2-HYDRAZINOQUINOLINE AS A NOVEL DERIVATIZATION AGENT FOR LC-MS-BASED METABOLOMIC INVESTIGATION OF KETOACIDOSIS IN STREPTOZOTOCIN-ELICITED DIABETES

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ABSTRACT

Short-chain carboxylic acids, aldehydes, and ketones are important intermediates and end products of many metabolic processes. Their levels in biofluids and tissues can reflect the status of specific metabolic reactions, the homeostasis of whole metabolic system, and the well-being of a biological entity. Traditionally, GC-MS has been widely used for analyzing carboxylic acids, aldehydes, and ketones in biological samples after chemical derivatization. However, due to poor compatibility of common GC columns with water, the derivatization process in GC-MS is often complicated and time consuming, especially for the metabolites in biofluids and tissues. Recently, new chemical derivatization techniques have been developed to enhance the sensitivity and performance of LC-MS for analyzing these metabolites. In this study, the use of 2-hydrazinoquinoline (HQ) as a novel derivatization agent for LC-MS analysis of carboxylic acids, aldehydes, and ketones in biological samples was explored, and the conditions for the derivatization reaction were optimized. The metabolites in urine, serum, and tissue extracts can be conveniently derivatized in a 60-min process. The formation of carboxylic acid derivatives is attributed to the esterification reaction between HQ and carboxyl group, while the production of aldehyde and ketone derivatives is through the formation of Schiff bases between HQ and carbonyl group. Compared to other known hydrazine derivatization agents, including 2-hydrazinopyridine, 2-picolylamine and dansyl hydrazine, HQ can react with a broader spectrum of intermediary metabolites in biological samples, and can achieve better chromatographic performance in reversed

phase LC system and higher ionization efficiency in electrospray source. Using this HQ-based approach, the metabolic disorder induced by streptozotocin-elicited diabetes was examined by the LC-MS-based metabolomics. The results showed the time-dependent separation of mouse urine samples from STZ treatment in a multivariate model of urinary metabolites. Both known and novel small-molecule biomarkers associated with STZ-induced ketoacidosis were conveniently identified and subsequently elucidated, reflecting the dramatic changes in nutrient (glucose, amino acid, and lipid) and energy metabolism after STZ treatment. Overall, HQ derivatization of carboxylic acids, aldehydes, and ketones can be an effective platform for the LC-MS-based metabolomic investigation of endogenous metabolism.

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Literature Review

[1] **Key Words:** Ketones, aldehydes, carboxylic acids; metabolite, metabolomics; chemical derivatization, GC-MS, LC-MS

Abbreviations: HBA, 3-hydroxybutyric acid; 4-ONE, 4-oxononenal; MDA, malondialdehyde; HNE, 4-hydroxynonenal; TCA, tricarboxylic acid cycle; PUFAs, Polyunsaturated fatty acids; DHA, docosahexaenoic acid; SCFAs, short-chain fatty acids; MSUD, maple syrup urine disease; BCAAs, branched chain amino acids; ADH, alcohol dehydrogenase; CYP2E1, cytochrome P-450 2E1; ALDH, aldehyde dehydrogenase; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; RPLC, reversed-phase liquid chromatography; NMR, Nuclear magnetic resonance spectroscopy; HPLC, high performance liquid chromatography; UPLC, ultra-performance liquid chromatography; UHPLC, ultra-high pressure liquid chromatography; EI, electron-impact; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; MDA, multivariate data analysis; RT, retention time; PCA, principle component analysis; PLS, partial least squares; OPLS, orthogonal partial least squares; PLS-DA, partial least squares-discriminant analysis; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PPARα, peroxisome proliferator-activated receptor α.

1.1 OVERVIEW OF SHORT-CHAIN KETONE, ALDEHYDE, CARBOXYLIC ACID METABOLITES.

Short-chain ketones, aldehydes, and carboxylic acids mainly refer to the metabolites that contain carbonyl (-C=O) and/or carboxyl (-COOH) groups but have no more than six carbon atoms in their structures (**Table 1.1**). These metabolites serve important functions in many biochemical processes inside the body and cells, especially in nutrient and energy metabolism, since they are substrates and products of numerous enzymatic reactions, intermediate metabolites in both anabolic and catabolic metabolism, building blocks for complex biomolecules, and causes of metabolic disorders and oxidative stress.

