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Letters to *Analytical Chemistry*

High-Performance Isotope Labeling for Profiling Carboxylic Acid-Containing Metabolites in Biofluids by Mass Spectrometry

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We have developed a new isotope labeling method, based on the use of isotope-coded *p*-dimethylaminophenacyl (DmPA) bromide as a reagent, combined with liquid chromatography−mass spectrometry (LC−MS) for high-performance metabolome analysis with a focus on profiling carboxylic acid-containing metabolites. Derivatization is simple, fast (1 h plus 30 min for quenching the reaction), and applicable to a wide range of carboxylic acids with a high yield and little or no side reaction products. This labeling method is demonstrated to be not only effective in introducing an isotope tag for accurate metabolite quantification but also improving the chromatographic retention of the metabolites in reversed-phase (RP) LC, enhancing ESI efficiency by 2−4 orders of magnitude, and facilitating the identification of metabolite peaks in LC−MS. In triplicate experiments of a 1:1 ratio of ^{13}C-/^{12}C-DmPA labeled human urine, we were able to detect 2671, 2546, and 2820 ion pairs from metabolites containing one or more carboxylic acid groups.

Metabolomics is a rapidly evolving field for studying biological systems and discovering potential disease biomarkers.[1] For any metabolomics application, metabolome analysis with adequate sensitivity and specificity is essential in defining the metabolome. Ideally, all metabolites present in a biological system are qualitatively and quantitatively profiled. Unfortunately, because of technical limitations, only a fraction of metabolites are currently analyzed by using techniques such as NMR and mass spectrometry (MS).[2,3] Because of limited metabolome coverage, many important metabolome networks and some subtle changes in the metabolome may not be revealed with current techniques. Herein we report a high-performance isotope labeling strategy, in combination with liquid chromatography (LC)−MS, for profiling the metabolites containing carboxylic acid moieties in a biological sample including body fluids such as urine.

Isotope labeling of chemicals has been widely used for quantitative analysis of targeted molecules such as drug metabolites by LC−MS.[4] By overcoming matrix and ion suppression effects, the use of an isotope internal standard for the analyte of interest often provides high accuracy of quantitative measurement. However, at present, it is not practical to generate individual isotope standards for all the metabolites of a biological system or the metabolome. An alternative approach is to use a chemical reaction to introduce a mass tag to all the metabolites with a common reactive moiety in a sample and apply the same derivatization reaction to the standards with a differential mass tag (e.g., ^{12}C-labeling for the sample and ^{13}C-labeling for the standards).[5] After the labeled sample and the mass-differentially labeled standards are mixed, LC−MS is carried out and, based on the accurate mass, retention time, and relative peak intensities of ion pairs detected in the mass spectra, tentative identification and absolute quantification of metabolites in the sample can be attained. With the use of additional information such as MS/MS spectral matches, positive metabolite identification can be made. Without the availability of standards, information on the relative quantification of metabolites in different samples can still be obtained.

While chemical derivatization is effective in introducing a mass tag to the metabolites for LC−MS,[5−13] it can also provide an opportunity to enhance the performance of the overall LC−MS

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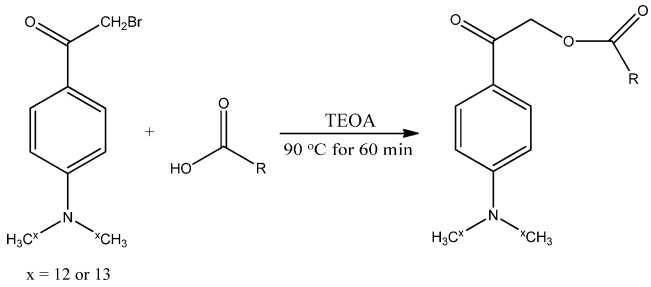

Figure 1. Reaction scheme for labeling carboxylic acid-containing metabolites using isotope coded *p*-dimethylaminophenacyl (DmPA) bromide (light chain, $x = 12$; heavy chain, $x = 13$).

analysis process. Within this context, a rational design of the tag to be attached to the metabolites becomes important. Considering that the LC−MS metabolome profiling work involves several sample handling and analysis steps, an ideal tag would provide concurrent improvement in the analytical performance of each step. In this work, we report a new isotope labeling method to tag carboxylic acid-containing metabolites (CAMs) and demonstrate its application for LC−MS metabolome profiling of complex samples. Global profiling of these metabolites is significant in metabolomics, as a large portion of the metabolome including a vast number of fatty acids belong to this class. For example, about 65% of the ~5000 known endogenous human metabolites contain at least one carboxylic acid group in a chemical structure.[14]

