Application Note

Clinical Research

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Determination of red blood cell fatty acid profiles in clinical research

Chemical ionization gas chromatography tandem mass spectrometry

Authors

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Abstract

Acknowledged as biomarkers in various human diseases, cellular fatty acid (FA) profiles are commonly analyzed by gas chromatography mass spectrometry (GC/MS), which is time-consuming. Therefore, a high-throughput analysis method is needed in clinical research studies. In this study, fatty acid methyl esters (FAMEs) were formed by derivatization after extraction of FA from red blood cells (RBCs). A gas chromatography tandem mass spectrometry (GC/MS/MS) FA profile method with ammonia-induced chemical ionization (CI) was developed for analysis in human RBCs. There were 703 RBC samples analyzed for FA profiles by GC/MS/MS. This analytical method was compared with classic single GC/MS using electron impact ionization (EI). Analysis by ammonia-induced CI allowed for further investigation of FAME by generating adequate amounts of molecular ions. This analysis determined specific fragments for 45 FA profiles for confident quantification and fragmentation. Typical analysis times using classic GC/MS can be up to 60 minutes, but this GC/MS/MS analytical method had a run time of nine minutes. Intra and inter assay variations were <10 % for all FAs analyzed. By combining ammonia-induced CI and GC/MS/MS analysis, high-throughput, robust and confident analysis of FA profiles in the clinical research laboratory can be achieved.

Introduction

To determine fatty acid (FA) profiles in the clinical research laboratory, specific and sensitive analytical methods are needed. Historical separation of FA profiles were performed by gas chromatography (GC) coupled with a flame ionization detector (FID), allowing researchers to analyze individual FAs in different matrices[1]. The introduction of mass spectrometry (MS) has since improved this analysis[2], but classic GC/MS analysis requires a long chromatographic separation to allow for confident identification and quantification. This study developed and verified a specific, fast, and sensitive analytical method for high-throughput analysis of FA in biological specimens such as red blood cells (RBCs). To do so, a combination of chemical ionization (CI) and gas chromatography tandem mass spectrometry (GC/MS/MS) for measurement of FA was used. Using these techniques delivers an improved analytical method over historical GC/MS FA analyses.

Experimental

GC/MS/MS with CI configuration and parameters

Parameter	Value
Instruments	Agilent 7890A GC (G3440A) with split/splitless inlet (G3452-67000) Agilent 7000 MS/MS with chemical ionization (CI) source (G7010BA)
Inlet temperature	250 °C
Source temperature	250 °C
Quadrupole temperature	150 °C
Injection volume	1 µL
Retention gap	Agilent 5 m, 0.25 mm uncoated precolumn
Analytical column	Agilent J&W CP-Sil 88 for FAME GC Column, 50 m × 0.25 mm, 0.20 µm, 7 inch cage (CP7488)
Carrier gas	Helium at 2.2 mL/min
CI Reagent	Ammonia at 1.3 mL/min
Column oven program	50 °C (hold 1 minute), then 120 °C/min to 70 °C, then 45 °C/min to 175 °C, then 35 °C/min to 230 °C (hold 3.5 minutes)
Stop time	9.00 minutes
MS Acquisition mode	MRM mode

GC/MS with EI configuration and parameters

Parameter	Value
Instruments	Agilent 7890A GC (G3440A) with split/splitless inlet (G3452-67000) Agilent 5975C MS with electron impact (EI) source (G3243A)
Inlet temperature	230 °C
Transfer line temperature	230 °C
Quadrupole temperature	150 °C
Analytical column	Agilent J&W CP-Sil 88 for FAME GC Column, 100 m, 0.25 mm, 0.20 µm, 7 inch cage (CP7489)
Carrier gas	Helium at 2.0 mL/min
Column oven program	120 °C (hold 5 minutes), then 5 °C/min to 220 °C (hold 5 minutes), then 4 °C/min to 240 °C (hold 10 minutes)
Stop time	45.00 minutes
MS Acquisition mode	SIM mode

Chemicals and reagents

Isopropanol, methanol, and hexane (GC grade) were purchased from Fisher Scientific (Schwerte, Germany). Water (LC/MS grade) and boron trifluoride (BF_3) (14 % in methanol) were purchased from Sigma-Aldrich (Hamburg, Germany). Natriumsulfate (Na_2SO_4) was purchased from Merck (Darmstadt, Germany).

