Chapter 14

Chemical Isotope Labeling LC-MS for Human Blood Metabolome Analysis

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Abstract

Blood is a widely used biofluid in discovery metabolomic research to search for clinical metabolite biomarkers of diseases. Analyzing the entire human blood metabolome is a major analytical challenge, as blood, after being processed into serum or plasma, contains thousands of metabolites with diverse chemical and physical properties as well as a wide range of concentrations. We describe an enabling method based on high-performance chemical isotope labeling (CIL) liquid chromatography-mass spectrometry (LC-MS) for in-depth quantification of the metabolomic differences in comparative blood samples with high accuracy and precision.

Key words Chemical isotope labeling, Dansylation, DmPA, LC-MS, Metabolomics, Blood

1 Introduction

Blood samples are being extensively used for discovery metabolomics with the goal of finding sensitive and specific biomarkers that are indicative of healthy and diseased states. Despite great advances in blood metabolomic profiling techniques in the past decades, in-depth quantitative analysis of the blood metabolome is still a major analytical challenge. Because of diverse chemical and physical properties of blood metabolites, a conventional strategy of increasing the metabolomic coverage is to use several analytical tools with different metabolite detectability to analyze the same sample. For example, a widely used metabolomic profiling platform includes the use of reversed-phase (RP) liquid chromatography-mass spectrometry (LC-MS) to analyze the hydrophobic metabolites and hydrophilic interaction (HILIC) LC-MS to analyze the relatively polar metabolites. For each separation, positive and negative ion modes of MS detection are separately carried out in order to ionize as many metabolites as possible. This platform is relatively easy to implement. However, the overall metabolomic coverage from the combined LC-MS analyses is still limited. In addition, untargeted

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metabolomic analysis using LC-MS without standards provides limited quantification accuracy and precision due to matrix and ion suppression effects.

An alternative strategy of performing metabolomic analysis is to classify the metabolites into several subgroups based on the presence of common functional moieties and then perform in-depth analysis of the individual chemical-group-submetabolomes [1]. The combined data from the submetabolomes would allow the analysis of the entire metabolome with high coverage. This divide-and-conquer strategy does not require many analytical measurements of the same sample. For example, when we examined the chemical structures of the endogenous human metabolites in the Human Metabolome Database (HMDB), we found that more than 95% of these metabolites contain one or more of the four functional groups: amine, carboxyl, hydroxyl, and carbonyl. Thus, in principle, if we could analyze all the metabolites within these four submetabolomes, a near-complete metabolomic profile could be generated.

Our laboratory has been developing a high-performance chemical isotope labeling (CIL) LC-MS approach to analyze the individual chemical-group-submetabolomes with very high coverage [1–3]. The idea is to use a rationally designed chemical labeling reagent to react with a common functional group within a submetabolome (e.g., amines) to form metabolite derivatives, followed by MS detection. The properties of the labeled metabolites are altered, compared to the original unlabeled metabolites, to such an extent that all the labeled metabolites can be retained on RPLC for efficient separation without the need of switching to another mode of separation column, ionized with high efficiency to improve overall detection sensitivity, and detected in positive ion mode only to avoid the need of running negative ion mode. For accurate relative quantification, a differential isotope labeling strategy using heavy and light isotope reagents is used. To this end, we have reported the use of ^{12}C-/^{13}C-dansyl chloride (DnsCl) for profiling the amine/phenol submetabolome [1], ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide for profiling the carboxylic acid submetabolome [2], base-activated ^{12}C-/^{13}C-dansyl chloride (b-DnsCl) labeling for profiling the hydroxyl submetabolome [3], and ^{12}C-/^{13}C-dansyl hydrazine labeling for profiling the ketone/aldehyde submetabolome including many sugars [4]. Each labeling method generates a submetabolome profile with very high metabolic coverage. For example, dansyl labeling of the amine/phenol submetabolome detects over 2000 metabolites routinely from a plasma sample.

