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Quantitative liquid chromatography–electrospray ionization-mass spectrometry analysis of amine-containing metabolites derivatized with cyanuric chloride and methylamine isotopologues

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ABSTRACT

Electrospray ionization (ESI) is the most useful interface for mass spectrometry associated with liquid chromatography (LC). However, analyses of polar metabolites become a challenge because the high polarity impairs the separation of metabolites in reversed-phase liquid chromatography and ionization of metabolites by ESI. In this article, we have used cyanuric chloride to couple the amine-containing molecules with methylamine and found that both resolution on LC chromatogram and ionization by ESI are greatly improved. Derivatives would be obtained in 2-h coupling reactions, and then resolved by LC–ESI-MS in 15 min for each sample. Most amino acids can be quantified with linear range from 1 nM to 1 μM and with an R^2 above 0.979. Although reversed-phase chromatography is suitable for resolving the derivatives, phenyl columns with methanol elution provide optimal separation and signal intensity. Moreover, most structural isomers are well separated following cyanuric chloride and methylamine derivatization. Instead of synthesizing a stable isotope-labeled cyanuric chloride, we can take advantage of using commercially available methyl-d3-amine for a novel stable isotope-coded derivatization method. Each metabolite can be directly quantified by the peak intensity ratio of each derivative isotopologue pair in a single LC–MS analysis. The coupling reactions are relatively easy and accessible to most investigators to generate multiple stable isotope-labeled derivatives of amine-containing compounds for a differential metabolomic analysis.

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1. Introduction

Metabolomics first defined as “the quantitative measurement of all low molecular mass metabolites in an organism’s cells at a specific time under specific environmental conditions” is the latest addition to the omics family [1,2]. Liquid chromatography–mass spectrometry (LC–MS) using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) is well suited to the analysis of metabolites, however with unsatisfactory ionization efficiency on occasion [3]. Therefore, pre-column chemical derivatization methods have been designed and applied to the LC–MS analysis of metabolites in biological samples with the following advantages: increased ionization efficiency, enhanced resolution on LC chromatogram and decreased matrix effects [4,5]. For examples, derivatization reagents such as methyl acetimidate [6], N-hydroxysuccinimide ester of N-alkylnicotinic acid [7], p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide [8], dimethylaminobutyryl succinimide [9], and dansyl chloride [10] are utilized to target amine-containing metabolites. Importantly, each derivatization reagent labeled with stable isotope is synthesized in house by the corresponding laboratory and utilized for quantitative applications. In practice, two samples labeled with two derivatization reagent isotopologues are mixed and subjected to the LC–MS analysis. The peak intensity ratio of each derivative isotopologue pair provides the relative quantification of each metabolite. This method, stable isotope-coded derivatization, is becoming one of the most powerful techniques

Abbreviations: APCI, atmospheric pressure chemical ionization; CyC, cyanuric chloride; DMEM, Dulbecco’s Modified Eagle Medium; ESI, electrospray ionization; GILISA, Global Isotope-Labeled Internal Standard Addition; GSIST, Quantification with Group Specific Internal Standard Technology; LOQ, limit of quantification; PFP, pentafluorophenylpropyl; TOF, time-of-flight; UPLC, ultra-performance liquid chromatography.

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for metabolite profiling study [11,12]. Unfortunately, the derivatization reagents labeled with stable isotope are only available to the investigators who can synthesize the reagents, but not to all investigators. Here we explore the applications of derivatization using cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) and methylamine in the quantitative analysis of amine-containing metabolites. Cyanuric chloride provides three reactive sites toward nucleophiles such as alcohols, thiols, amines and phenols [13–15]. Cyanuric chloride has been used to couple enzymes to solid supports [16,17], to cross-link proteins [18,19], and to synthesize chiral derivatizing reagents for enantioresolution of chiral compounds [20–22]. We showed in this communication that cyanuric chloride and methylamine isotopologues can be used to derivatize amine-containing metabolites resulting in metabolite derivative isotopologues. This novel stable isotope-coded derivatization method can be applied to quantitative analysis of amine-containing compounds.

2. Experimental

2.1. Materials

Cyanuric chloride (99%, C95501), amino acid standards (amino acids mixture, A9906), L-lysine, methylamine hydrochloride, methyl-d3-amine hydrochloride, and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) medium and fetal bovine sera were purchased from Invitrogen (Waltham, MA, USA). Acquity UPLC® BEH C18 1.7 μm column, 2.1 mm × 100 mm (Waters, Miliford, USA), Kinetex 2.6 μm pentafluorophenylpropyl (PFP) column, 150 mm × 2.1 mm (Phenomenex, Torrance, CA, USA), and Inertsil® HPLC Ph-3 2 μm column, 2.1 mm × 100 mm (GL Science Inc., Torrance, CA, USA) were purchased from the company in parentheses. Methanol and acetonitrile of LC–MS grade were purchased from J.T. Baker (Avantor Performance Materials Inc., Center Valley, PA, USA). HepG2 cells were obtained originally from American Type Culture Collection (Manassas, VA, USA).