A certified 37 fatty acid methyl ester (FAME) mix (TraceCERT) was purchased from Sigma-Aldrich (Hamburg, Germany). Another FAME mix was purchased from NuChekPrep (Elysian, MN, USA), and all other FAMEs, including the internal standard C17:1 (heptade-cenoate), were purchased from Larodan (Malmö, Sweden).

Aliquots of EDTA-anticoagulated blood specimens were used from samples submitted for routine laboratory analysis. The study was explained thoroughly to the subjects and informed consent using de-identified laboratory data was obtained, in accordance with the Helsinki II Declaration.

MRM parameters for FAME analysis

Table 1. Analyte parameters.

Fatty acid	Common name	RT (min)	Precursor ion (*m/z*)	Quantifier ion (*m/z*)	CID (V)	Qualifier ion (*m/z*)	CID (V)
C6:0		2.90	147.8	59.0	21		7
C8:0		3.90	175.8	159.1	5	57.0	25
C10:0		4.36	203.8	187.1	5	57.0	30
C11:0		4.58	217.8	201.2	3	57.0	25
C12:0		4.79	231.8	215.5	3	57.0	30
C13:0		4.99	245.8	229.2	5	57.0	30
C14:0		5.19	259.8	243.2	5	57.0	35
C14:1t		5.29	257.8	241.2	3	191.1	11
C14:1c		5.35	257.8	241.2	3	191.1	11
C15:0		5.38	273.8	257.2	5	57.0	35
C15:1		5.54	271.8	255.2	9	205.1	11
C16:0		5.57	287.7	271.2	5	57.0	35
C16:1t		5.67	285.9	269.2	3	237.2	9
C16:1c		5.71	285.9	269.2	3	237.2	9
C17:0		5.77	301.8	285.3	7	103.0	25
C17:1		5.92	299.8	283.3	3	251.2	9
C18:0		5.98	315.8	299.3	4	71.0	9
C18:1n9t		6.06	313.8	297.3	4	265.2	9
C18:1n9c		6.12	313.8	297.3	7	265.2	9
C18:2n6t		6.21	311.9	295.2	3	263.2	9
C18:2n6c	Linoleic acid	6.32	311.9	295.2	7	263.2	9
C20:0		6.40	343.8	327.3	3	85.0	25
C18:3n6		6.49	309.9	293.2	3	261.2	3
C20:1n9		6.56	341.8	325.3	3	293.3	9
C18:3n3	α-Linolenic acid	6.60	309.9	293.2	3	261.2	3
C21:0		6.64	357.8	341.3	7	57.0	35
C18:4n3		6.74	308.0	291.0	3	259.0	3
C20:2		6.81	339.9	323.3	3	291.2	35
C22:0		6.91	371.9	355.3	7	103.0	30
C20:3n9		7.00	337.9	321.3	3	289.2	3
C20:3n6		7.02	337.9	321.3	5	289.2	3
C22:1n9		7.10	369.9	353.3	3	321.3	11
C20:3n3		7.14	337.9	321.3	3	289.2	3
C23:0		7.21	385.8	369.3	6	71.0	30
C20:4n6	Arachidonic acid	7.21	335.9	319.3	3	287.2	3
C22:2		7.42	367.9	351.3	3	319.3	7
C24:0		7.54	399.8	383.4	3	103.0	29
C20:4n3		7.55	336.0	319.2	5	287.2	9
C20:5n3	Eicosapentaenoic acid	7.62	333.9	317.3	3	285.2	3
C22:3		7.63	366.0	348.8	5	317.1	5
C24:1n9		7.79	397.9	381.4	3	349.3	11
C22:4n6		7.98	364.0	347.3	5	297.1	5
C22:5n6		8.20	362.0	345.0	3	313.0	5
C22:5n3		8.54	362.0	345.0	3	313.0	5
C22:6n3	Docosahexaenoic acid	8.82	359.9	343.2	3	311.2	3