Figure 1 shows the high-performance CIL LC-MS workflow for quantitative metabolomics. A control sample is prepared by mixing small aliquots of individual samples to form a pool, followed by heavy (e.g., ^{13}C-dansyl) labeling. This ^{13}C-labeled control is

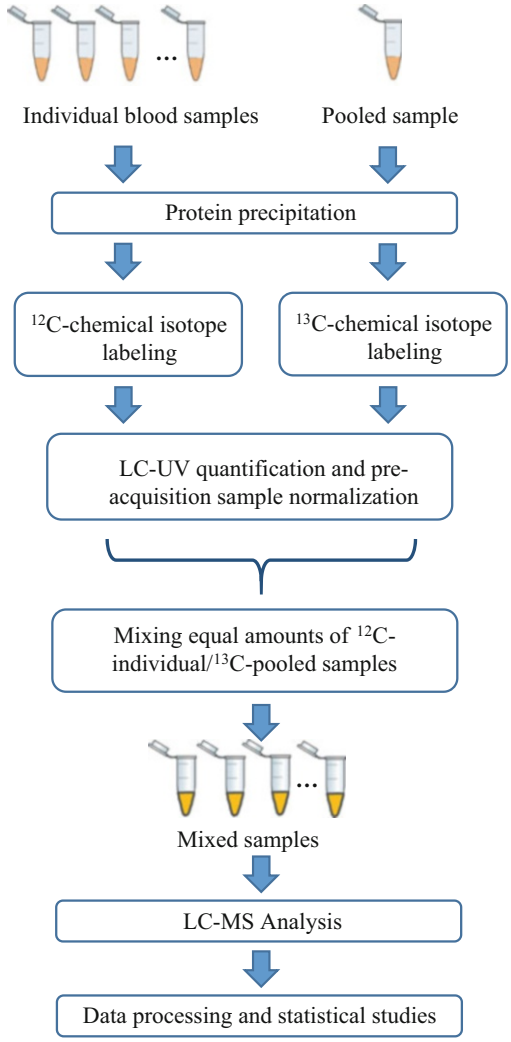

Fig. 1 CIL LC-MS workflow

spiked into all the light or ^{12}C-labeled individual samples and thus serves as a global internal standard [5]. The ^{12}C-labeled metabolite and its corresponding ^{13}C-labeled counterpart in a ^{12}C-/^{13}C-mixture are detected as a peak pair in MS, and their peak area ratio reflects the concentration difference of the metabolite in an individual sample vs. the control. Since the same ^{13}C-labeled control is used for preparing all individual ^{12}C-/^{13}C-mixtures, the peak ratio values can be used for relative quantification of individual metabolites in different samples. We have developed the required data processing software for analyzing the CIL LC-MS data [6]. In addition, over 650 metabolite standards have been individually labeled with the proper labeling reagents for positive metabolite identification [7]. In the library of standards, each labeled

metabolite contains triplet parameters: molecular mass, LC retention time, and MS/MS spectrum. Library search program has been developed for fully automated search for rapid metabolite identification [7]. For putative metabolite identification or structure match, accurate mass search is used to match the detected labeled metabolite masses to the metabolites in metabolome databases.

In this chapter, we describe the updated protocols for performing dansyl and DmPA labeling of metabolites and LC-MS analysis of labeled metabolites for human blood metabolomics.

2 Materials

All chemicals and reagents were purchased from Sigma-Aldrich Canada (Markham, ON, Canada) unless otherwise noted. The chemical isotope labeling reagents, including ^{12}C- and ^{13}C-dansyl chloride and ^{12}C- and ^{13}C-DmPA bromide, are available on http://mcid.chem.ualberta.ca. All solutions and LC mobile phases were prepared using LC-MS grade solvents (*see* **Note 1**), and the solutions were stored at room temperature. Blood samples were collected from participants with standard operating procedures after they reviewed and signed the informed consents. All human samples were processed in a Containment Level II laboratory and stored in a Level I or regular chemistry laboratory after being processed. The waste was disposed following the waste disposal regulations. All the studies involving human subject have been reviewed and approved by the University of Alberta Health Research Ethics Board.

