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PCI-GC–MS–MS approach for identification of non-amino organic acid and amino acid profiles

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ABSTRACT

Alkyl chloroformate have been wildly used for the fast derivatization of metabolites with amino and/or carboxyl groups, coupling of powerful separation and detection systems, such as GC–MS, which allows the comprehensive analysis of non-amino organic acids and amino acids. The reagents involving *n*-alkyl chloroformate and *n*-alcohol are generally employed for providing symmetric labeling terminal alkyl chain with the same length. Here, we developed an asymmetric labeling strategy and positive chemical ionization gas chromatography—tandem mass spectrometry (PCI-GC–MS–MS) approach for determination of non-amino organic acids and amino acids, as well as the short chain fatty acids. Carboxylic and amino groups could be selectively labelled by propyl and ethyl groups, respectively. The specific neutral loss of C_3H_8O (60 Da), $C_3H_5O_2$ (74 Da) and $C_4H_8O_2$ (88 Da) were useful in the selective identification for qualitative analysis of organic acids and amino acid derivatives. PCI-GC–MS–MS using multiple reaction monitoring (MRM) was applied for semi-quantification of typical non-amino organic acids and amino acids. This method exhibited a wide range of linear range, good regression coefficient (R^2) and repeatability. The relative standard deviation (RSD) of targeted metabolites showed excellent intra- and inter-day precision (<5%). Our method provided a qualitative and semi-quantitative PCI-GC–MS–MS, coupled with alkyl chloroformate derivatization.

1. Introduction

Gas chromatography–mass spectrometry (GC–MS) is considered as the golden standard method in volatile, low-boiling metabolite analysis due to its high chromatography resolution and high reproducible chromatography [1–3]. Pre-column derivatization has been developed as an effective method of rendering highly polar materials sufficiently volatile and narrowing the boiling point window [4,5]. Metabolite derivatization based on alkylation agents has been widely used for extending coverage of detectable metabolites class for GC–MS technique [6–9]. Especially, alkyl chloroformate are excellent reagents for the fast derivatization of metabolites with amino and/or carboxyl groups in aqueous media without the requirement of heating [10]. We have successfully performed methyl chloroformate (MCF) derivatizaiton for nonamino organic acids and amino acids in our previous study [11]. However, this method is unsuitable for short chain fatty acids (SCFAs), especially the acetic acid [12,13]. Recently, the use of propyl chloroformate derivatiztion for modification of short fatty acids and branched chain amino acids before EI-GC–MS analysis has been described [14]. The electron ionization (EI) ion source has been widely used. EI spectra at 70 eV provide informative fragment ions, which are reproducible for database searches, but less information about the molecular ion [15–17]. Positive chemical ionization (PCI) is a soft ionization method through molecular reaction between small molecules and the reactant ions produced by ionization of methane [18,19], retaining the molecular ion and other relative high molecular weight fragments that could be used as a precursor ion for determination of metabolites in tandem mass spectrometry (MS–MS) [20].

Most of the derivatizing regents mentioned above provided terminal alkyl chain with the same length by using *n*-alkyl chloroformate and *n*-alcohol combinations. This symmetric labeling method was widely used. In this study, we developed an asymmetric labeling strategy based on PCI-GC–MS–MS for determination of non-amino organic acids and amino acids, as well as the short chain fatty acids. Carboxylic acid and amino group could be selective labelled by propyl and ethyl groups, respectively. Special ions corresponding to different neutral losses were shown in mass spectra that can be used for qualitative research. PCI-GC–MS–MS with

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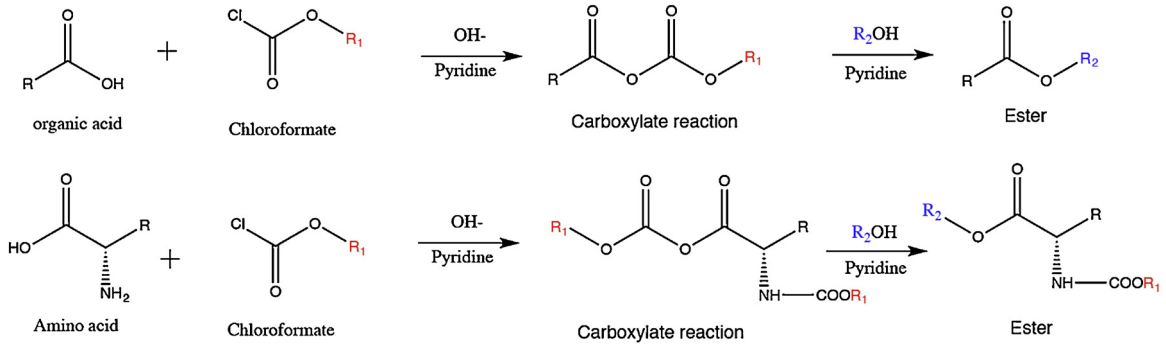

Fig. 1. Reaction scheme of organic acids and amino acids, treated with alkyl chloroformate derivatization.

multiple reaction monitoring (MRM) was applied for quantification of typical non-amino organic acids and amino acids.

