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원 저

지방산대사이상질환 스크리닝을 위한 TBDMS 유도체화 후 GC-MS를 이용한 혈장 중 유리지방산의 분석

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A Sensitive Determination of Plasma Free Fatty Acids Following *Tert*-butyldimethylsilyl Derivatization using Gas Chromatography-Mass Spectrometry for Screening of Fatty Acid Oxidation Disorders

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Purpose: A sensitive gas chromatography mass spectrometry (GC-MS) method was developed for screening of fatty acid oxidation disorders.

Methods: The assay utilized a simple protein precipitation with sulfosalicylic acid followed by *tert*-butyl dimethylsilyl (TBDMS) derivatization of hydroxyl functional group by N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA).

Results: Calibration curves of spiked pooled plasma showed a linear relationship in the range of 0.01 ng -2 mg with correlation coefficient value greater than 0.98. Limits of detection (LOD) and limits of quantification (LOQ) were found in the range of 0.9-8.8 ng and 9-88 ng, respectively.

Conclusion: The new developed method might be useful for a rapid, sensitive screening of inherited fatty acid oxidation disorders. In addition, the method expected to be one of the alternative method for screening newborns of metabolic disorders in the laboratories where expensive MS/MS is unavailable.

Key words: Free fatty acids, Gas chromatography-mass spectrometry, *tert*-butyldimethylsilyl derivatives, Fatty acid oxidation disorders

Introduction

Fatty acids are basic components of naturally occurring lipids in animals and plants which are important for energy metabolism and cell signalling [1-3]. The FAs profiling is important during lipid analysis either in biological matrices from human or plant samples like food, extracts or oils[4]. Study of individual fatty acids profiling is important for the diagnosis of fatty acid disorders and inherited errors of metabolism like mitochondrial fatty acid disorder and peroxisomal disorders[5,6]. Thus, there is a need of development of simple and sensitive analytical method to determine fatty acids[7]

Gas chromatography is the widely used techniques to measure fatty acids. James et al. initially measured fatty acids directly in an underivatized form. The drawbacks like sample loss in the injector, column and the problem with the integration

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limits the use of fatty acids in underivatized form [8]. James et al. again reduces the disadvantages of previous method by preparing the methyl esters of fatty acids. Further, this method has increased the sensitivity[7]. FAs do not possesses chromophores so they cannot be measured by UV-Visible techniques[8]. Pre- and post-column derivatization with suitable chromophores is required to enhance the sensitivity and selectivity of fatty acid detection by UV detector. The use of capillary column coupled with flame ionization detector (FID) provides a high resolution of free fatty acids analysis. Furthermore, HPLC coupled with evaporative light scattering detection (ELSD) and HPLC with corona charged aerosol detection was developed to measure FFAs[9,10]. The coupling of HPLC with mass spectrometry provides the efficiency of analysis as well as structural elucidation [11]. A major advantage of HPLC over GC is the lower temperature during analysis which prevent the risk of isomerization of unsaturated fatty acids. The various other methods to measure fatty acid includes HPLC-fluorimetric detection[12] CE-UV detection[13,14], HPLC-UV coupled with 2-nitrophenylhydrazines[15], high performance liquid chromatography mass spectrometry (HPLC-MS/MS) [16-18], Hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-ESI-MS/MS) [19].

Free fatty acids are generally analyzed after various derivatization before GC-MS analysis such as trimethylsilyl, pentafluorobenzoyl[20], or esterification derivatives[21] etc. Silylation for low molecular weight dicarboxylic acids provide low detection limits and satisfactory reproducibility compared to esterification derivatization[22]. Trimethylsilyl (TMS) derivatives are prone to hydrolysis compared to corresponding TBDMS derivatives indicating that the TBDMS derivatives are hydrostatically more stable[23]. Here we describe a rapid and sensitive analytical method for the determination of free medium and long chain fatty acids in human plasma using GC-MS. .