2.1 Human Blood Sample Collection

1. We recommend BD Vacutainer 10 mL serum collection tubes (*see* **Note 2**).
2. We recommend VACUETTE 5 mL plasma collection tubes.

2.2 Dansylation Labeling

1. 250 mM $NaHCO_3/Na_2CO_3$ buffer solution: Weigh 26.5 g of anhydrous Na_2CO_3 and 21.0 g of anhydrous $NaHCO_3$ in a clean 1 L glass bottle. Measure 1 L of water in a 1 L volumetric flask, and transfer the water to the glass bottle to dissolve the solid (*see* **Note 3**).
2. 250 mM NaOH solution: Weigh 1.0 g of NaOH and transfer it to a Nalgene lab quality bottle. Measure 100 mL of water in 100 mL volumetric flask, and transfer the water to the plastic bottle. Dissolve the NaOH.
3. 425 mM formic acid solution: Dilute 1.60 mL of formic acid to 100 mL with (50/50, *v/v*) acetonitrile/water.
4. 20 mg/mL dansyl chloride solution: Before the chemical isotope labeling experiment, 20 mg/mL ^{12}C-dansyl chloride

(^{12}C-DnsCl) solution is prepared by dissolving 20 mg of ^{12}C-DnsCl in 1 mL of acetonitrile, and 20 mg/mL ^{13}C-dansyl chloride (^{13}C-DnsCl) solution is prepared by dissolving 20 mg of ^{13}C-DnsCl in 1 mL of acetonitrile.

2.3 DmPA Labeling

1. 0.5 M triethanolamine solution: Dissolve 7.46 g of triethanolamine in 100 mL of acetonitrile.
2. 10 mg/mL DmPA bromide solution: Before the chemical isotope labeling experiment, 10 mg/mL ^{12}C-DmPA bromide (^{12}C-DmPABr) solution is prepared by dissolving 10 mg of ^{12}C-DmPABr in 1 mL of acetonitrile, and 10 mg/mL ^{13}C-DmPA bromide (^{13}C-DmPABr) solution is prepared by dissolving 10 mg of ^{13}C-DmPABr in 1 mL of acetonitrile.

2.4 LC-MS Analysis

1. Mobile phase A: 1 mL of formic acid and 50 mL of acetonitrile are transferred into a 1 L volumetric flask and then diluted to 1 L with water.
2. Mobile phase B: 1 mL of formic acid is transferred into a 1 L volumetric flask and then diluted to 1 L with acetonitrile.
3. HPLC column: Agilent reversed-phase Eclipse plus C18 column (2.1 mm × 100 mm, 1.8 μm particle size, 95 Å pore size) for LC-MS analysis.

3 Methods

3.1 Human Serum Samples

Ten milliliters of venipuncture blood are collected into a BD Vacutainer 10 mL serum collection tube. An individual sample is allowed to clot spontaneously at room temperature for 1 h and then centrifuged at 1,500 × *g* for 15 min to separate serum and cells. The supernatant (serum) is divided into multiple 250 μL aliquots in 1.5 mL microcentrifuge tubes for analysis or storage in a −80 °C freezer. Also, a pooled serum sample is prepared by mixing equal volumes of individual serum samples. This pooled sample is processed with ^{13}C-labeling reagents to serve as the internal standard during the quantitative analyses (*see* **Note 4**).

3.2 Human Plasma Samples

Blood should be collected by venipuncture into a VACUETTE 5 mL plasma collection tube with lithium heparin (*see* **Note 5**). The tube is then inverted for ten times to ensure blood mixing with the inner tube coating material. The sample is left on the bench for 30 min and then centrifuged at 2,100 × *g* for 30 min at 4 °C. The plasma sample is divided into multiple 250 μL aliquots in 1.5 mL microcentrifuge tubes for analysis or storage in a −80 °C freezer. Also, a pooled plasma sample is prepared by mixing equal volumes of individual plasma samples.

