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Original Article

Development and optimization of a gas-chromatographic separation method of fatty acids in human serum

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ABSTRACT

In this paper, we present the development and optimization of an efficient GC-MS method for the separation of fatty acids in human serum collected from a healthy volunteer and from an adrenoleukodystrophic (ALD) patient. The method was optimized in every step, meaning derivatization, extraction of obtained fatty acid methyl-esters (FAME) and GC separation. For the separation, we used a non-polar stationary phase (DB-5ms column) and helium as carrier gas at 1 ml/min, under a temperature gradient (100-170-280°C). The method was applied for the separation and identification of 16 fatty acids and other 3 lipidic compounds. Very long chain fatty acids, such as C26:0, were identified in single ion monitoring (SIM) mode of the mass spectrometric detector.

Keywords: fatty acids, gas chromatography, mass spectrometry

INTRODUCTION

Fatty acids are one of the most common compounds present in living organisms, be they plants, animals or humans. They appear as essential components of the cellular membrane, being the lipidic part of the glycolipids, which are playing important roles in the biology, life and protection of the cell. Moreover, their role is very important in many metabolic pathways or other essential routes in the organism. They have an essential role in the pro-inflammatory/anti-inflammatory processes [1,2] and their modified profiles can indicate different conditions, such as diabetes mellitus [2–4], different types of cancer [5,6], but also neurologic or psychiatric diseases [7,8]. In the same time, the amount and the quality of fatty acids ingested by a human being can have a positive and/or a negative influence on the total fatty acid profile in the organism [1,2] and/or it can be reflected in different tissues or fluids.

The analysis of fatty acids in serum/plasma is usually realized by gas-chromatography coupled with mass spectrometry, this being the top choice method [9], due to its high efficiency, relative low analysis times, coupled with the specificity and sensitivity of the mass spectrometer. Alternative methods like HPLC-MS [10–12] or CE-MS [13,14] have been used, especially because they do not necessarily need the derivatization step, thus a lower total procedure time can be achieved.

While GC-MS remains the top choice method for the analysis of fatty acids, literature gives a very large number of methods for their analysis, which vary a lot and can be very messy. The aim of this present work was to develop and fully optimize a straight GC-MS method for the analysis of fatty acids in human serum, with a focus on the separation not only of common fatty acids found in human serum, but especially focusing on very long chain fatty acids (VLCFA), up to 26 carbon atoms, which are normally found in trace levels. For this purpose, our objectives were to optimize not only the GC-MS separation conditions, but also the sample pretreatment procedure.

MATERIALS AND METHODS

Materials: Standards for fatty acids, docosanoic acid (C22:0), tetracosanoic acid (C24:0), hexacosanoic acid (C26:0) and heptadecanoic acid (C17:0) were purchased from Sigma-Aldrich® (Germany) and were of GC analytical purity grade. Methanolic hydrochloric acid 3N and hexane were purchased from Sigma-Aldrich® (Germany) and

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were also of GC analytical grade. Human serum was prepared after collecting 10 ml of blood from the only patient diagnosed with adrenoleukodystrophy registered to the Endocrinology Clinic in the city of Cluj-Napoca, Romania (universitary center with over 300.000 citizens and over 1 million students each year) and, after clotting, the serum was separated by centrifugation. Serum from one healthy male volunteer was prepared following the same procedure. All serum samples were stored at -20°C until sample preparation. All human individual (pacient and healthy volunteer) involved in this study signed an informed consent form.

Apparatus and methods

Derivatization and extraction of FAME: Stock solutions of each fatty acid standard, including internal standard C17:0, were prepared by dissolving around 1.5 mg of fatty acid in 10 ml methanolic HCl. From each stock solution, an exact volume containing 10 µg of fatty acid was measured and diluted to 2 ml methanolic HCl in a glass capped vial. The solutions were placed in an Ecocell BMT oven, at 70°C, for 3 h. After solutions were cooled down at room temperature, they were extracted twice with 1 ml hexane, each extraction being done for 10 minutes in an Elmasonic S10H ultrasonic bath. The resulting hexane solution was completely evaporated under nitrogen stream and the residue was dissolved in 1 ml hexane. All solutions were filtered through a 0.2 µm PTFA syringe filter before injection through the GC-MS system. When using internal standard, analysis solutions were mixed with solution of C17:0 prepared in the same way, in order to keep a concentration of 1 µg/ml of C17:0 in each solution. In case of serum samples, the same procedure was applied, starting with 200 µl serum which was mixed with 2 ml methanolic HCl, followed by the two-step extraction procedure, evaporation and redissolution in hexane. Serum samples were mixed with 1 µg/ml C17:0 solution.

