Analytica Chimica Acta xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of versatile isotopic labeling reagents for profiling the amine submetabolome by liquid chromatography–mass spectrometry

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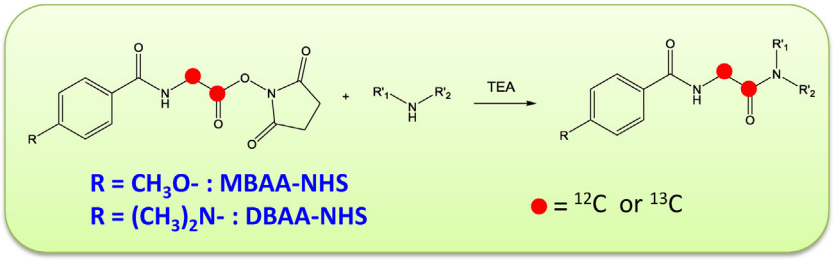
HIGHLIGHTS

- Two new reagents were developed for chemical isotope labeling mass spectrometry (MS).
- They could be used to label amine-containing metabolites in a metabolomic sample.
- The labeled metabolites could be detected with much improved sensitivity in MS.
- One of the reagents could also help generate useful MS/MS spectra for structural analysis.
- These reagents should be useful for quantitative metabolomics.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 February 2015
Received in revised form 6 April 2015
Accepted 11 April 2015
Available online xxx

Keywords:
Mass spectrometry
Liquid chromatography
Isotope labeling
Amine-containing metabolites
Metabolomics

ABSTRACT

Metabolomic profiling involves relative quantification of metabolites in comparative samples and identification of the significant metabolites that differentiate different groups (e.g., diseased vs. controls). Chemical isotope labeling (CIL) liquid chromatography–mass spectrometry (LC–MS) is an enabling technique that can provide improved metabolome coverage and metabolite quantification. However, chemical identification of labeled metabolites can still be a challenge. In this work, a new set of isotopic labeling reagents offering versatile properties to enhance both detection and identification are described. They were prepared by a glycine molecule (or its isotopic counterpart) and an aromatic acid with varying structures through a simple three-step synthesis route. In addition to relatively low costs of synthesizing the reagents, this reaction route allows adjusting reagent property in accordance with the desired application objective. To date, two isotopic reagents, 4-dimethylaminobenzoylamido acetic acid *N*-hydroxylsuccinimide ester (DBAA-NHS) and 4-methoxybenzoylamido acetic acid *N*-hydroxylsuccinimide ester (MBAA-NHS), for labeling the amine-containing metabolites (i.e., amine submetabolome) have been synthesized. The labeling conditions and the related LC–MS method have been optimized. We demonstrate that DBAA labeling can increase the metabolite detectability because of the presence of an electrospray ionization (ESI)-active dimethylaminobenzoyl group. On the other hand, MBAA labeled metabolites can be fragmented in MS/MS and pseudo MS^3 experiments to provide structural information on metabolites of interest. Thus, these reagents can be tailored to quantitative profiling of the amine submetabolome as well as metabolite identification in metabolomics applications.

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http://dx.doi.org/10.1016/j.aca.2015.04.021
0003-2670/

1. Introduction

Differential chemical isotope labeling (CIL) is a method that introduces a chemical tag (e.g., ^{12}C-reagent) and its isotopic analog (e.g., ^{13}C-reagent) into targeted analytes in a sample and a comparative control, respectively. After mixing the labeled sample and control, the mixture is analyzed by liquid chromatography–mass spectrometry (LC–MS). A differentially labeled metabolite is detected in a mass spectrum as a pair of peaks with mass difference defined by the isotope mass difference of the tag(s). The intensity ratio of the peak pair can be determined to provide relative quantification of the metabolite in the sample vs. the control. Absolute quantification is also possible if the concentration of the metabolite in the control is known, i.e., using a metabolite standard. CIL LC–MS has been shown to provide high accuracy and precision for metabolome profiling [1–4], which can be attributed to simultaneous measurement of the co-eluted analytes and their isotope counterparts to overcome ion suppression, instrument drift and other technical problems. Furthermore, chemical derivatization provides a useful means of improving the separation of polar or ionic metabolites in reversed phase (RP) LC as well as enhancing the ionization efficiency in electrospray ionization (ESI) by introducing a relatively hydrophobic and easily chargeable ESI-active tag [1,5,6].

To date, several CIL reagents have been developed to quantify organic amines, carboxylic acids, carbonyl compounds and some other types of metabolites with varying degrees of performance [1,4,5,7–24]. These research activities are mainly driven by the need of dealing with different classes of metabolites; a universally applicable derivatization reagent for LC–MS is not available at the present. On the other hand, chemical labeling can be used as a way of dividing the entire metabolome into several submetabolomes according to their functional groups (e.g., amine submetabolome, acid submetabolome, etc.), thereby reducing the complexity of a metabolomic sample [5,25]. This divide-and-conquer approach requires the use of robust chemical labeling reagents for profiling submetabolomes with different intended purposes (e.g., untargeted quantification, targeted quantification, chemical identification, etc.).