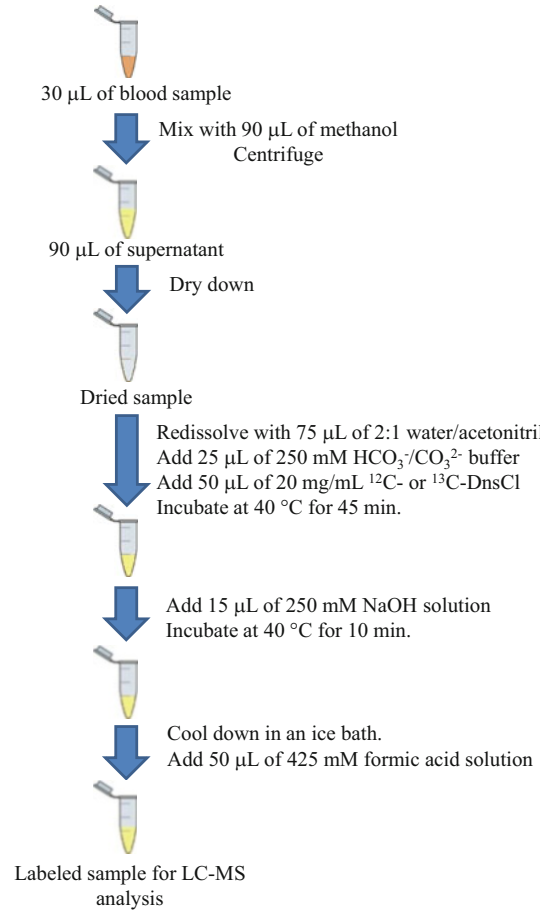

Fig. 2 Dansylation labeling scheme

3.3 Dansylation Labeling Reaction

The workflow of dansylation labeling is shown in Fig. 2.

1. Frozen serum or plasma samples are thawed (*see* **Note 6**) in an ice bath and then centrifuged at 15,000 × *g* for 15 min.
2. Thirty microliters of supernatant are transferred into a micro-centrifuge tube and mixed with 90 µL of methanol.
3. Store the mixture at −20 °C for 2 h to precipitate the proteins. After this, the mixture is centrifuged at 15,000 × *g* for 15 min.
4. Take 90 µL of supernatant and dry using a Speed-Vac centrifugal evaporator.
5. Redissolve to 75 µL with 2:1 water/ACN. Then add 25 µL of 250 mM sodium carbonate/sodium bicarbonate buffer to the sample to generate a basic environment for the dansylation reaction.
6. Vortex, spin down, and mix with 50 µL of freshly prepared ^{12}C-DnsCl solution (20 mg/mL) (for light labeling) or ^{13}C-DnsCl solution (20 mg/mL) (for heavy labeling).