Materials and Methods

1. Materials

Decanoic acid (C10), lauric acid (C12), cyristic acid (C14), myristoleic acid (C14:1), palmitoleic acid (C16), stearic acid (C18), octadecenoic acid (C18:1), and linoleic acid (18:2) were purchased from TCI Co. (Tokyo, Japan) (Fig. 1). Decanoic acid-d_3, lauric acid-d_3, and 13C-stearic acid were used as internal standard and purchased from Sigma Aldrich (MA, USA). N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was used as derivatizing reagent and purchased from Sigma Aldrich (MA, USA). All other chemicals and solvents were of analytical grade and purchased from J. T. Baker. Distilled water was prepared using a Millipore-Milli QTM. Thermo vap (TAITEC model DTU-2C) was used for evaporation and derivatization of samples. A centrifuge (Eppendorf model 5424) (Hamberg, Germany) was used for centrifuging the sample in different steps.

2. Gas chromatography-mass spectrometry

Hewlett-Packard-6890 Series gas chromatograph coupled with 5973 network mass selective detector was used as the GC/MSD system. Separation of 8 free fatty acids with TBDMS derivative was accomplished using HP 5 capillary column (30 m×0.251 mm I.D, 0.33 μm). Helium was used as a mobile phase carrier gas at the flow rate of 0.8 ml/min.

The column oven temperature was programed

at 80℃ for 2 min, then constantly increased to 290℃ with 5℃/min increment. The injection port and transfer line temperature was set at 280℃ and 300℃ respectively. 1 μL sample was injected with a flow rate 0.8 ml/min and split ratio 1:10. Electorn voltage was 70 eV for electron impact ionization. Spectra were observed for eight free fatty acids in scan mode (range: 50-550 m/z) and SIM mode was utilized for quantification (Fig. 1).

3. Preparation of standard solution

10 mg/10 mL (1,000 ppm) stock solution of C10, C12, C14, C16, C16:1, C18, C18:1, and 18:2 were prepared by dissolving the standard compound in n-hexane and stored at -20℃. Each stock solution was further diluted to 10, 40, 80, 160, and 200 μg/mL working standard solution for generating calibration curves. Decanoic acid-d_3, dodecanoic acid-d_3, and 13C-octadecanoic acid were also prepared as 10 mg/10 mL stock solution (1,000 ppm). All the solutions were stored at -20℃ until the analyses.

4. Sample preparation and derivatization

Whole blood samples were collected and centrifuged at 3,000 rpm for 20 minutes. The plasma was separated and stored at -70℃ deep freezer to prevent the enzymatic degradation of FFAs. The plasma samples were collected and stored at -20℃ until analysis.

A 100 μL of plasma specimen was collected in eppendorf tube. 20 μL of 30% sulfosalicylic acid was then added for protein precipitation. 10 μL of each internal standard was added. The mixture was vortex mix for 1 min and centrifuged at 5,000 rpm for 3 mins. The precipitate was discarded and the upper layer was transferred to glass tube. Two mL of methylene chloride was added, vortex mixed and centrifuged at 2,000 rpm for 3 minutes. The lower layer was then transferred to a new glass tube and a tea spoonful moisture free sodium chloride was added. The organic solvent was evaporated to dryness at 80℃ under gentle stream

