFA standards (acetic, propionic, butyric and lactic acids; Aldrich) were dissolved in methanol to obtain the final concentration of each acid of 0.1%. Aliquot (5 µl) of the mixture was placed on the tip of pyrolytic filament, then an equal volume of 1% or 10% solution of TMAH (Aldrich) in methanol was added, and the pyrolysis coupled with gas chromatography/mass spectrometry analysis of the product formed (Py-GC/MS) was carried out. Lakcid ® and Lakcid forte® (Aldrich), LAB containing pharmaceutical preparations, were analyzed in the similar way, except that a drop of a methylating reagent was applied on the tip of pyrolytic wire coated with the dry bacteria lyophilizate.

Py-GC/MS analyses were performed in a Pye Unicam Curie point pyrolyser coupled directly to a Hewlett Packard 5890 series II gas chromatograph interfaced to a Hewlett Packard 5890A MS-Engine quadrupole mass spectrometer. The pyrolyses were carried out at 480, 610 or 770°C (Curie point of the pyrolytic filaments used) for 4s. The pyrolysis cell was kept at 50, 120, 135, 150, 170 or 220°C. GC separations were carried out on Rtx®--5MS (Restek) fused-silica capillary column (5% diphenyl, 95% dimethyl polysiloxane, 60 m x 0.32 mm i.d., 0.5 µm film thickness) with helium as a carrier gas. The splitting ratio of 1 : 20 was used. The GC oven temperature was programmed from 30°C (isothermal for 5 min) to 150°C at a rate of 2°C/min, then to 250°C at a rate of 10°C/min; the final temperature was held for 10 min. The parameters of mass spectrometer were as follows: the ion source temperature 176°C, the quadrupole temperature 100°C, ionization energy 70 eV. Mass spectra of the separated compounds were compared to standard spectra of the Wiley 8th Edition library database.

RESULTS AND DISCUSSION

Pyrolytic derivatization of SCFA with the use of TMAH leads to the formation of fatty acid methyl esters. Generally, carboxylic groups of SCFA are involved in the esterification reaction. In the case of α-hydroxy acids, however, ether bonds can also be formed via hydroxy groups of these compounds.

As can be seen from figure 1A, when the mixture of SCFA standards was pyrolyzed in the presence of 1% TMAH, methyl esters of acetic (3.3 min), propionic (4.9 min) and butyric (7.5 min) acid were formed, while lactic acid was derivatized to two different products, namely 2-hydroxy propanoic acid methyl ester (8.1 min; main product) and 2-methoxy propanoic acid methyl ester (9.6 min; formed with lower efficiency). When 10% TMAH was used, however, only one product of lactic acid derivatization (2-methoxy propanoic acid methyl ester) was detected in the pyrolysate apart from acetic, propionic and butyric acid methyl esters (Fig. 1B). These results show that esterification of SCFA carboxyl groups occurs irrespective of TMAH concentration. For complete derivatization of SCFA hydroxyl groups, however, higher concentration of the methylating reagent should be used.

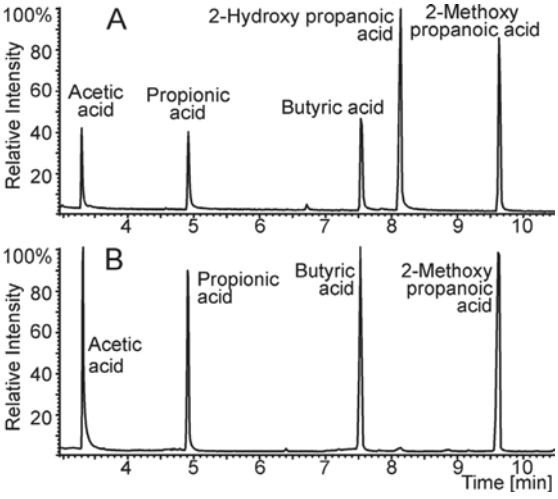

Fig. 1. Pyrogram of standard SCFA methyl esters obtained at optimal pyrolysis parameters (Curie point: 480°C, pyrolysis cell: 150°C) using 1% (A) or 10% (B) TMAH in methanol as a derivatizing reagent.

Ryc. 1. Pirogram estrów metylowych wzorcowych SCFA otrzymanych w optymalnych warunkach pirolizy (punkt Curie: 480°C, komora pirolizera: 150°C) przy użyciu 1% (A) lub 10% (B) roztworu TMAH w metanolu jako odczynnika derywatyzującego.

For optimization, the mixture of SCFA standards was pyrolytically methylated at variable pyrolysis parameters, such as: Curie temperature (T_c) of the pyrolytic filaments used, and a temperature of the pyrolysis cell (T). The results obtained indicate that pyrolytic derivatization of SCFA to methyl esters was

Table I. The efficiency of pyrolytic methylation of standard SCFA at various Curie temperatures

Tabela I. Wydajność metylacji pirolitycznej wzorcowych SCFA w różnej temperaturze Curie

T_c [°C]	Area under the peaks x 10^5			
	acetic acid	propionic acid	butyric acid	lactic acid
	$\bar{X} \pm SD$ [a]	$\bar{X} \pm SD$ [a]	$\bar{X} \pm SD$ [a]	$\bar{X} \pm SD$ [a]
770	nd	3 ± 0.2	2.4 ± 0.9	5.3 ± 0.8
610	11 ± 9	16 ± 6	49 ± 18	109 ± 75
480	544 ± 38	483 ± 37	462 ± 18	453 ± 23

[a] Mean ± standard deviation (n = 5).