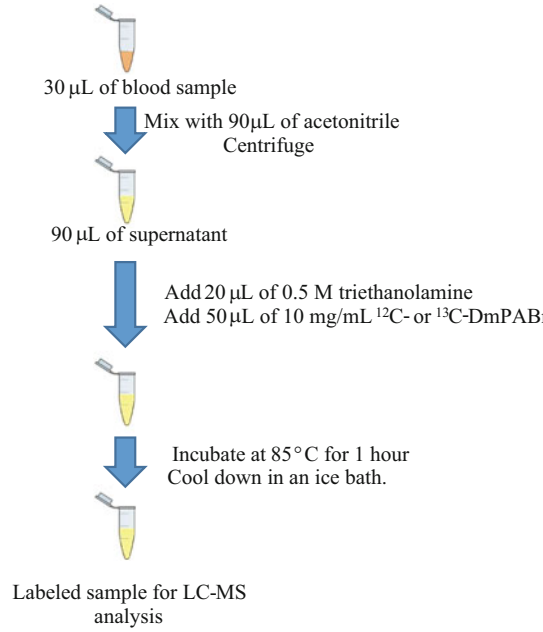

Fig. 3 DmPA labeling scheme

7. After 45 min incubation at 40 °C, 10 µL of 250 mM NaOH are added to the reaction mixture to quench the excess dansyl chloride. The solution is then incubated at 40 °C for another 10 min.
8. Finally, add formic acid (425 mM) in 1:1 ACN/H_2O to consume excess NaOH and to make the solution acidic. The labeled samples are now ready for LC-MS analysis or stored at a –80 °C freezer (*see* **Note 7**).

3.4 DmPA Labeling Reaction

The workflow is shown in Fig. 3.

1. Frozen serum or plasma samples are thawed in an ice bath and then centrifuged at 15,000 × *g* for 15 min.
2. Transfer 30 µL of supernatant into a microcentrifuge tube and mix with 90 µL of acetonitrile.
3. Store the mixture at −20 °C for 2 h to precipitate the proteins. After this, the mixture is centrifuged at 15,000 × *g* for 15 min.
4. Take 90 µL of supernatant, and mix with 20 µL of 0.5 M triethanolamine and 50 µL of freshly prepared ^{12}C-DmPA bromide solution (10 mg/mL) (for light labeling) or ^{13}C-DmPA bromide solution (10 mg/mL) (for heavy labeling).
5. Incubate the mixture at 85 °C for 1 h (*see* **Note 8**).
6. Finally, the samples are cooled down in an ice bath and are ready for LC-MS analysis. The labeled samples can also be stored in a –80 °C freezer for future use.

3.5 LC-UV Quantification and Pre-acquisition Sample Normalization

Variations in total sample amount in different samples must be minimized in order to detect the concentration differences of individual metabolites caused by the biological or clinical factors being studied. An LC-UV method [8] can be applied to determine the total concentration of dansylated amine/phenol submetabolome based on the UV absorption of the dansyl group (*see* **Note 9**).

1. For the LC-UV setup, a Waters ACQUITY UPLC system with a photodiode array (PDA) detector can be used for the quantification of dansyl-labeled metabolites for sample amount normalization.
2. Four microliters of the labeled serum or plasma are injected onto a Phenomenex Kinetex C18 column (2.1 mm × 5 cm, 1.7 μm particle size) for a fast step-gradient run.
3. Solvent A is 0.1% (v/v) formic acid in 5% (v/v) ACN/H_2O, and solvent B is 0.1% (v/v) formic acid in ACN. The gradient starts with 0% B for 1 min and is increased to 95% B within 0.01 min and held at 95% B for 1 min to ensure complete elution of all labeled metabolites. The flow rate used is 0.45 mL/min.
4. The peak area, which can represent the total labeled metabolite concentration in the sample, is integrated using the Empower software (6.00.2154.003).
5. Based on the quantification results, the ^{12}C- and ^{13}C-labeled samples are mixed in equal mole amounts for LC-MS analysis. When a ^{12}C-labeled individual sample is mixed with the ^{13}C-labeled pooled sample, each peak pair ratio in the LC-MS result represents the relative concentration of one specific metabolite in the individual sample, with respect to the average concentration of the sample set.

3.6 LC-FTICR-MS

1. The LC-Fourier transform ion cyclotron resonance (FTICR)-MS analysis is performed using an Agilent 1100 series binary system (Agilent, Palo Alto, CA) connected to a 9.4 T Apex-Qe FTICR-MS (Bruker, Billerica, MA). The MS data are acquired in the positive ion mode with an electrospray ionization (ESI) source. An Agilent reversed-phase Eclipse plus C18 column (2.1 mm × 100 mm, 1.8 μm particle size, 95 Å pore size) is used for chromatographic separation (*see* **Note 10**). The mobile phase A is 0.1% formic acid in ACN/H_2O (5/95, v/v), and the mobile phase B is 0.1% formic acid in ACN.
2. All dansyl-labeled samples are analyzed with a 32-min gradient: 0 min (20% B), 0–3.5 min (20–35% B), 3.5–18 min (45–65% B), 18–21 min (65–95% B), 21–24 min (95–99% B), and 24–32 min (99% B). The column is re-equilibrated with the initial mobile phase conditions for 15 min before injecting the next sample. The flow rate is 180 μL/min, and the injection volume is 5.0 μL.