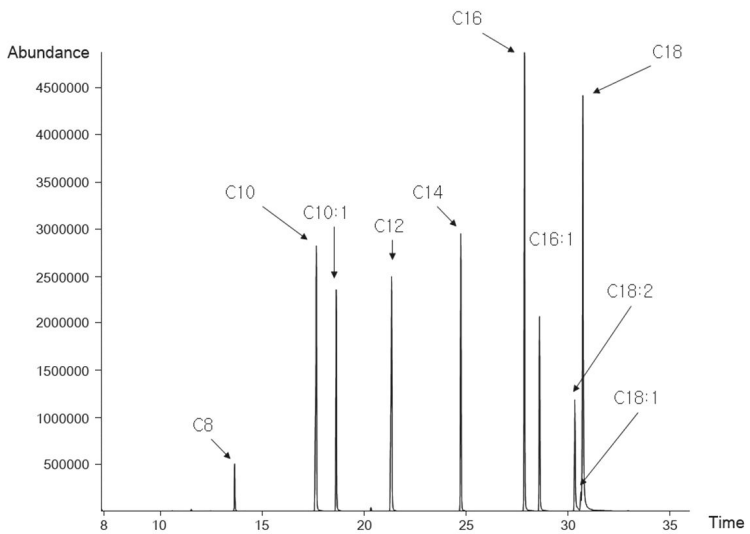

Fig. 1. GC-MS chromatograms of standard free fatty acids (C8-C18:1) following TBDMS derivatives.

of nitrogen.

The TBDMS derivative of hydroxyl functional groups was achieved by adding 20 μL of MTBSTFA and 30 μL of toluene. After gentle vortex mixing for 1 min, the mixture was reacted at 80℃ for 30 min. The complete TBDMS derivative of FFAs were cooled for 5 min and transferred to GC-vial for analysis using GC-MS.

5. Linearity, precision, and accuracy

The calibration curve was constructed by plotting the peak area intensity with five different concentration levels of fatty acids 10, 40, 80, 160 and 200 μg/mL and measured in five replicates. Limit of detection (LOD) is the lowest concentration that can be detected and calculated at signal-to-noise ration greater than 3. Limit of quantitation (LOQ) is the lowest concentration that can be quantitate and calculated at signal-to-noise ratio greater than 10. The accuracy of the method was calculated by the following formula: [measured concentration-apparent concentration]/[apparent concentration]×100%. The precision was determined as the relative standard deviation (RSD).

Results

Each standard solution (1 ppm) was initially injected in GC-MS in a scan mode. Mass spectrum for TBDMS derivative of each compound was obtained as molecular ion $[M]^+$. The molecular ion was used for the identification of the compound investigated. The subsequent product ions were monitored on the basis of fragmentation pattern and are used for the confirmation of compounds. Loss of specific ion m/z 57 was most abundantly observed for each compounds. Formula weight (FW), $[M]^+$ion and the retention time were evaluated for selected ion monitoring (SIM) mode (Fig. 1). The ions having maximum intensity in the spectra was chosen as a quantification ion (QI) that does not interfere with other ions from matrices (Fig. 1). Either $[M]^+$ion or $[M-57]^+$ion was chosen for confirmation ion (CI). Retention time (RT), molecular weight (M.W), molecular ion $[M]^+$, characteristic fragmented ion (M-57) are presented in Table 1.

The chromatogram from SIM mode (Fig. 2) shows the clear separation of each compounds investigated. GC-MS mass spectrum for each compound was obtained (Fig. 2).

The linear range of the method was observed

Table 1. Retention Time, Molecular Ion (M^+), Common Fragment Ion (M-57) and SIM Ions of FFAs Following TBDMS Derivatives

Free fatty acids	Retention time (min.)	M.W.	M^+	(M-57)	Quantification ion, Confirmation ion
Caprylic acid (C8)	13.95	144	258	201	201, 258
Capric acid (C10)	17.90	172	286	229	229, 286
Trans-2-decylenic acid (C10:1)	18.90	170	284	227	227, 284
Lauric acid (C12)	21.80	200	257	257	257, 314
Myristic acid (C14)	25.00	228	342	285	285, 342
Palmitic acid (C16)	28.10	256	370	313	313, 370
Palmitoleic acid (C16:1)	28.78	254	368	311	311, 368
Stearic acid (C18)	31.00	286	398	341	341, 398
Octadecenoic acid (C18:1)	30.80	284	396	339	339, 396
Linoleic acid (C18:2)	30.50	282	394	337	337, 394

over the concentration range of 0.01-2,000 μg/mL ($r^2 \geq 0.99$) (Table 2). Calibration curves were plotted by the peak area ratio of analyte to IS against the analyte concentration using control human pooled plasmas spiked at various concentrations.