EXPERIMENTAL SECTION

Synthesis of *p*-Dimethylaminophenacyl (DmPA) Bromide. Chemical reagent information is provided in Supplemental Note N1 in the Supporting Information. The synthesis of DmPABr is based on a two-step procedure modified from those described in the literature (see Supplemental Scheme S1 and Note N1 in the Supporting Information).[15−17] The first step is the dimethylation reaction,[15,16] where an isotope tag is introduced to the reagent using $^{13}C_2$-dimethyl sulfate for the heavy chain reagent or $^{12}C_2$-dimethyl sulfate for the light chain reagent. The second step involves bromation and debromation.[17] The synthesis procedures were optimized for reactions with ~1 g of starting material. Two semipreparative reversed-phase separation and normal phase flush chromatography were used to obtain the pure labeling reagents. The purity of the labeling reagent synthesized was >99.5% determined by high-performance liquid chromatography ultraviolet detection (HPLC UV) and MS. The structure and purity were further confirmed by NMR.

Labeling Reaction. Figure 1 shows the reaction scheme for labeling carboxylic acids using the isotope reagents, ^{13}C- or ^{12}C-DmPABr. For labeling human urine samples, urine in 50% (v:v) of acetonitrile was centrifuged for 10 min at 12 000 rpm. About 50 μL of human urine in acetonitrile (v:v) or 50 μL of carboxylic acid standards in acetonitrile (1.2 mM each) were mixed with an equal volume of 750 mM of triethanolamine (TEOA) in a reaction vial. The solutions were vortexed, spun down, and then mixed with 50 μL of freshly prepared ^{13}C-DmPA (20 mg/mL) (for heavy labeling) or ^{12}C-DmPA (20 mg/mL) (for light labeling). The derivatization reaction proceeded for 60 min at 90 °C in a water bath. After 60 min, the mixtures were vortexed, spun down, and 100 μL of triphenylacetic acid (30 mg/mL) was added to consume the excess labeling reagent, ^{12}C-/^{13}C-DmPA, at 90 °C in TEOA for 30 min. The solutions were vortexed and spun down. The labeling reactions were carried out in sealed glass vials with Teflon lined caps. After labeling, the ^{13}C-labeled mixtures were combined with their ^{12}C-labeled mixtures for LC−MS analysis. We note that the labeled sample was usually analyzed within 2 weeks. No degradation products for the labeled standards were observed after storing the labeled samples in −20 °C for up to 2 weeks.

LC−MS. The LC−MS setup for analyzing the labeled samples is similar to the one described elsewhere[5] and is briefly described in Supplemental Note N1 in the Supporting Information.

RESULTS AND DISCUSSION

Derivatization of carboxylic acids can be done with a variety of chemical reactions for analytical applications and, among them, phenacyl bromide (PBr) has been used to label the acids to improve the performance of HPLC and UV detection.[18] Our labeling chemistry is based on this reaction. However, to tailor our needs, we designed a new reagent that allows the introduction of a mass tag and concurrent improvement in LC−MS analysis. Figure 1 shows the structure of the reagent, *p*-dimethylaminophenacyl (DmPA) bromide, and the reaction scheme for labeling the carboxylic acid to form isotope mass-coded derivatives. Triethanolamine (TEOA) was used as a base catalyst for the reaction. The mass difference of the ^{13}C-/^{12}C-labeled products with one tag has a nominal mass of 2 Da.

We have also constructed a standard library of 113 carboxylic acid-containing metabolites (CAMs) by labeling individual compounds with DmPABr one-by-one (see Supplemental Table T1 in the Supporting Information). The reaction was found to be complete within 60 min, and the yield was in the range of 95−99%. Supplemental Table T2 in the Supporting Information shows the reproducibility and reaction yield of 10 standards; the average CV was 4.3% with a range from 2.2% to 7.2% for four repeated experiments. This labeling reaction has high specificity toward the acids. We tested a variety of compounds with different functional groups, such as alcohols, thiols, amides, amines, ketones, and aldehydes, and did not see reaction products. The reaction can be performed in an aprotic solvent such as acetonitrile, acetone, or *N*,*N*-dimethylformamide (DMF). A small amount of water (up to 20%) does not significantly affect the reaction yield. To quench the reaction, various acids were tested and it was found that triphenylacetic acid could be used to effectively consume the excess amount of DmPABr and the labeled product eluted often as the last peak in reversed-phase (RP) LC, thereby avoiding the