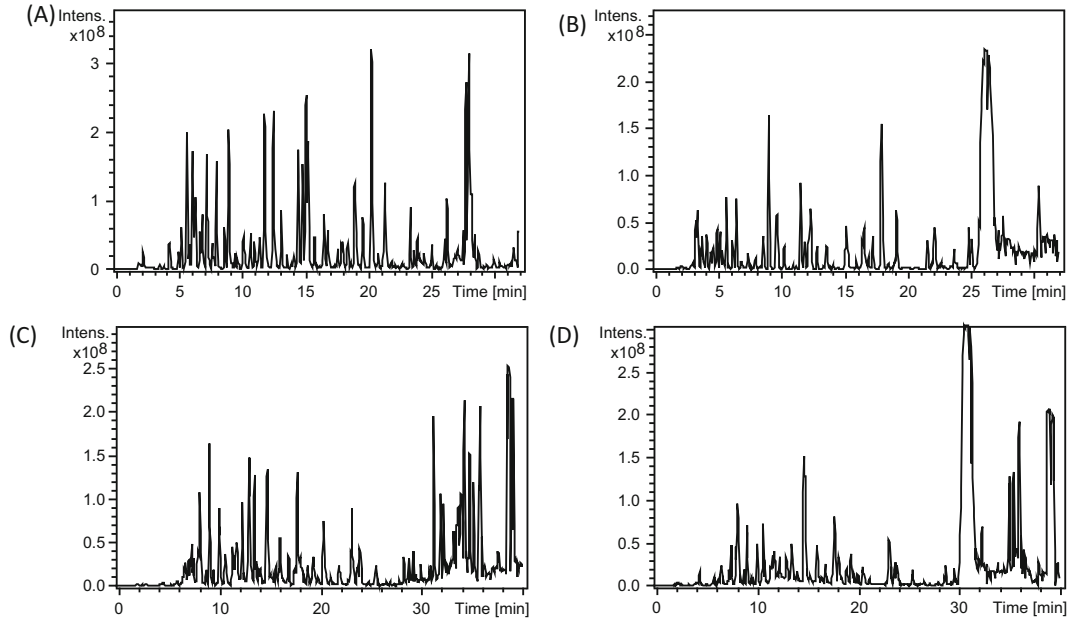

Fig. 4 Representative LC-MS chromatograms from (**a**) a dansyl-labeled human serum sample, (**b**) a dansyl-labeled human plasma sample, (**c**) a DmPA-labeled human serum sample, and (**d**) a DmPA-labeled human plasma sample (acquired by LC-FTICR-MS)

3. The 40-min gradient for all DmPA-labeled samples is 0 min (20% B), 0–9 min (20–50% B), 9–22 min (50–65% B), 22–26 min (65–80% B), 26–28 min (80–98% B), and 28–40 min (98% B). The column is re-equilibrated with the initial mobile phase conditions for 15 min before injecting the next sample. The flow rate is 180 μL/min, and the injection volume is 2.5 μL.
4. The MS conditions used for FTICR-MS are as follows: nitrogen nebulizer gas, 2.3 L/min; dry gas flow, 7.0 L/min; dry temperature, 190 °C; capillary voltage, 4,200 V; spray shield, 3,700 V; acquisition size, 256 k; scan range, 200–1100; and ion accumulation time, 1 s.
5. Figure 4 shows the representative base peak chromatograms of (A) a dansyl-labeled serum, (B) a dansyl-labeled plasma, (C) a DmPA-labeled serum, and (D) a DmPA-labeled plasma. In Fig. 5, the peak pair of dansyl-serine is given as an example for a representative mass spectrum. The peak with m/z of 339.0957 is the ^{12}C-dansyl-labeled serine from the individual sample, and the peak with m/z of 341.1032 is the ^{13}C-dansyl-labeled serine from the pooled sample.

