


Analysis of short-chain fatty acids (SCFAs) by LC-MS/MS coupled with chemical derivatization

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ABSTRACT

Short-chain fatty acids (SCFAs) are common compounds in living organisms. Due to the rigid molecular structure and stable chemical properties, multiple ion monitoring (MIM) mode was always used to detect SCFAs in LC/MS analysis. This approach could not avoid the matrix effects and also limited sensitivity and accuracy. Therefore, a LC-MS/MS method coupled with chemical derivatization was developed for the determination of SCFAs in plasma samples. Using chemical derivatization technology, the tertiary amine group is labelled in the structure of the SCFAs therefore enabling the SCFAs to be detected in positive mode and the ionization in the electrospray ionization (ESI) source to be increased. In addition, the labelled SCFAs can produce high intensity and stable fragment ions, which improves the detection sensitivity and accuracy.

INTRODUCTION

SCFAs are a subset of fatty acids that are formed primarily in the gut by microbiota and play a major role in gut and immune homeostasis. Due to their chemical stability, the non-specific MIM mode has typically been used to detect SCFAs by LC-MS analysis in negative ion mode. This analysis is confounded by matrix interference and is limited in sensitivity and accuracy.

Here, an LC-MS/MS method coupled with chemical derivatization was developed to selectively and sensitively analyze SCFAs in plasma samples. Using a tertiary amine-based derivatizing agent, SCFAs can be detected in positive ion mode. In addition, 3 MRM transitions were used for the quantitation attributed to the high intensity and stable fragment ions produced by the labelled SCFAs at following collision-induced dissociation (CID). As a result, the labeled SCFAs produce high-intensity, stable fragment ions, which improve the overall assay sensitivity and accuracy. The endogenic SCFAs could be quantified from trace plasma (less than 1 µL). Furthermore, a stable neutral loss ion was observed after derivatization, which indicated that this method could be used for the targeted screening of carboxylic acid compounds in samples.

MATERIALS AND METHODS

Sample preparation:
For the analysis of plasma samples, the SCFAs were first extracted from plasma using acetonitrile (ACN) precipitation by adding 40 µL of ACN to a 10 µL plasma sample. After vortexing, the mixture was centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The supernatant was evaporated to dryness at 40°C under a nitrogen stream.
The chemical derivatization process was described previously[1] and the chemical reaction is shown in Figure 1. Briefly, the pre-treated plasma samples were dissolved in 100 µL of ACN and the reaction was performed by adding 10 µL CMPI (20 µmol/mL) and 20 µL TEA (20 µmol/mL). After mixing, 20 µL of DMBA (20 µmol/mL) was added for the chemical derivatization. The reaction solutions were incubated at 40°C for 1 h with shaking at 1500 rpm and then evaporated under a stream of nitrogen gas until dry. Finally, the dried samples were dissolved in 1 mL water and diluted 500-fold with water for the LC-MS/MS analysis.

HPLC conditions:
An Exion LC system with a Phenomenex Kinetex C18 (100 mm × 2.1 mm, 2.6 µm) column was used for the analysis at a flow rate of 0.3 mL/min. The mobile phase A was 0.1% formic acid in water and mobile phase B was ACN. A 6-minute gradient was used (Table 1). The column temperature was 40 ℃ and a 1 µL sample was injected for the analysis.

MS/MS conditions:
A SCIEX Triple Quad 4500 system with Turbo V source and electrospray ionization (ESI) probe was used. MRM (multiple reaction monitoring) scanning mode was employed in ESI positive mode in MS detection for the quantification of labeled SCFAs. The optimized MRM parameters are shown in Table 2. The optimized MS parameters included: IS voltage, 5500 V; curtain gas, 35 psi; GS1, 60 psi; GS2, 65 psi; source temperature, 550°C and CAD gas, medium.

Figure 1. schematic of the chemical derivatization of SCFAs

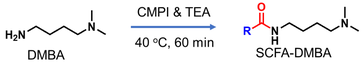

Table 1. chromatographic gradient

Time (min)	%A	%B
0.00	95.0	5.0
0.50	90.0	10.0
3.00	5.0	95.0
4.00	5.0	95.0
4.10	95.0	5.0
6.00	95.0	5.0

Table 2. Optimized MRM parameters for the quantitation of acetic acid (AA), butyric acid (BA), pentanoic acid (PA) after chemical derivatization.

ID	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (eV)
AA-DMBA-1*	159.1	100.1	10	19
AA-DMBA-2	159.1	114	10	17
AA-DMBA-3	159.1	72.1	10	21
BA-DMBA-1*	187.1	100	15	23
BA-DMBA-2	187.1	142.1	15	19
BA-DMBA-3	187.1	72.1	10	24
PA-DMBA-1*	215.1	170.2	20	21
PA-DMBA-2	215.1	100.1	20	25
PA-DMBA-3	215.1	72.1	15	26

*: Transition used for quantitation

RESULTS

Derivatized SCFAs were detected in positive ion mode with increased ionization efficiency compared to the underivatized SFCAs in negative ion mode. The labeled SCFAs produced 2 stable fragment ions at m/z 100 and 72 and underwent a neutral loss of 45 Da, generating 3 unique ions with high intensity (Figure 2). These 3 MRM transitions were used for the SCFA quantitation, which improved the assay sensitivity and accuracy. Furthermore, due to the increased carbon number in labeled SCFAs, the retention of SCFAs was enhanced by using a reversed-phase chromatographic column and the matrix interference was reduced.