Separation of FAME: An Agilent GC 7894 system coupled with a mass spectrometer detector 5975C inert XL EI/CI MSD was used for the separation of FAME. The column used for the analysis was DB-5ms (5%-phenyl-arylene-95%-dimethyl poly siloxane stationary phase), with following characteristics: 60 m length, 0.32 mm I.D., 0.5 µm film. Helium 6.0 was used as mobile phase (carrier gas) at a flow of 1 ml/min. The solvent delay was set at 4 minutes. The initial inlet temperature was set at 250°C. The oven temperature gradient was set as following: initial oven temperature was set at 100°C and was kept for 3 min, then the temperature was increased at 170°C with 25°C/min, where it was kept for 5 min, then it was increased until 280°C with 2°C/min, where it was kept for 10 min. The MS interface temperature was set at 230°C. The MS detector was set both in Scan mode (scanned masses were in a range between 50 and 550) and in single ion monitoring (SIM) mode, where the monitored m/z was set as the molar mass of the analyzed fatty acids: m/z= 284.5 for C17:0 ($M\pm1e^-$), m/z=354.6 for C22:0 ($M\pm1e^-$), m/z=382.65 for C24:0 ($M\pm1e^-$) and m/z=410.7 for C26:0 ($M\pm1e^-$).

RESULTS AND DISCUSSION

Optimization of derivatization and extraction procedure: The gas-chromatographic separation of fatty acids is usually done after their derivatization to methyl esters (FAME), due to their higher volatility. The FAMEs were obtained in an acid catalyzed reaction, using methanol as derivatization agent and hydrochloric acid as catalyst. The temperature of reaction and reaction time were optimized, following two procedures indicated in literature [15]: 100°C for 2h and 70°C for 3h. In case of the last procedure of derivatization reaction, better results were obtained in terms of signal-to-noise ratio, indicating a higher reaction yield, when analyzing both serum samples and standard samples.

The obtained FAMEs were extracted through a liquid-liquid phase extraction in hexane, following a two-step procedure. The extraction technique was selected to be ultrasonication, while, as expected, vortex did not return such good extraction output. The ultrasonication time was tested as follows: 2 min, 5 min, 10 min, 20 min. An ultrasonication time of 10 min gave the best extraction output, being verified after separation of a C22:0 standard, using the internal standard C17:0, where at 10 min, the highest relative response was obtained. An ultrasonication time higher than 10 min seemed to lead to degradation of the analyzed fatty acids, giving a lower relative response (Fig. 1.).

Optimization of separation parameters: Separation of fatty acids in serum was firstly tested using a temperature gradient described in literature [16], slightly modified. Basically, the initial temperature was set at 70°C and kept for 1 minute, then the temperature was increased to 120°C, with 25°C/min, and kept for 5 min, then the temperature was increased with 3°C/min up to 280°C and kept for 20 min. The separation was tested using the MS detector in Scan mode (m/z=50-550).

At first sight, this separation gave quite good results, but the temperature gradient was optimized, in terms of analysis time and resolution, in the same time, by using two gradients: initial

temperature was adjusted at 100°C, then it was increased with 25°C/min up to 170°C, then increased with 2°C/min and with 5°C/min, respectively, up to 280°C, isotherm for 10 min. The temperature of 170°C was determined to be the temperature of the oven just before the separation of the first FAMEs (C14:0). After the detection of C14:0, the gradient was set at a lower rate of increase of temperature in order to obtain a better resolution of the separated FAME. The last isotherm set at 280°C was kept for 10 min, because a complete baseline is obtained only after the last 10 isotherm minutes.

The best results expressed as resolution of separation and also as analysis time were given by the last gradient studied, where in the last part, the temperature was increased at a range of 5°C/min (Fig. 2). In this case, it seems that a lower analysis time does not affect the resolution of separation, but on the contrary.

In total, 16 fatty acids could be identified in the human serum from the healthy volunteer and 15 fatty acids could be identified in the human serum collected from the adrenoleukodystrophic patient, by using the NIST mass spectral database. Other 3 lipidic compounds were identified in the mixtures and also the internal standard, heptadecanoic acid, C17:0, which was added to the serum samples. All compounds identified in the human serum with indication of retention times are presented in Table I below.

It is to be noticed the presence of trace levels (Fig. 3) of C23:0, tricosanoic acid, which is a fatty acid with an odd number of carbon atoms in its chain, in both healthy volunteer and ALD serum. As literature reports, this type of fatty acids should not be synthetized by the human body. In the same time, the identification of this fatty acid should be done using analytical standards to precisely confirm its presence in the human serum.