The regression equation and correlation coefficients were presented in Fig. 3. The calibration curve showed an excellent linearity with a correlation coefficient of greater than 0.99 for all compounds except 0.9832 and 0.9841 for C16 and C18:1 respectively. This wider linear range allow

Table 2. Linear Range, LOD, and LOQ for Free Fatty Acids

Free fatty acids	Linear Range (μg /mL)	LOD (ng)	LOQ (ng)
C10	0.01-2,000	3.3	33
C10:1	0.01-2,000	2.4	24
C12	0.01-2,000	2.2	22
C14	0.01-2,000	1.8	18
C16	0.01-2,000	0.9	9
C16:1	0.01-2,000	2.2	22
C18	0.01-2,000	2.8	28
C18:1	0.01-2,000	8.8	88
C18:2	0.01-2,000	6.6	66

Abbreviations: LOD, 3X signal-to-noise ratio, LOQ, 10X signal-to-noise ratio.

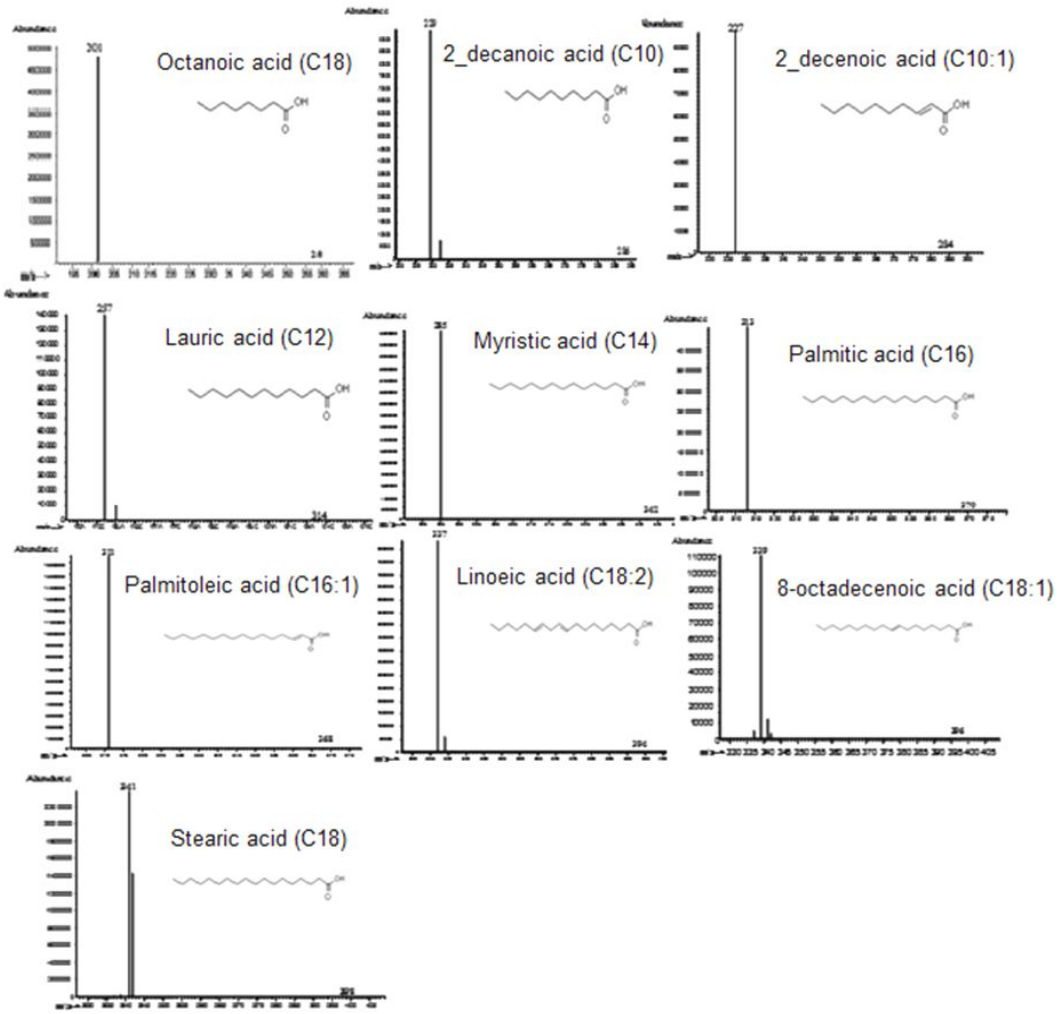

Fig. 2. Mass spectra of free fatty acids (C8-C18:1) following TBDMS.