2. Experimental method

2.1. Chemicals and reagents

Standards used for method optimization and validation, e.g., Acetic acid, Propionic acid, 1-Propanol (PrOH), Butyric acid, Isobutyric acid, Valeric acid, Isovaleric acid, Valine, Isoleucine and Leucine were obtained from Sigma-Aldrich and prepared in the ultrapure water from a Milli-Q system (Millipore, USA). Methyl-, Ethyl- and Propyl- chloroformate (MCF, ECF and PCF) was obtained from American International Chemical, Inc (United States). All other chemicals and reagents used for the experiments were of analytical grade. 2-ethylbutyric acid and 4-chlorophenylalanine were used as internal standards (IS) for final adjustment on quantity of short-chain fatty acids and branched-chain amino acids present in the biological samples, respectively.

2.2. Sample preparation and derivatization

Stool samples were collected from a healthy human volunteer at Hong Kong Baptist University Clinical Centre. Written informed consent was obtained from volunteer before stool collection. 100 mg of stool sample was homogenized at 4 °C. The sample was then centrifuged 5 min at 13200*g* speed in an Eppendorf centrifuge. A 300-μl aliquot of supernatant samples was transferred into a 10 mL glass tube. Primary stock solutions were prepared in water at a concentration of 5 mM for each standard. The stock solution was further diluted to a series of concentrations, ranging from 5 to 5000 μM. The calibration curve was constructed by plotting the peak-area ratio of each standard to IS versus concentration. Each solution or supernatant samples were mixed with 300 μl of NaOH (1 M), 160 μl of 1-Propanol and 40 μl of pyridine in a 10 mL glass centrifuge tube. The derivative reaction was started by adding 100 μl of MCF, ECF or PCF into the tube, and the pooled mixture was then shaken for 60 s using a vortex. After the successive derivatization steps, 500 μl of dichloromethane was added and shaken for 10 s for extracting the MCF, ECF or PCF derivatives. After adjusted the pH value with the 200 μl of $NaHCO_3$ (50 mM), the dichloromethane layer containing derivatives were isolated and dried by anhydrous Na_2SO_4 and subsequently subjected to PCI-GC–MS analysis.

2.3. Instrumental conditions

GC-PCI-MS analysis was performed with an Agilent 7890B gas chromatography coupled with 7000C triple quadrupole mass spectrometer. The column used for all analysis was a HP-5MS capillary column coated with 5%-Phenyl)-methylpolysiloxane (30 m × 250 μm i.d., 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA). Solvent delay was set for 2 min. The measurements were made with PCI in the full scan mode (*m*/*z* 90–650). Splitless injection was used with the Agilent 7693 autosampler system. For the GC-PCI-MS–MS analysis, the MS was operated in the positive ionization mode, methane (Grade 4.0, 99.99% pure) was used as reactant gas at an apparent pressure of 1.0×10^4 Torr in the ionization source. The MS interface, source and quadrupole temperatures were 290 °C, 320 °C and 150 °C, respectively. Multiple reaction monitoring was used with a dwell time of 100 ms per ion.

The oven temperature was initially held at 50 °C for 2 min. Thereafter the temperature was raised with a gradient of 6 °C/min until 180 °C. Afterward, the temperature was raised with a gradient of 6 °C/min until 260 °C and then increased to 300 °C at a rate of 20 °C/min. This temperature was held for 2 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The peak abundances of MCF, ECF or PCF derivatives were used to quantify the concentrations of the amino acids and organic acids in the samples. The majority of the metabolites detected were identified by commercially available compound libraries: NIST, and reference compounds available. GC–MS data was processed by Agilent MassHunter Qualitative/Quantitative Analysis (Version B.07.00) softwares.

3. Results and discussion

3.1. Optimization of derivatization reagents

Chloroformate derivatization method was developed and employed for profiling analysis of organic acids (Fig. 1), especially short chain organic acids. 1-Propanol, which function as a key derivatization solvent. Therefore acetic acid derivatives could be separated from the solvent peak and can be measured precisely [14]. To our knowledge, Methyl-, Ethyl- and Propyl- chloroformates are widely used in alkyl chloroformates derivatization procedures, converting amino and organic acids into volatile carbamates and esters [21–23]. Methyl-, Ethyl- chloroformates yielded higher derivatization efficiency than Propyl- chloroformates [14]. In this study, alkyl chloroformates with different lengths of alkyl groups, including Methyl-, Ethyl-, Propyl-, were compared. 1-Propanol was essential derivatization solvent, participating in the alkyl chloroformates triggered derivatization reaction and providing the alkyl-group for organic acids. As showed in Fig. 2, short chain fatty acid derivatives including Propyl acetate (2.08 min), Propyl propionate (3.90 min), Propyl butyrate (6.00 min) and Propyl valerate (8.40 min) were smoothly produced and well separated after above mentioned three alkyl chloroformates derivatization procedures. Meanwhile, alkyl chloroformates derivatization procedures