Another remark that should be done is that the separation of an androstenol derivative, which has a close retention time to the the time of the internal standard, is to be noted. Actually, the two compounds, the hormone and C17:0, did not separate by using the first gradient (Rs=0), the resolution improved when using the second gradient (with a range of 2°C/min), but the resolution did not come to baseline, while when using the last gradient (with a range of 5°C/min) the separation of the two compounds was with a baseline resolution. It can be seen in Fig. 4. that the concentration of this hormone is much higher in the serum of ALD patient than in serum of healthy volunteer.

The group of octadecanoic acid was not separated with baseline resolution by any gradient that was used, but a good resolution could be achieved. Moreover, any significant improvement in the resolution of these fatty acids could not be seen by changing the temperature gradient (data not shown).

As it could be discovered up-above, two very long chain fatty acids (VLCFA), C22:0 and C24:0, could be identified using the NIST mass spectral database, but C26:0 could not be detected in any case. For this reason, we prepared analytical standard solutions, containing also the internal standard C17:0, and analyzed them in single ion monitoring (SIM) mode of the detector. The monitored ions were set as being the molecular masses of the separated FAME ± $1e^-$ (numerically translated to the molecular masses of FAME, ions seen in the molecular peak in the spectra). Three methods of analysis were set following pairs of monitored ions: correspondent ion of C17:0 (internal standard, analyzed every time) making pair with the correspondent ion of C22:0, another pair with the correspondent ion of C24:0 and the other pair with the correspondent ion of C26:0.

Although the temperature gradient using a rate of 5°C/minute seemed to be the optimal one, due to the higher resolution obtained in case of C17:0 and adrenostenol derivative, it seems that in case of C24:0 and C26:0 there is an overlapping of the two peaks. In this case, when we focus on the separation of the VLCFA, the only gradient that gave a separation of C24:0 and C26:0 was 100°C - 170°C – (2°C/min) up to 280°C (isotherm 10 min). In this case, a resolution of Rs=1.1 is still kept in case of internal standard and androstenol derivative.

Using this last temperature gradient, the VLCFA were detected at the following retention times: 40.524 min for C22:0, 47.994 min for C24:0 and 55.412 min for C26:0, while the retention time of the internal standard C17:0 was of 21.241 min (Table II).

CONCLUSIONS

In this paper, we presented the development and optimization of a GC-MS separation method of fatty acids in human serum, after their methyl transesterification. The method was optimized in each essential stage. The developed method can be applied for the separation of FA with a chain length of up to 24 carbon atoms just by scanning the ions or, for FA with very long chains, such as C24:0 or C26:0, single ion monitoring mode of the detector can be used.

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TABLE 1. LIPIDS IDENTIFIED IN HUMAN SERUM

Lipid	Tr (healthy vol.) (min.)	Tr (ALD) (min.)
C14:0	11.713	11.713
C16:1	15.719	15.719
C16:0	16.178	16.182
C17:0	18.269	18.265
Androst-2-ene-1,17-diol-4-methyl-(1-alpha,4-alpha,5-beta)	18.418	18.419
C18:2	19.640	19.663
C18:1(E)	19.755	19.771
C18:1(Z)	19.859	19.866
C18:0	20.259	20.261
C20:4	22.718	22.720
C20:5	22.832	-
C20:3	23.047	23.045
C20:2	23.386	23.386
C20:0	23.936	23.931
DHA (C22:6)	26.056	26.054
C22:0	27.340	27.347
C23:0	28.977	28.977
C24:0	30.597	30.613
cholesta-3,5-diene	32.974	32.966
cholest-5-en-3-ol (3-beta)	37.044	37.113

Lipids identified in human serum of healthy volunteer and adrenoleukodystrophic patient with indication of retention times. Separation conditions: column DB-5ms, He 1 ml/min, 1 µl splitless, gradient 100°-170°-(5°C/min)-280°c. MS in Scan mode (m/z=50 to 550).

TABLE 2. RETENTION TIMES OF VLCFA

VLCFA	Tr (min) 5°/min	Tr (min) 3°/min	Tr (min) 2°/min
C22:0	27.340	33.850	40.524
C24:0	30.613	39.013	47.994
C26:0	30.617	39.033	55.412

Retention times of VLCFAs obtained after their separation in SIM mode of the detector. Separation conditions: DB-5ms column, He 1ml/min, 1 µl injection volume, splitless, temperature gradient 100°-170°-(5°C/min or 3°C/min or 2°C/min)-280°C. MS in SIM mode (m/z=284.5 and 354.6 for C22:0; m/z=284.5 and 382.65 for C24:0; m/z=284.5 and 410.7 for C26:0).