us to determine the amount of free fatty acids in normal plasma as well as fatty acid oxidation disorders.

LOD ranged from 0.9 to 8.8 ng and LOQ ranged from 9 to 88 ng (Table 2). LOD and LOQ from our method permits the successful detection and quantification of FFAs . Intra-day and inter-day assay was measured (Table 3, 4).

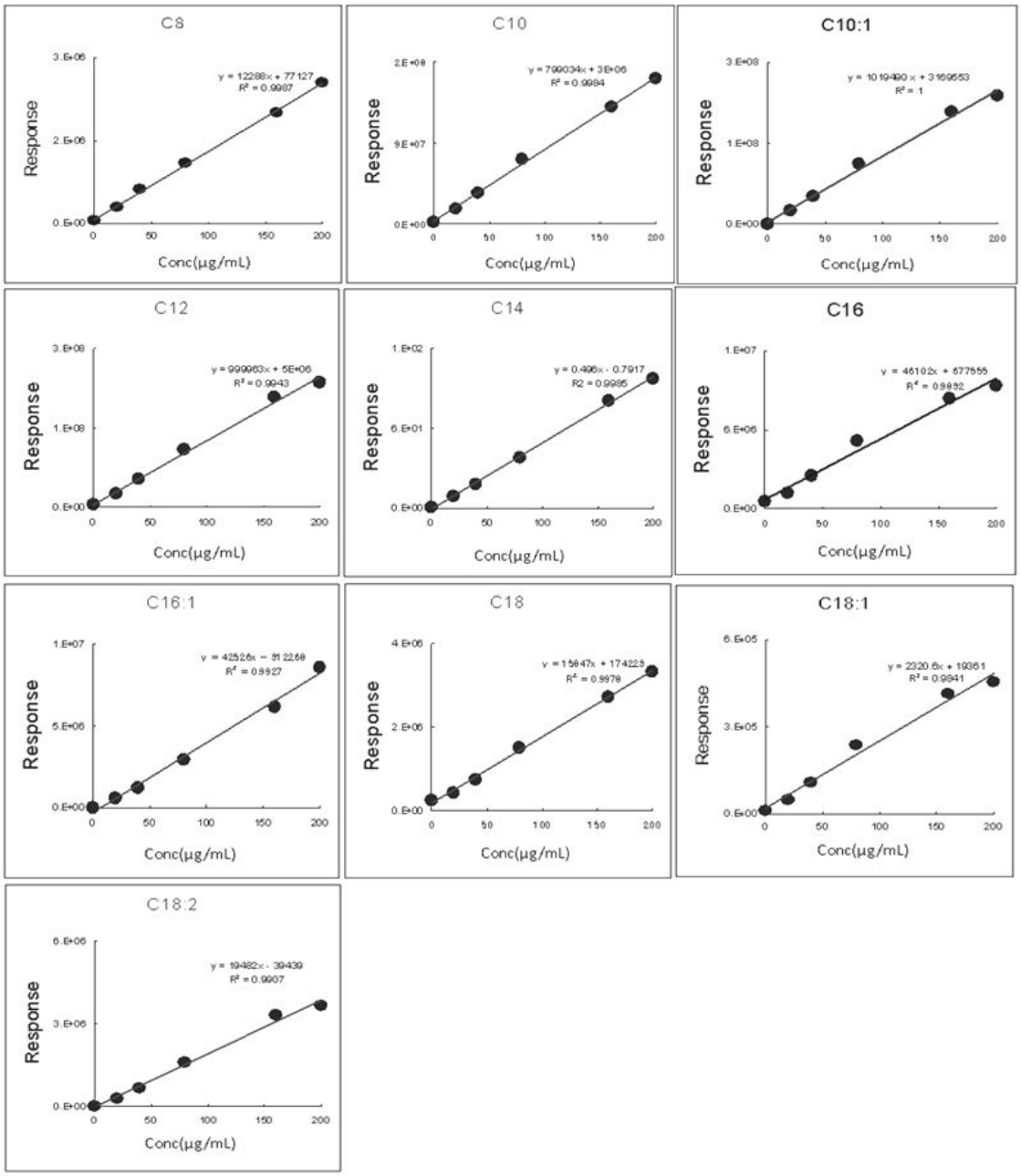

Fig. 3. Calibration curve of free fatty acids (C8-C18) standard solution spiked to plasma.

Table 3. Intra-day Precision and Recovery for QC Samples Spiked in Plasma (n=5)

		C10	C10:1	C12	C14	C16	C16:1	C18	C18:1	C18:2
20 μg/mL	Mean±S.D	19.58±1.7	19.29±1.8	19.06±2.9	19.05±0.5	18.93±0.7	15.68±0.2	19.72±2.8	15.16±2.1	20.52±2.2
	RSD (%)	3.3	9.5	15.4	2.7	3.9	2.6	4.4	7.9	10.6
	Recovery (%)	97.9	96.5	95.3	95.3	94.7	78.4	98.6	75.8	102.6
40 μg/mL	Mean±S.D	33.22±1.1	33.22±1.2	36.27±1.6	37.08±0.8	33.76±3.8	37.59±1.8	31±3.2	38.16±1.1	39.07±2.6
	RSD (%)	12.2	3.7	4.4	2.2	5.2	4.7	10.4	2.8	6.7
	Recovery (%)	83.1	83.1	90.7	92.7	84.4	94.0	77.5	95.4	97.7
100 μg/mL	Mean±S.D	97.62±3.2	94.3±2.4	86.44±0.9	100.36±4.4	93.9±2.0	79.53±2.1	100.3±4.4	88.83±2.4	89.42±4.7
	RSD (%)	3.3	2.6	1.1	4.4	2.2	2.6	4.4	2.7	5.3