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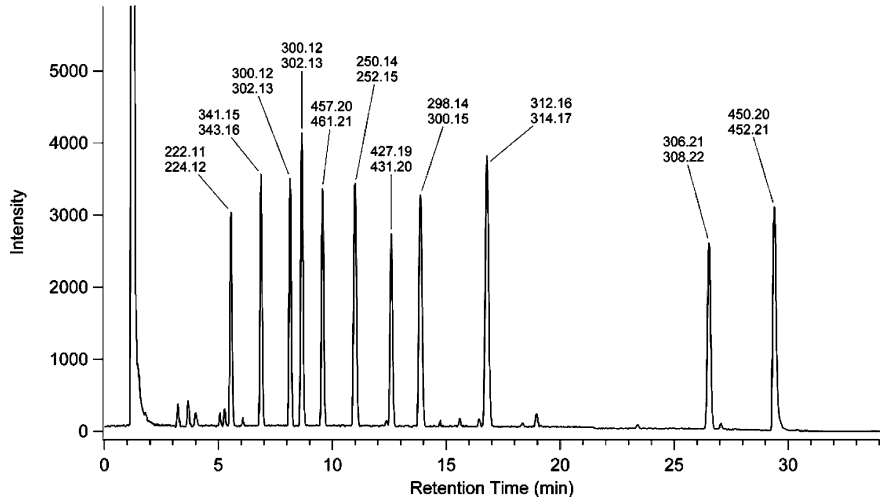

Figure 2. Base-peak ion chromatogram of a mixture of 10 carboxylic acid standards (6 pmol each injected). The peaks labeled with masses from left to right are (1) acetic acid, (2) hippuric acid, (3) 4-hydroxybenzoic acid, (4) 3-hydroxybenzoic acid, (5) malic acid, (6) butyric acid, (7) malonic acid, (8) phenylacetic acid, (9) hydrocinnamic acid, (10) octanoic acid, and (11) triphenylacetic acid (triphenylacetic acid was added to consume the remaining labeling reagent and quench the reaction). The standard mixture was labeled with either ^{13}C-DmPABr or ^{12}C-DmPABr, and a 1:1 mixture of the labeled compounds was analyzed by RPLC quadrupole time-of-flight mass spectrometry (QTOFMS).

interference of the analyte peaks from the quenching reagent. We note that the reaction product of the hydrophobic quenching reagent, triphenylacetic acid, has low solubility. The majority of the product remained as solid and would not be extracted to the sample and thus not injected into the column. This could avoid column overloading. Furthermore, derivatized triphenylacetic acid eluted when there was around 90−95% ACN in the mobile phase. Under this condition, the ionization efficiency of the derivatized triphenylacetic acid was low, and thus their signal intensity was low.

Figure 2 shows a base-peak ion chromatogram (BPC) of a mixture of 10 carboxylic acids labeled with ^{13}C-/^{12}C-DmPABr obtained by LC−MS using the QSTAR mass spectrometry (6 pmol each injected). Figure 3 shows the chromatograms of a human urine sample labeled with ^{13}C-/^{12}C-DmPABr (Figure 3A) and the same sample without labeling (Figure 3B). In each case, a sample amount of equivalent to 30 nL of urine was injected into a 2.1 mm RPLC column combined with the Bruker Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. The molecular region of a representative mass spectrum obtained from the labeled urine sample is shown in Figure 3C.

There are several important features shown in Figures 2 and 3 that demonstrate the merits of performing DmPA derivatization for acid analysis. First of all, DmPA derivatization improves the metabolite separation by RPLC. While many unlabeled acids are not retained on a RP column, the labeled analytes have sufficient interaction with the column to be separated by RPLC. For the standard mixture shown in Figure 2, except the last two acids, the unlabeled acids eluted out in the void or in a short retention time of less than 5 min (data not shown). For the unlabeled urine sample, most peaks were observed at or near the void volume (Figure 3B); many other acids expected to retain on the column were not observed due to low signal intensities (see below). However, the labeled acids were separated over the gradient elution time. The addition of the DmPA tag containing a hydrophobic aromatic ring to a polar and hydrophilic acid increases hydrophobicity, thereby enhancing its retention on a RP column.