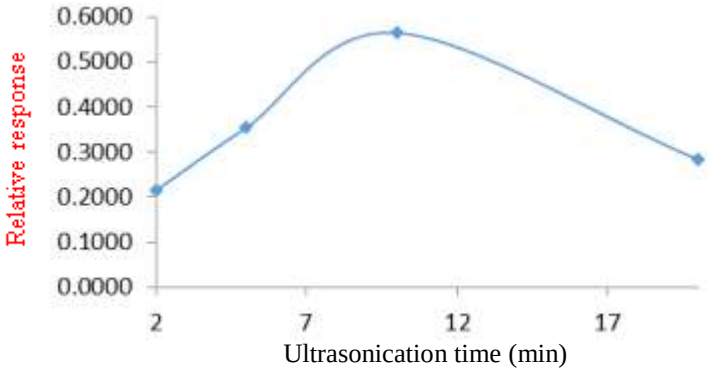

Fig. 1. Optimization of extraction times – 2+2, 5+5, 10+10, 20+20 minutes of ultrasonication extraction times (two-step). Chart represents relative response of C22:0 standard peak height as function of extraction time, using C17:0 as internal standard, obtained after GC-MS separation. Separation parameters: DB-5ms column, He 1ml/min, 1 µl injection volume, split ratio 2:1, gradient 100-170-280°C, MS in SIM mode (monitored m/z: 284.5 and 354.6).

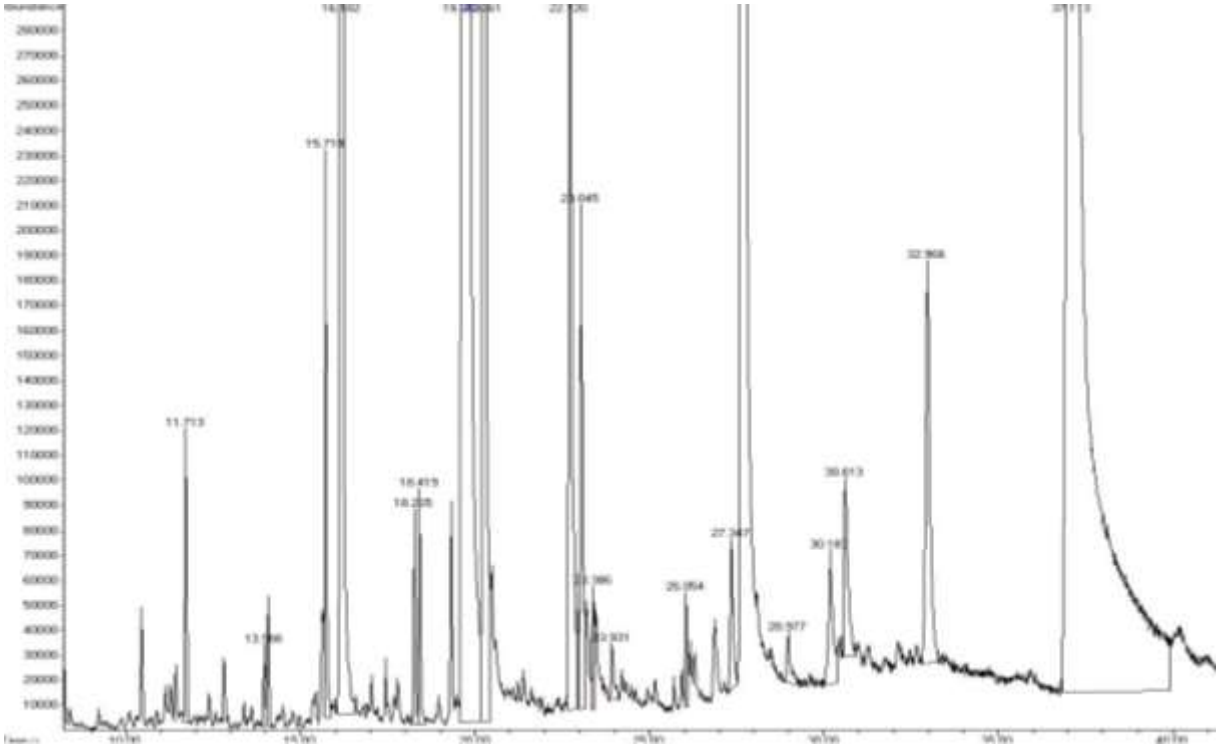

Fig. 2. Separation of lipids in human serum from ALD patient with indication of retention times. Separation conditions: column DB-5ms, He 1 ml/min, 1 µl injection volume, splitless, gradient 100°-170°-(5°C/min)-280°C. MS in Scan mode (m/z=50 to 550).