	Recovery (%)	97.6	94.3	86.4	100.4	93.9	79.5	100.3	88.8	89.4

Abbreviations: S.D, standard deviation; RSD, precision calculated as relative standard deviation; Recovery, calculated as (mean calculated concentration/ nominal concentration)×100.

Table 4. Inter-day Precision and Recovery for QC Samples Spiked in Plasma (n=10)

		C10	C10:1	C12	C14	C16	C16:1	C18	C18:1	C18:2
20 μg/mL	Mean±S.D	20.64±1.1	20.09±1.9	18.98±0.2	19.14±0.4	19.73±1.0	18.54±1.0	21.65±1.2	19.07±1.0	20.42±0.8
	RSD (%)	5.6	9.5	1.1	2.3	5.1	5.5	5.5	5.4	3.9
	Recovery (%)	103.2	100.5	94.9	95.7	98.7	92.7	108.3	95.4	102.1
40 μg/mL	Mean±S.D	37.2±1.3	36.62±2.9	36.8±1.9	37.2±3.0	37.33±2.4	36.74±1.8	36.55±1.5	35.63±2.6	37.91±2.5
	RSD (%)	3.6	8.1	5.3	2.0	8.1	4.8	4.0	7.4	6.5
	Recovery (%)	93.0	91.6	92.0	93.0	93.3	91.9	91.4	89.1	94.8
100 μg/mL	Mean±S.D	96.34±1.0	96.15±6.1	86.43±1.2	95.83±3.1	92.09±3.2	82.88±3.6	98.31±3.7	82.52±4.3	97.42±5.4
	RSD (%)	1.1	6.3	1.4	3.3	3.5	4.4	3.8	5.3	5.5
	Recovery (%)	96.3	96.2	86.4	95.8	92.1	82.9	98.3	82.5	97.4

Abbreviations: S.D, standard deviation; RSD, precision calculated as relative standard deviation; Recovery, calculated as (mean calculated concentration/ nominal concentration)×100.