Sample preparation

For FA extraction from blood erythrocytes, 0.5 mL of whole blood and 10 mL of 0.9 % saline were mixed and centrifuged at 2,500 g for five minutes. After the supernatant was discarded, this washing procedure was repeated once more. Afterwards, the cells were hemolyzed by adding 1 mL of distilled water, and stored for at least 30 minutes at refrigerator temperature. The FA extract was then mixed with 5 mL internal standard (IS) solution, and centrifuged at 2,500 g for five minutes. The IS solution contained 0.2 mg/mL of FAC17:1 in hexane/isopropanol (3:2) and 3 mL Na_2SO_4 solution (6.7 %). The hexane phase was then transferred to a clean glass tube and evaporated to dryness with nitrogen. To resolve the FA, 1 mL BF_3 in methanol (14 %) was added and incubated for 10 minutes at 100 °C for esterification. Then, 1 mL water and 3 mL hexane were added to the samples after cooling to room temperature, and centrifuged (2,500 g for five minutes). The hexane phase was transferred to a clean vial, and evaporated with nitrogen. The final FAME sample was in 250 mL hexane, and could be stored at −25 °C. The samples were diluted 1:20 with hexane before analysis.

Data analysis

Agilent MassHunter software was used for data acquisition (Waldbronn, Germany). To properly identify and quantify the FAME, two fragment ions were used, one for quantification, and one for confirmation. A mixture of 45 FAMEs (Table 1) was used in the calibration standard. Individual FA concentrations were calculated as relative percentage with the evaluated FA set at 100 % or as absolute values. MassHunter Quantitative Software 5.0 and MassHunter Qualitative Software 5.0 were used for data analysis. The ratio of the peak area of analyte to the internal standard area were used to calculate calibration curves.

Method parameters

To determine the linearity and accuracy of the GC/MS/MS analytical method, a dilution series of a 45 FAME standard mix in both hexane and pooled human erythrocytes was used. Method accuracy was also evaluated using the 45 FAME standard mix at three different concentration ranges. To evaluate intra-assay precision, 10 independent sample work-ups of aliquots of one human blood pool were analyzed. The inter assay precision was determined in the same way, but with sample work-ups on different days. The concentration was determined using a calibration standard prepared on the day of analysis. Relative standard deviation (RSD) was calculated for precision. Ten replicates of a human blood sample were evaluated for analytical sensitivity determination. Limits of detection (LOD) and lower limits of quantification (LLOQ) were calculated based on the signal-to-noise (S/N) ratio for selected analytes. The S/N ratios were calculated with MassHunter Qualitative software.

Results and discussion

GC/MS analysis

To compare the two ionization methods, EI and CI, for the FAME analysis, docosahexaenoic acid methyl ester spectra (DHA, C22:6n3) are shown. In Figure 1A, the GC/MS spectrum using EI shows numerous low mass fragments. Some of the fragments, such as *m/z* = 67, *m/z* = 79, and *m/z* = 99 are characteristic of LC-PUFAs, however they are not compound-specific. Typically, when using EI fragmentation, the molecular ion of the FAME is not detectable. Conversely, when using CI, the dominating peak of the GC/MS spectrum is the molecular ion ($[M+H]^+$), *m/z* = 343. (Figure 1B). The other dominate peak is the ammonia adduct ($[M+NH_4]^+$), *m/z* = 360, since ammonia was used as reactant gas for CI. Both fragments are considered compound-specific, as the loss of ammonia ($[M+NH_4]^+ \rightarrow [M+H]^+$) in MS/MS mode can be used as a quantification transition.