3.7 LC-QTOF-MS

1. For LC-quadrupole time-of-flight (QTOF)-MS, an Agilent 1100 series binary system (Agilent, Palo Alto, CA) and an Agilent reversed-phase Eclipse plus C18 column (2.1 mm × 100 mm,

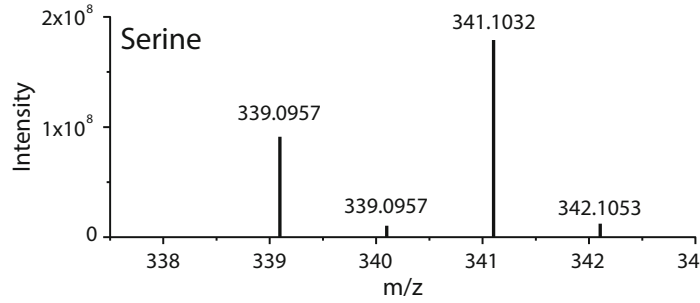

Fig. 5 Molecular ion region of a peak pair (labeled serine)

1.8 μm particle size, 95 Å pore size) are used. The flow is loaded to the electrospray ionization (ESI) source of a Bruker maXis impact high-resolution QTOF mass spectrometer (Bruker, Billerica, MA). All MS spectra are obtained in the positive ion mode.

2. LC solvent A is 0.1% (v/v) formic acid in 5% (v/v) ACN/H_2O, and solvent B is 0.1% (v/v) formic acid in ACN. The gradient elution profile for dansyl-labeled samples is as follows: 0 min (20% B), 0–3.5 min (20–35% B), 3.5–18 min (45–65% B), 18–21 min (65–95% B), 21–24 min (95–99% B), and 24–32 min (99% B). The column is re-equilibrated with the initial mobile phase conditions for 15 min before injecting the next sample. The flow rate is 180 μL/min and the injection volume is 10.0 μL.
3. The 40-min gradient for running the DmPA-labeled samples is: 0 min (20% B), 0–9 min (20–50% B), 9–22 min (50–65% B), 22–26 min (65–80% B), 26–28 min (80–98% B), and 28–40 min (98% B). The column is re-equilibrated with the initial mobile phase conditions for 15 min before injecting the next sample. The flow rate is 180 μL/min and the injection volume is 5.0 μL.
4. The MS conditions used for QTOF-MS are as follows: end plate offset, 500 V; capillary voltage, 4,500 V; nebulizer, 1.0 bar; dry gas, 8.0 L/min; dry temperature, 230 °C; transfer time, 40 μs; and prepulse storage, 10 μs.

3.8 Data Processing and Statistical Analysis

The data processing workflow is shown in Fig. 6 (*see* **Note 11**). A software tool, IsoMS, is used to process the raw data generated from multiple LC-MS runs by peak picking, peak pairing, peak-pair filtering, and peak-pair intensity ratio calculation [6].

1. Align peak pairs detected from multiple samples using IsoMS-Align.
2. Fill missing ratio values using the zero-fill program [9].