Discussion

In a previous report[20-24], we verified that the TBDMS derivatives of FFAs possess superior GC and MS properties. In most of their El mass spectra, $[M-57]^+$high- mass ions generated by preferential cleavage of the labile *tert.*-butyl function from molecular ions constituted base peaks with a few exceptions[24]. Thus, for profiling analysis of FFA mixtures in SIM acquisition mode, $[M-57]^+$ions were selected as the single ion to be monitored for all FFAs. Under the present GC-MS condition, the retention time of each drug was very precise with reproducibility of 0.20% or better. Therefore, the start time of SIM activation was programmed from 8.0 to 35.0 min to set up six groups of ions to be monitored (Table 1). Among the dwell times tested, 80 ms was found to yield the highest ion abundance for most of the FFAs.

Previous report presented that determination of trace FFAs in plasma was difficult due to the presence of interfering biogenic organic acids at much higher concentrations. Therefore, plasma matrix effects on SIM detection were examined with plasma extracts prepared from blank plasma using our developed method in partition mode. Those were recovered more efficiently within a shorter time (20 min), and required smaller amounts of plasma (100 μL).

The typical total ion chromatogram of blank

plasma extracts obtained in scanning mode shows a good plasma fatty acid profile (data not shown). When the same extract was analyzed in SIM mode, complete resolutions with no interferences are more clearly depicted in the individual ion chromatograms (Fig. 2).

For method validation, accuracy was determined as the ratio of back-calculated value to the nominal standard concentration and precision as the % CV of peak areas from replicate analyses for intra-day (n=5) and for inter-day (n=5). Accuracy was calculated as: [measured concentration]/[nominal concentration]×100%. Precision and recovery of the method were evaluated on standard spiked plasma at low (20 μg/mL), medium (40 μg/mL), and high (100 μg/mL) quality control concentrations. The precision was calculated in terms of relative standard deviation (RSD) and evaluated as 1.1 to 15.4% for intraday assay and 1.1 to 9.5 % for inter-day assay. The precision was found in accordance with the validation criteria i.e. 1 to 15%. Recovery of FFAs ranged from 75.8 to 102.6% for intra-day assay and 82.5 to 108.3 % for inter-day assay (Table 3, 4).

Fatty acid β-oxidation (FAO) plays a pivotal role in energy homoeostasis, but it competes with glucose as the primary oxidative substrate. The mechanisms behind this so-called glucose-fatty acid cycle operate at the hormonal, transcriptional and biochemical levels. Inherited defects for most of the FAO enzymes have been identified and characterised and are currently included in neonatal screening programmes. Symptoms range from hypoketotic hypoglycaemia to skeletal and cardiac myopathies. The pathophysiology of these diseases is still not completely understood, hampering optimal treatment. Studies of patients and mouse models will contribute to our understanding of the pathogenesis and will ultimately lead to better treatment.

Korean patients with Glutaric aciduria type 2 plasma, one of the fatty acid oxidation disorders was analyzed, which characterized clinically by fatal neonatal acidosis, hypoglycemia and a strong 'sweaty-feet' odour. Biochemical features showed a massive urinary excretion of glutaric and lactic acids. In free fatty acid profile, the excretion of C8 and C10 fatty acid was elevated compared to normal. In organic acid profile, it showed abnormal excretions of adipic, C8:1 dicarboxylic acid, suberic, C10:1 dicarboxyllic acid, sebacic, and hexanoic acid. In addition, the plasma level of glutaric acid was greatly elevated

Conclusion

A sensitive, and selective analytical method of FFAs was developed using GC-MS/SIM for the screening of fatty acid oxidation disorder. The method was validated and this method could provide useful tool for the screening of fatty acid oxidation disorders where the places does not facilitate MS/MS.

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