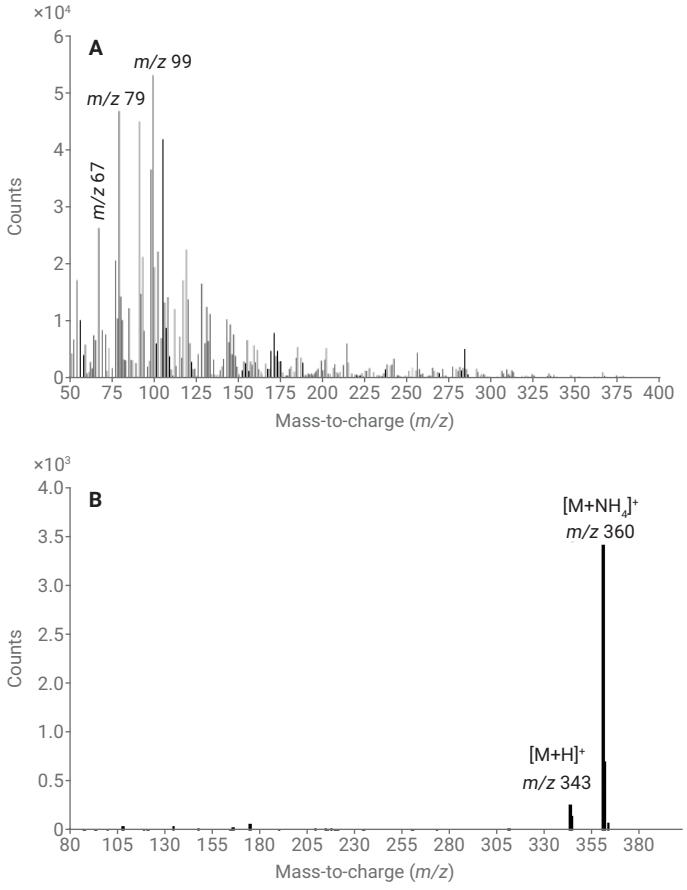

Figure 1. GC/MS spectra of DHA methyl ester, comparison of ionization methods. A) EI spectrum B) CI spectrum.

Since only analytes with adequate proton affinity were ionized, the CI ionization mode reduced the chemical background level compared to the EI ionization mode.

A method using CI and GC/MS/MS in MRM mode was optimized for FA analysis with an analysis time less than nine minutes. Table 1 shows the analyte-specific parameters. To achieve proper identification and quantification, precursor ions, retention times, and quantitative and qualitative fragment ions were determined. Figure 2 shows that no significant background peaks were observed. Figure 2A shows a GC/MS/MS chromatogram of a FAME standard. Figures 2B and 2C show two sections of overlapping peaks enlarged. Figure 2B shows three slightly overlapping analyte signals, while Figure 2C shows an example of two completely overlapping analyte ions. No interfering peaks were found that would hamper the quantification of the analytes.