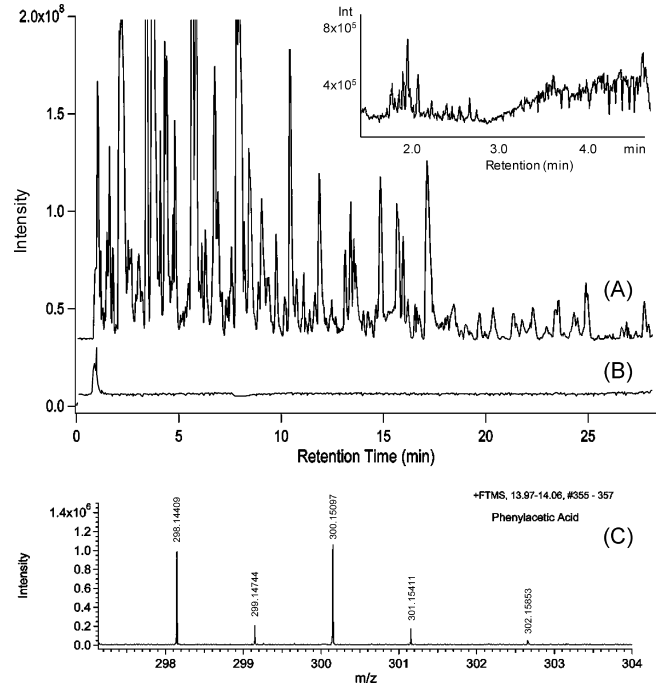

Figure 3. Base-peak ion chromatograms of (A) a labeled human urine sample obtained in positive ion mode and (B) a urine sample without labeling obtained in negative ion mode that was optimized for detecting carboxylic acids (the expanded view of the chromatogram at the earlier elution is shown in the inset). The urine sample was labeled with either ^{13}C-DmPABr or ^{12}C-DmPABr, and the labeled urine samples were mixed in 1:1. A sample with an amount equivalent to about 30 nL of the original urine was injected into RPLC FTMS for analysis. (C) The molecular ion region of an ESI mass spectrum displaying a pair of ^{13}C-/^{12}C-DmPABr labeled metabolite ions. Some of the metabolites tentatively identified and their retention times from part A are provided in Supplemental Table T3 in the Supporting Information.

The major advantage of using RPLC for separating the metabolites is that it has superior separation efficiency to other modes of separation such as hydrophilic interaction (HILIC) chromatogra-

phy. In addition, the use of one column, i.e., RPLC, instead of using different columns to handle the different polarity of the metabolites of the same sample, saves the analysis time.

Second, DmPA derivatization enhances the ESI efficiency significantly. As Figure 2 shows, 10 chromatographic peaks from the standards are observed with similar responses, despite large differences in chemical structures of these acids. The injection of the same amount of the mixture without derivatization did not produce many detectable signals, even in the optimized negative ion mode. For the 113 carboxylic acid standards, DmPA derivatization was found to enhance the detection sensitivity by about 2−4 orders of magnitude, depending on the compound structure (as an example, Supplemental Figure S1 in the Supporting Information shows the calibration curves of two standards before and after derivatization obtained by LC−FTICR-MS). The signal enhancement can be attributed to several factors. One is related to the increased propensity of being charged for the labeled acid due to the presence of the dimethyamine moiety attached to the aromatic ring of the tag where a tertiary amine can be readily formed. The labeled acid has higher hydrophobicity than its unlabeled counterpart, making it easier to stay on the surface of the droplets during ESI. An elution solvent with higher organic solvent content where a labeled acid is eluted out, as opposed to the unlabeled one eluted at void or high water content solvent, also enhances the ionization efficiency. Note that the first large peak eluted at or near the void volume in Figure 2 was mainly from the base catalyst, TEOA. The small peaks shown were most likely from the unknown impurities present in the standards that were also labeled with DmPA. The ESI signal enhancement is quite dramatic in the analysis of biological samples such as urine. As Figure 3A,B illustrates, only a few peaks are observed in the analysis of unlabeled urine (Figure 3B and the expanded chromatogram in the inset) while many peaks are detected from the labeled urine sample (Figure 3A).

The third advantage of DmPA derivatization is that a proper isotope mass tag can be readily attached to a carboxylic acid-containing metabolite, and the labeled metabolite does not display any isotope effect on RPLC. Co-elution of the analyte and its internal standard is a key to achieve accurate quantification by LC−MS. Otherwise, ion suppression can cause significant variation in detectability of analyte and standard, resulting in quantitative errors. The ^{13}C-/^{12}C-labeled metabolites coelute perfectly. The relative amount of two comparative samples, one labeled with ^{13}C-DmPA and another with ^{12}C-DmPA, can be determined directly from the peak ratio of the ion pair in the mass spectra (e.g., Figure 3C), not in the chromatogram. For example, a linear relation ($R^2 > 0.995$) was found in the LC−MS analysis of differential isotope labeled carboxylic acid standards, such as malic acid, where the amount ratios of ^{13}C-/^{12}C-labeling were 5:1, 1:1, 1:5, 1:10, and 1:20. The relative standard derivation (RSD) on the ratios of the ion pairs in replicate analysis was less than 6%, indicating that good reproducibility could also be achieved.