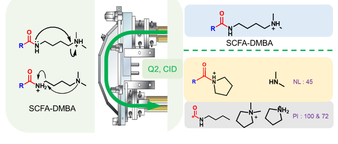

Figure 2. Fragmentation behavior after chemical derivatization

Acetic acid (AA), butyric acid (BA) and pentanoic acid (PA) were employed as the standard compounds for method development. Good linear correlations were obtained with the coefficient of determination (R^2) ranging from 0.9930 to 0.9967 and with a linear range extending from 0.1-200 ng/mL for the quantitation of the 3 SCFAs (Figure 3). The LOQs for AA, BA and PA were 0.1 ng/mL, 0.05 ng/mL and 0.01 ng/mL, respectively.

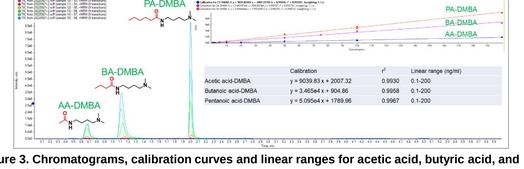

Figure 3. Chromatograms, calibration curves and linear ranges for acetic acid, butyric acid, and pentanoic acid.

The repeatability and accuracy of the developed method were assessed by recoveries and relative standard deviations (RSDs), respectively. The RSD of 8 continuous injections at the 0.1 ng/mL concentration was <3.6% (Figure 4). The recoveries obtained ranged from 85.0% to 113.4% at 3 different concentrations (0.1, 10 and 200 ng/mL) (Table 3).

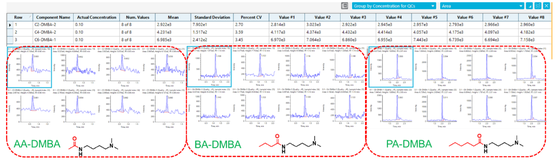

Figure 4. Chromatograms and stability of 8 continuous injections at 0.1 ng/mL.

Table 3， Limit of quantitation (LOQ), accuracy and repeatability of acetic acid, butyric acid and pentanoic acid.

	Linear range	LOQ	Accuracy (%)			Repeatability (RSD%, n=8)
	ng/mL	ng/mL	0.1 ng/mL	10 ng/mL	200 ng/mL	0.1 ng/mL
Acetic acid-DMBA	0.1-200	0.1	111.5	97.4	100.7	2.7
Butanoic acid-DMBA	0.1-200	0.05	85.0	113.4	99.1	3.6
Pentanoic acid-DMBA	0.1-200	0.01	107.7	99.4	97.2	3.6

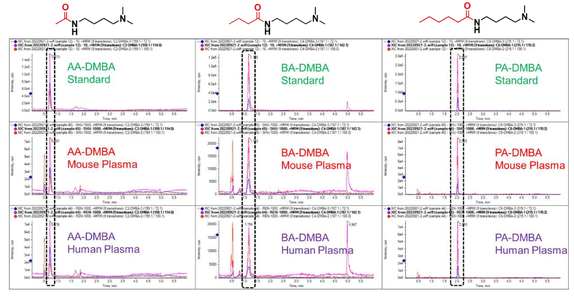

Figure 5. Chromatograms of acetic acid (AA), butyric acid (BA) and pentanoic acid (PA) in standard, human and mouse plasma samples after chemical derivatization.

We quantified the content of AA, BA and PA in human and mouse plasma samples. The SCFAs could still be detected after plasma was diluted 50000-fold, which indicates that the endogenic SCFAs could be quantified from trace plasma (less than 1 µL). The quantitation results are shown in Figure 5 and Table 4. The concentrations of the 3 SCFAs ranged from 120.5 mg/L to 490.2 mg/L.

Table 4， Quantitation results of acetic acid (AA), butyric acid (BA) and pentanoic acid (PA) in human and mouse plasma samples

	Human plasma (mg/L)	Mouse plasma (mg/L)
Acetic acid	484.8	490.2
Butanoic acid	104.5	120.5
Pentanoic acid	158.3	173.4

CONCLUSIONS

A method was developed to achieve high sensitivity and selectivity for the determination of SCFAs in plasma. Using a tertiary amine-based derivatizing agent, SCFAs can be detected in positive ion mode. The labeled SCFAs produce high-intensity, stable fragment ions, which improve the overall assay sensitivity and accuracy. Using chemical derivatization technology, the endogenic SCFAs could be quantified from trace plasma (less than 1 µL). Furthermore, a stable neutral loss ion was observed after derivatization, which indicated that this method could be used for the targeted screening of carboxylic acid compounds in biological samples.

REFERENCES

1 1. Huang Y.Q., Wang Q.Y., Liu J.Q., Hao Y.H., Yuan B.F., Feng Y.Q., Isotope labelling – paired homologous double neutral loss scan-mass spectrometry for profiling of metabolites with a carboxyl group, Analyst, 2014, 139, 3446

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