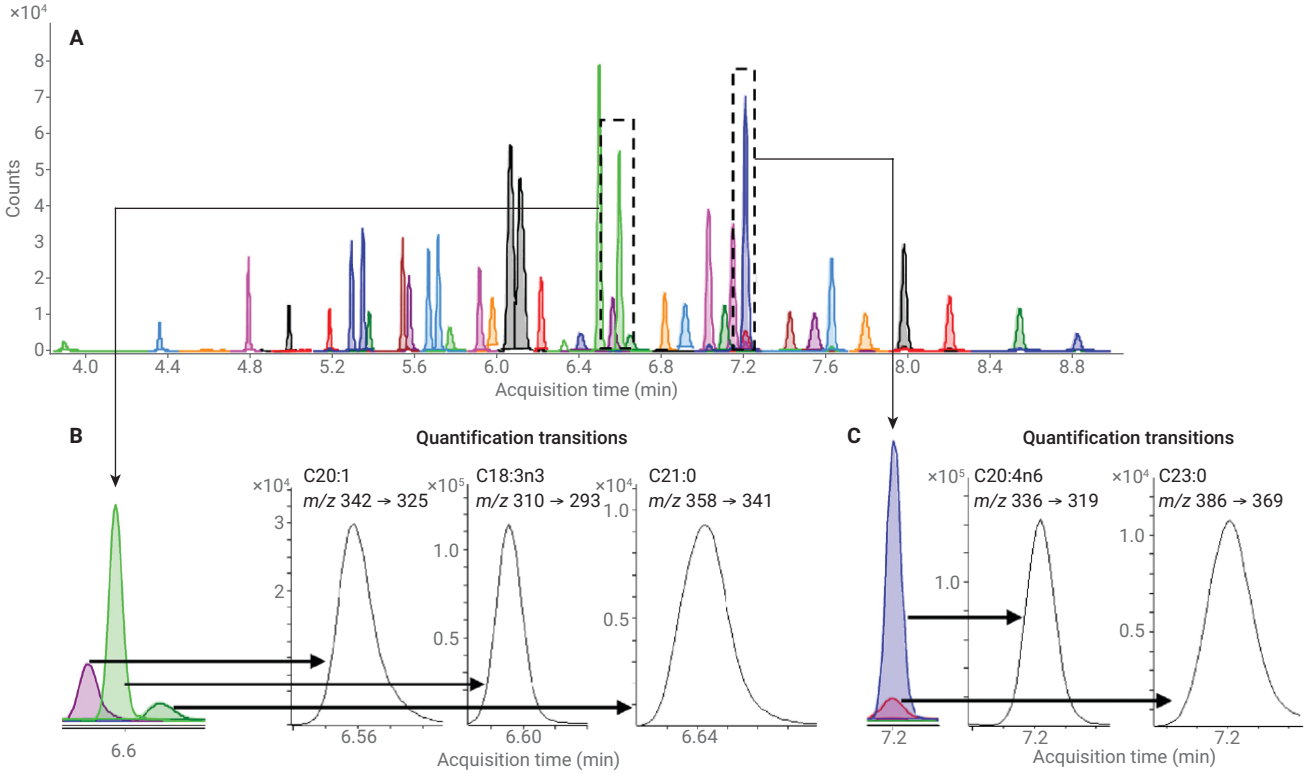

Figure 2. A) GC/MS/MS chromatogram of 45 FAME B) Enlargement of the chromatogram at 6.5–6.7 minutes and correspondent quantification transitions. C) Enlargement of the chromatogram at 7.1–7.3 minutes and correspondent quantification transitions.

Method verification

To assess the linearity of the GC/MS/MS method, a dilution series of a FAME standard mix ranging from 5 ng/mL to 20 mg/mL in hexane was analyzed. All analytes had a coefficient of determination (R^2) better than 0.995. A dilution series of 45 FAMEs in matrix was also measured. All analytes had R^2 better than 0.992, and no matrix interferences were observed. The accuracy, LOD, and LLOQ were also evaluated with the FAME standard at three different concentrations. The accuracy was within 90–110 % for all analytes, and the LOD and LLOQ were in the low ng/mL range (Table 2). RSD was calculated for precision using 10 FAs in a 10-fold measurement of a human RBC pool. Intra-assay RSD and inter assay RSD were lower than 10 % for the 10 selected FAs (Table 2).

Table 2. Method parameters for 10 selected FAs. Intra- and inter day precision data are shown as % of total FA.

Fatty acid	Intra-day			Interday			LOD		LOQ	
	Mean		RSD	Mean		RSD	ng/mL		ng/mL	
C16:0	25.2	± 1.4	5.3	25.7	± 2.1	8.0	6.3	± 1.5	20.8	± 2.1
C18:0	15.9	± 0.7	4.7	15.0	± 0.8	5.4	4.9	± 1.2	16.3	± 2.0
C18:1*cis*	18.2	± 0.8	4.5	19.5	± 1.0	5.3	4.2	± 0.5	13.6	± 1.6
C18:2*cis*	10.7	± 0.3	2.6	11.8	± 0.3	2.4	4.6	± 0.4	15.2	± 1.2
C20:3n6	1.5	± 0.1	5.3	1.6	± 0.1	6.5	0.9	± 0.1	2.7	± 0.8
C20:4n6	17.3	± 0.9	5.1	15.8	± 1.6	10.0	0.9	± 0.1	3.1	± 0.4
C20:5n3	0.4	± 0.1	7.1	0.5	± 0.1	8.7	1.9	± 0.2	5.9	± 0.8
C22:4n6	3.5	± 0.3	8.5	3.7	± 0.3	9.0	1.3	± 0.1	4.3	± 0.8
C22:5n3	2.2	± 0.2	9.1	2.0	± 0.2	8.6	1.6	± 0.2	5.3	± 0.9
C22:6n3	4.1	± 0.3	6.2	4.1	± 0.3	7.8	2.2	± 0.2	7.3	± 1.1

Conclusions

This study developed an analytical GC/MS/MS method for the determination of FA profiles in biological matrices through derivatization to FAME. There were 45 FA profiles quantitated in nine minutes with good analytical sensitivity and selectivity. Other parameters were researched, including sample preparation procedure, stability of the method, and GC and MS/MS conditions. This method was found to be robust and have a short analysis time. It has broad applicability for FA analyses.

References

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