Finally, DmPA isotope labeling facilitates the identification of metabolite peaks among many spectral features observed in LC−MS (a feature refers to a mass spectral peak with a specific m/z at a given retention time). There are usually many peaks detected in an LC−MS run (e.g., for the labeled urine samples, over 15 000 features could be seen), and many of these peaks are from impurities present in the solvents, column, tubing, and interface, salts and adducts, electric noises, column coatings, etc. Identification of the true metabolite peaks can be done by analyzing a mixture of an equal amount of a sample differentially labeled with ^{13}C- and ^{12}C-DmPABr, such as the urine sample results shown in Figure 3A and Supporting Table T3 in the Supporting Information. Perfect coelution of the ion pairs and their characteristic mass difference of $2 \times n$ Da for metabolites with n tags or acidic groups with <2 ppm errors in mass difference can be used to identify the metabolite peaks. Other nonmetabolite peaks and unlabeled metabolites would show up as singlet peaks. For example, the ion pair at 298.144 09 and 300.150 97 shown in Figure 3C has a mass difference error of −0.53 ppm. The mass difference of 2.007 88 Da indicates this metabolite has one carboxylic acid. With the comparison of the retention time and accurate mass of the standards, the ion pair was identified to be from phenylacetic acid. While the use of ion pairing of differentially labeled samples to assist in distinguishing the metabolite peaks from the others is not unique to DmPA labeling, this labeling method does provide two additional benefits. One is related to the reduction of interference from low mass ions in LC−MS. The addition of a tag (162 Da) to a metabolite increases the molecular ion mass, effectively shifting away from the low mass region of the mass spectra where high background signals are usually observed at $m/z < 200$. Another benefit is related to the increased stability of the labeled metabolites where, in general, only the molecular ions are observed in the mass spectra. Dissociation of molecular ions of unlabeled metabolites in the interface and during ion translation to the mass analyzer can cause ambiguity in assigning the mass spectra peaks; a number of peaks observed may be from the fragment ions of the metabolites, not the intact metabolite ions. In triplicate experiments of the 1:1 ratio of ^{13}C-/^{12}C-DmPA labeled human urine, 2671, 2546, and 2820 ion pairs were detected (see the Supporting Information, Table T3, for the list). Using the 113 carboxylic acid standard library, we identified 51, 43, and 48 metabolites (see Table T3 in the Supporting Information). This example illustrates that a large number of metabolites can be profiled from human urine using the DmPA labeling LC−FTICR-MS method. Of course, positive identification of these ion pairs remains to be challenging. Future work on MS/MS analysis of these labeled ions may result in identifying more metabolites. We also note that in the case of the urine sample, we could not determine if there were any side reactions contributing to the overall number of peaks observed, as the identities of most of the peaks were unknown.

In summary, we have developed a new isotope labeling method for high-performance metabolome analysis with a focus on global profiling of carboxylic acid-containing metabolites. This labeling method is demonstrated to be not only effective in introducing an isotope tag for accurate metabolite quantification but also improving the chromatographic retention of the metabolites in RPLC, enhancing ESI efficiency by 2−4 orders of magnitude, and facilitating the identification of metabolite peaks in LC−MS. This method along with other high-performance labeling methods, such as dansylation, that are targeted at amine-,[5] phenol-,[5] and ketone/aldehyde-containing metabolites should cover the majority of the metabolome. We believe that high-performance isotope labeling

LC−MS should open the possibility of carrying out comprehensive metabolome profiling experiments on any biological sample, thereby increasing the power of metabolomics for investigating subtle changes in the metabolome for biological studies and disease biomarker discovery. Finally, we note that design of multiplex isotope labeling reagents based on a similar reaction scheme of DmPABr is possible and will be reported in the future.

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SUPPORTING INFORMATION AVAILABLE

Synthesis of the isotope-coded DmPABr reagents (Scheme S1), information on chemical reagents, reagent synthesis, and LC−MS conditions (Note N1), list of standard acids (Table T1), list of 10 standards showing labeling reaction reproducibility and yield (Table T2), list of ion pairs detected from the human urine samples and names of the positively identified metabolites by LC−FTMS (Table T3), and calibration curves of two standards (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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