Table II. The effect of the temperature of pyrolysis cell on the yield of pyrolytically generated standard SCFA methyl esters

Tabela II. Wpływ temperatury komory pirolizera na wydajność pirolitycznego tworzenia estrów metylowych SCFA

T [°C]	Area under the peaks x 10^5			
	acetic acid	propionic acid	butyric acid	lactic acid
	$\bar{X} \pm SD$ [a]	$X \pm SD$ [a]	$X \pm SD$ [a]	$\bar{X} \pm SD$ [a]
220	nd	49 ± 9	61 ± 11	53 ±5
170	251 ± 21	351 ± 24	342 ± 23	403 ± 86
150	512 ± 35	502 ± 56	541 ± 19	554 ± 90
135	568 ± 2	551 ± 12	362 ± 28	332 ± 20
120	555 ± 45	535 ± 33	224 ± 91	316 ± 77
50	118 ± 9	148 ± 16	109 ± 06	75 ± 1

[a] Mean ± standard deviation (n = 5).

the most efficient with the use of pyrolytic filaments with T_c of 480°C (Table I). When the filaments with higher Curie temperatures (610°C and 770°C) were applied, significantly decreased yields of SCFA derivatives were observed, in particular more volatile acetic and propionic acid methyl esters. Urakami et al. [10] also achieved the best results when the pyrolysis of lactic/glycolic acid copolymer was carried out at relatively low temperature (500°C).

Another series of experiments was performed to establish an optimal temperature of the pyrolysis cell. The results presented in Table II indicate that the yield of SCFA methyl esters was the highest when the pyrolysis cell was kept at 150°C. At lower temperatures (135°C, 120°C), the yield of acetic and propionic acid methyl esters increased slightly, while the levels of less volatile esters of butyric and lactic acids were substantially diminished. The yield of all the SCFA methyl esters formed decreased dramatically when both too low (50°C) and too high (220°C) temperature of the pyrolysis cell was used. Such results are undoubtedly connected with the boiling points of the analyzed SCFA methyl esters (the higher boiling point, the

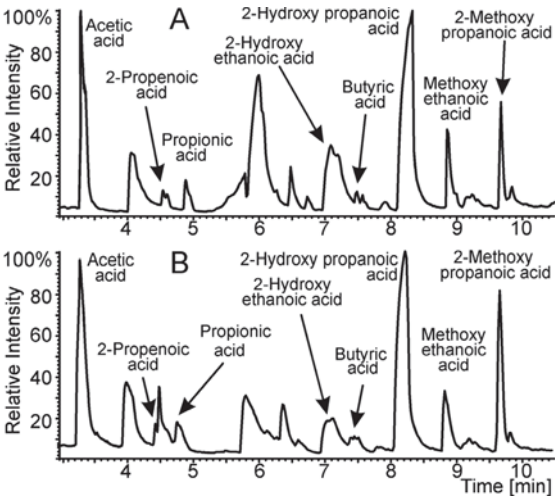

Fig. 2. Pyrogram of SCFA methyl esters obtained during pyrolytic methylation of LAB lyophilizates Lakcid® (A) and Lakcid forte® (B), performed at the optimized conditions (Curie point: 480°C, pyrolysis cell: 150°C, 10% TMAH in methanol as a derivatizing reagent).

Ryc. 2. Pirogram estrów metylowych SCFA otrzymanych podczas metylacji pirolitycznej liofilizatów LAB: Lakcid® (A) i Lakcid forte® (B) w zoptymalizowanych warunkach (punkt Curie: 480°C, komora pirolizera: 150°C, odczynnik derywatyzujący: 10% roztwór TMAH w metanolu).

higher optimal temperature of the pyrolysis cell).

The optimized conditions of pyrolytic methylation procedure (T_c of 480°C, pyrolysis cell kept at 150°C, 10% methanolic solution of TMAH used as a derivatizing agent) have been applied for Py-GC/MS analysis of LAB-containing preparations: Lakcid (Fig. 2A) and Lakcid forte (Fig. 2B). SCFA profiles of the studied bacterial lyophilizates involve short-chain α-hydroxy acids (glycolic and lactic acid), monocarboxylic saturated SCFA (acetic, propionic and butyric acids) and some unsaturated structures (acrylic acid). It was found that acidic metabolites of LAB in the studied preparations were dominated by lactic acid.

CONCLUSION

Pyrolytic methylation with the use of TMAH as a derivatizing agent is suitable for qualitative GC/MS analysis of bacterial SCFA profile in commercially available LAB lyophilizates.

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