2.2. Cyanuric chloride coupling reaction

Cyanuric chloride was prepared in acetone as 0.1 M solution, and methylamine or methyl-d3-amine was prepared in ultrapure water as 1 M solution. The first step of reaction was carried out by mixing 20 μl of sample with 10 μl of 0.1 M cyanuric chloride (forming precipitates at first but disappearing in a few minutes), and then adding 5 μl of 0.5 M $NaHCO_3/Na_2CO_3$, pH 9.4 to activate the reaction. Inappropriate sequence of the steps could lead to loss of reactive cyanuric chloride and thus incomplete reaction. Premature mixing of cynuric chloride with alkaline buffer completely impaired the following reaction due to hydrolysis of the reactive sites on cyanuric chloride. The mixture was agitated at 25 °C for 2 h and then added with 10 μl of 1 M methylamine or methyl-d3-amine for further reaction at 25 °C for 1 h. After completing the coupling reaction, 5 μl of 10% formic acid was added to stop the reaction and stabilize the derivatives. Each sample was centrifuged at 14,000 × *g* for 10 min before being transferred into an insert vial for sampling in an autosampler. The derivatives were found to be stable at 4 °C for at least 2 days.

2.3. Effect of pH in coupling reactions

Reaction buffers consisting of 0.5 M NaH_2PO_4/Na_2HPO_4 of pH 6.2, 6.7, 7.3, 7.9, and 8.7 were used to examine the effect of pH on the coupling reactions. The reactions were carried out with the same procedure as mentioned above by replacing $NaHCO_3/Na_2CO_3$ by H_2NaPO_4/Na_2HPO_4 buffer. Negative and positive controls were created by addition of 5 μl of ultrapure water and 5 μl of 0.5 M $NaHCO_3/Na_2CO_3$, respectively.

2.4. LC–ESI-MS analysis

The LC–ESI-MS system consisted of an ultra-performance liquid chromatography (UPLC) system (Ultimate 3000 RSLC, Dionex, Sunnyvale, CA, USA) and an electrospray ionization (ESI) source of quadrupole time-of-flight (TOF) mass spectrometer (maXis HUR-QToF system, Bruker Daltonics, Bremen, Germany). The sample was kept in an autosampler at 4 °C. Separation was performed with reversed-phase liquid chromatography on a BEH C18 column, Kinetex PFP column, or Ph-3 column. The elution started from 70% mobile phase A (0.1% formic acid in ultrapure water) to 30% mobile phase B (0.1% formic acid in methanol or acetonitrile), held at 30% B for 0.5 min, raised to 90% B in 7.5 min, held at 90% B for 1.5 min, and then lowered to 30% B in 0.5 min. The column was equilibrated by pumping 30% B for 3 min until next run. The flow rate was set 0.2 ml/min. LC–ESI-MS chromatograms were acquired under following conditions: capillary voltage of 4500 V in positive ion mode, dry temperature at 190 °C, dry gas flow maintained at 8 l/min, nebulizer gas at 1.4 bar, and acquisition range of *m*/*z* 100–1000.

2.5. Data process

Data were acquired by HyStar and micrOTOF control software (Bruker Daltonics, Bremen, Germany) and processed by DataAnalysis and TargetAnalysis software (Bruker Daltonics, Bremen, Germany). Each identified metabolite was elected by matching the theoretical *m*/*z* value and isotope pattern derived from the chemical formula, and each metabolite signal was summed from the area of peaks in the extracted ion chromatogram.

2.6. Cell culture

Culture medium was Dulbecco's Modified Eagle Medium (DMEM), high glucose medium containing 10% fetal bovine serum (FBS). HepG2 cells were cultured with 5% CO_2 atmosphere at 37 °C. Before harvest, the cells were rinsed with 2 ml of phosphate buffered saline (PBS), three times. After discarding the residual PBS, the cells were stored at −70 °C until the extraction of metabolites.

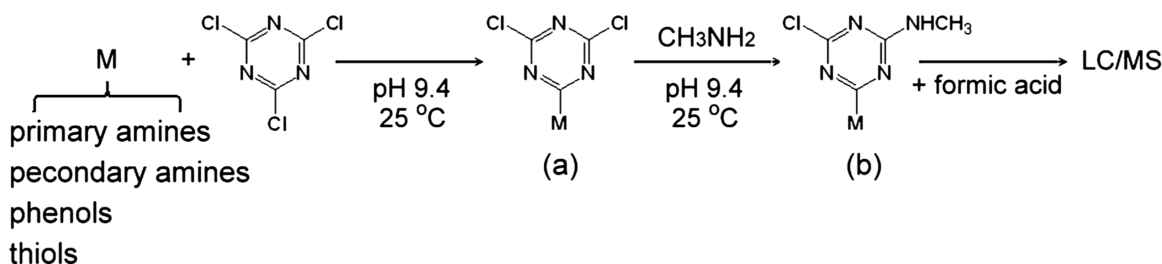

Fig. 1. General labeling flow for compounds containing primary, secondary amines, thiols and phenol groups by cyanuric chloride coupling reactions.

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