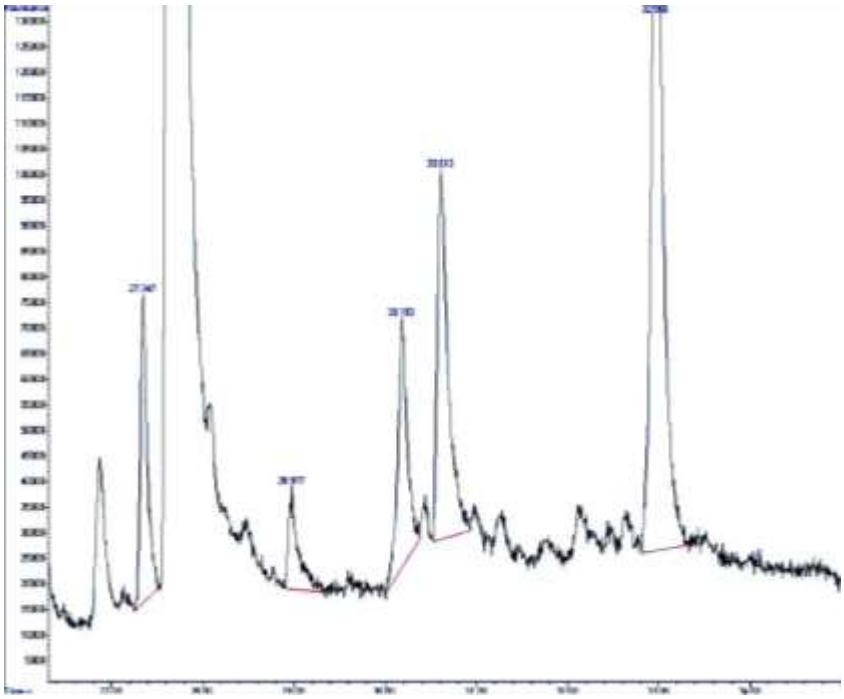

Fig. 3. Detail image of separation of VLCFA. Separation of C23:0 at 28.977 min. Separation conditions: DB-5ms column, He 1ml/min, 1 µl injection volume, splitless, gradient 100°-170°-(5°C/min)-280°C. MS in Scan mode (m/z=50 to 550).

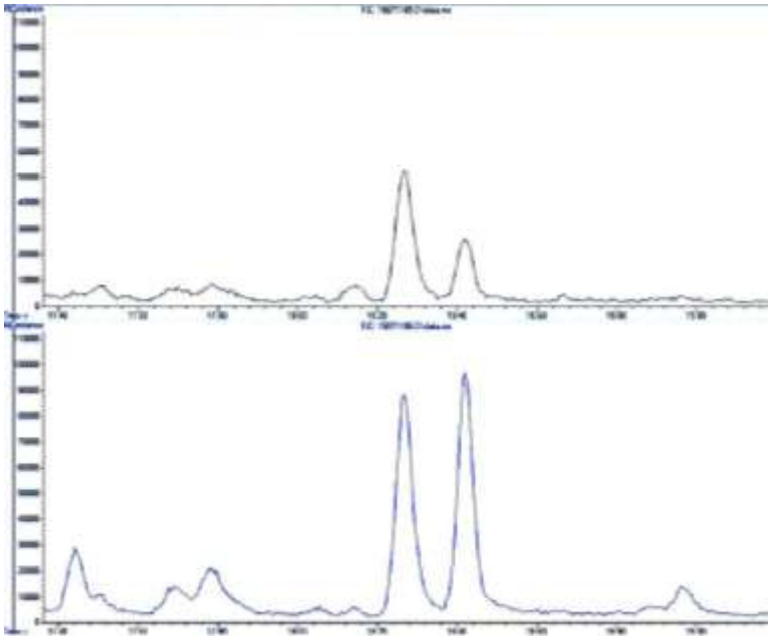

Fig. 4. Detail with separation of internal standard C17:0 and androst-2-ene-1,17-diol-4-methyl-(1-alpha,4-alpha,5-beta) in serum from healthy volunteer (black) and in serum from ALD patient (blue). Separation conditions: DB-5ms column, He 1ml/min, 1 µl injection volume, splitless, gradient 100°-170°-(5°C/min)-280°C. MS in Scan mode (m/z=50 to 550).

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