Development of a proper chemical labeling reagent is not an easy task, because multiple factors including reagent synthesis, labeling efficiency, analytical performance, applicability and cost need to be carefully considered. For example, Guo and Li developed a CIL method based on $^{12}C_2$- and $^{13}C_2$-dansyl chloride (DnsCl) [1]. Although the method allows for rapid and accurate quantification of amine-containing metabolites [26–29], metabolite identification, particularly for unknown metabolites, is a challenge due to the lack of structural information in the MS/MS spectra of dansyl labeled metabolites. Tsukamoto et al. developed H_6-/D_6-7-(*N*,*N*-dimethylaminosulfonyl)-4-(aminoethyl)-piperazino-2,1,3-benzoxadiazole (H_6-/D_6-DBD-PZ-NH_2) to profile fatty acids in rat plasma samples [8] and Shimbo et al. reported the use of *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide (TAHS) and D_3-TAHS for amino acid quantification [30]. Because deuterium was used in the labeling reagents, isotopic effect on retention time in RPLC was observed (i.e., H- and D-labeled same metabolite eluted out at different time, thereby subjecting to different matrix effect and ion suppression). Abello et al. described multiplex reagents for analyzing amine-containing metabolites in human cells based on pentafluorophenyl-activated ester of ^{13}C-containing poly(ethylene glycol) chains (PEG-OPFP) [10]. The family of these CIL reagents could be used to quantify three samples in parallel, but the reagents did not provide much signal enhancement in LC–MS. Yang et al. developed H_3-/D_3-*N*-hydroxysuccinimide ester of *N*-alkylnicotinic acid (H_3/D_3-C_n-NA-NHS) to measure concentrations of amino acids in rat urine by which the sensitivity of labeled amino acids was enhanced by up to 1000-fold [11]. While this reagent is useful for targeted analysis of amino acids, it may not be suitable for untargeted analysis, because of the deuterium isotopic effect and the use of an ionizable tag in solution which would limit efficient separation of labeled metabolites by RPLC, requiring the use of different columns for separating different classes of labeled metabolites.

To increase the versatility of CIL LC–MS for metabolomics, we have developed a general strategy based on the use of $^{12}C_2$-glycine and $^{13}C_2$-glycine to construct a family of CIL reagents that can be tailored to different applications. In this work, we report two CIL reagents, $^{12}C_2$-/$^{13}C_2$-4-dimethylamino-benzoylamido acetic acid *N*-hydroxysuccinimide ester ($^{12}C_2$-/$^{13}C_2$-DBAA-NHS) and $^{12}C_2$-/$^{13}C_2$-4-methoxybenzoylamido acetic acid *N*-hydroxysuccinimide ester ($^{12}C_2$-/$^{13}C_2$-MBAA-NHS), that can be used to improve detection and identification of amine-containing metabolites while maintaining efficient retention and separation on RPLC. The synthesis of these reagents and their analytical performance in profiling the amine submetabolome of biofluids such as human urine are described.

2. Experimental

2.1. Chemicals and urine samples

All chemicals and reagents were purchased from Sigma–Aldrich (Oakville, ON) unless otherwise noted. $^{13}C_2$-glycine was purchased from Cambridge Isotopes Laboratories (Andover, MA). Stock solutions of twenty amino acid standards (20 mM each) were prepared in H_2O:ACN (50:50 (v/v)) and stored at 4 °C. To aid the dissolution of amino acids, 6 M HCl could be added drop-by-drop (less than a few microliter) to a solution to completely dissolve an amino acid. A pooled amino acid (1 mM each) was prepared by mixing aliquots of twenty stock solutions. The 300 mM triethylamine (TEA) solution was prepared by dilution of 500 μL of LC-grade TEA in 28.5 mL acetonitrile. The 300 mM formic acid (FA) solution was prepared by adding 1.13 mL of formic acid into 100 mL of H_2O. Both solutions were stored at 4 °C.

The study of human urine samples was approved by the Ethics Approval Board of the University of Alberta. Human urine sample collection and processing were performed according to a previous reported protocol [6].

2.2. CIL reagent synthesis

Fig. 1(A) shows the three-step synthesis pathway to prepare the reagents and their isotopic analogs. *N*,*N*-Dicyclohexylcarbodiimide (DCC) (1.25 g) and *N*-hydroxyl succinimide (NHS) (0.67 g) were added into a solution of 4-methoxybenzoic acid (0.92 g) for preparing MBAA-NHS or 4-dimethylaminobenzoic acid (0.99 g) for preparing DBAA-NHS in 40 mL DMF at 25 °C. The mixture was stirred for 24 h and then filtered to give a clear solution. Glycine (0.75 g) or its isotopic counterpart ($^{13}C_2$-glycine) was dissolved in a mixture of H_2O (20 mL) and triethylamine (2.7 mL). The solution was quickly added into the filtrate from the first reaction. After 30 min, the mixture was neutralized by formic acid to give a white solid, 4-methoxybenzoylamido acetic acid or 4-dimethylbenzoylamido acetic acid, which was filtered out and washed by cold acetone (3 × 10 mL). The product could be directly used in the next step without purification. The third step was exactly the same as the first reaction. 4-Methoxybenzoylamido acetic acid (0.90 g) or 4-dimethylbenzoylamido acetic acid (0.98 g) was dissolved in 40 mL DMF. NHS (0.50 g) and DCC (0.89 g) were added into the solution which was stirred for 24 h at 25 °C. The final product was purified by recrystallization with Hexane:*i*-PrOH (3:1 v/v). The

Please cite this article in press as: R. Zhou, et al., Development of versatile isotopic labeling reagents for profiling the amine submetabolome by liquid chromatography–mass spectrometry, Anal. Chim. Acta (2015), http://dx.doi.org/10.1016/j.aca.2015.04.021