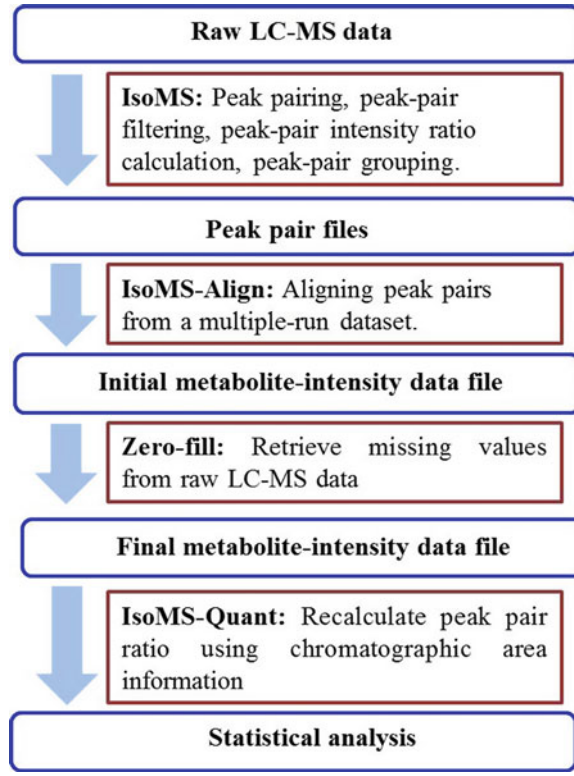

Fig. 6 IsoMS data processing workflow

3. Finally, use IsoMS-Quant to determine the chromatography-peak-intensity ratio of a ^{12}C-/^{13}C-pair [10]. The final metabolite relative concentration file can be exported to SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden) for multivariate statistical analysis (*see* **Note 12**).

3.9 Metabolite Identification

Positive metabolite identification is performed based on mass and retention time matching to a labeled standard library [3, 7]. Putative identification is done based on accurate mass matches to the metabolites in the Human Metabolome Database (HMDB) (8,021 known human endogenous metabolites) and in the evidence-based metabolome library (EML) (375,809 predicted human metabolites with 1 reaction) using MyCompoundID (www.MyCompoundID.org) [11]. The mass accuracy tolerance window is set at 10 ppm for database search.

4 Notes

1. HPLC grade organic solvents or purified deionized water are found to be adequate for preparing solutions for dansylation.

2. Serum tubes without clot activators are recommended for better reproducibility of the analysis results.
3. Ultra-sonication may be needed to dissolve the solid.
4. Normally the pooled sample is made by mixing equal mole amounts of all the individual samples from the sample set being studied. However, if some of the samples are in very limited amount, they can be skipped from contributing to the pooled sample; the pooled sample works as an internal reference for relative quantification, and thus not including a few samples within a group in the pooled sample does not affect the results. In addition, a preprepared universal serum/plasma sample can also be used as the internal standard, which enables inter-study comparisons.
5. EDTA plasma and citrate plasma can also be used for the CIL LC-MS analysis without significant matrix effect.
6. Samples used in one study should experience the same number of freeze-thaw cycles. This rule also applies to the samples after labeling.
7. The labeled samples can be stored at 4 °C for 1 week without significant degradation. However, −80 °C freezer is recommended for long-term storage.
8. The leftover amount of unreacted DmPA is very small, and thus it does not affect the metabolomic profiling of blood. If it interferes with the detection of a specific compound with similar retention time, triphenylacetic acid can be used to quench the excess amount of DmPA.
9. To reduce the effect of real samples on the lifetime of a C18 column, all samples should be centrifuged at 15,000 × *g* for 10 min before analysis. A pre-column filter should also be helpful, particularly for running blood samples.
10. Generally, the standard deviation of the distribution of total amine-/phenol-containing metabolite amounts among human blood samples is less than 15%. If a variance of as large as 50% is allowed in a specific study, post-acquisition normalization methods can be used, instead of the pre-acquisition normalization. Nevertheless, the LC-UV quantification is recommended for profiling blood samples for increased accuracy.
11. The data processing packages, as well as metabolite identification libraries, are available on the MyCompoundID website: http://www.mycompoundid.org.
12. Other metabolomic statistical tools, such as MetaboAnalyst, can also be used for the analysis.

Acknowledgment

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