1.1.1 Ketone metabolites

Ketone metabolites are organic compounds with the structure RC(=O)R', where R and R' represent carbon-containing substituents (**Table 1.1**). Compared to alkyl groups, the carbonyl group in ketones is more polar due to the electronegativity of oxygen atom. This chemical property makes ketones more reactive than aliphatic hydrocarbons and more prone to various non-enzymatic and enzymatic reactions, such as the formation of hemiacetals and Schiff bases, as well as oxidation, reduction, and transamination reactions.

The term "ketone bodies" is widely used to describe acetone, acetoacetate, and 3-hydroxybutyric acid (HBA), the three metabolites generated from ketogenesis. Among them, acetone and acetoacetate are real ketones, while HBA is actually a carboxylic acid. Acetoacetate is the intermediate metabolite in ketogenesis, from which acetone is generated through decarboxylation reaction catalyzed by acetoacetate decarboxylase while HBA is produced by the NADH-dependent reduction catalyzed by HBA dehydrogenase (*1*). When the availability of glucose is restricted, the ketone bodies become an important sources of metabolic energy (*2*). The metabolism of ketone bodies interfaces with many metabolic pathways such as tricarboxylic acid cycle, fatty acid β-oxidation, lipogenesis, sterol biosynthesis, glucose metabolism, and mitochondrial electron transport chain (*3*). For example, acetoacetate found to have a metabolic role in regulating the branched-chain amino acids decarboxylation in skeletal muscle (*4*). Therefore, abnormal levels of ketone bodies are the biomarkers of metabolic diseases (*5*). Besides acetone and acetoacetate, many metabolites, such as fructose, carry the keto carbonyl group. Keto acids and keto aldehydes are two important groups of bioactive ketone metabolites. Transamination of amino acids is a major source of keto acid metabolites, while keto aldehydes, such as 4-oxononenal (4-ONE), can be produced through lipid peroxidation (*6*).

1.1.2 Aldehydes metabolites

Aldehyde metabolites are organic compounds containing a formyl group, with the structure R-CHO (**Table 1.1**). Similar to ketones, aldehydes are also prone to various chemical and metabolic reactions. Because the carbonyl group in aldehydes is at the end of carbon chain, in general, aldehydes are more reactive than their ketone isomers due to the lack of steric hindrance. The reactivity to DNA makes some aldehydes potential carcinogens, while the formation of aldehyde-protein adducts has negative impacts on normal functions of affected proteins. Therefore, the detection and measuring of DNA and protein adducts of reactive aldehydes have been widely used to monitor the toxic effects of chemical exposure or diseases (*7, 8*). Among numerous aldehyde metabolites in the body that are originated from exogenous and endogenous sources, glucose, formaldehyde, acetaldehyde, malondialdehyde (MDA), and 4-hydroxynonenal (HNE) are the most well-known ones. Low-level formaldehyde is commonly produced as a metabolite in demethylation reactions, while acetaldehyde is a bioactive metabolite in alcohol metabolism and tobacco consumption, responsible for several adverse effects of alcohol intoxication (*9, 10*). MDA and HNE are generated as the secondary products of free radical-triggered lipid peroxidation (*11-13*). Functioning as signaling molecules and cytotoxic species, MDA, HNE and related reactive aldehydes have been shown to play important roles in the pathogenesis of multiple chronic diseases, including diabetes (*14*). Besides the toxic effects associated with reactive aldehydes, aldehyde-containing metabolites, especially semialdehyde species, are also important intermediates in intermediary metabolism. For example, succinate semialdehyde is involved in the

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formation of succinate via succinic semialdehyde dehydrogenase. Its deficiency leads to increased amounts of 2,4-dihydroxybutyric acid, and its lactone, as well as 3-hydroxypropionic acid, which are related to the alpha-oxidation of 4-hydroxybutyric acid (*15*). Aspartate semialdehyde is involved in both the lysine biosynthesis and homoserine biosynthesis pathways. Aspartate β-semialdehyde dehydrogenase plays an important role in an essential aspartic biosynthetic pathway found in bacteria, fungi and the higher plants (*16*).

1.1.3 Carboxylic acid metabolites

Carboxylic acid metabolites, which are commonly called organic acids, are organic compounds with single or multiple -COOH groups (**Table 1.1**). Compared to the carbonyl group in ketones and aldehydes, the carboxyl group in carboxylic acid is much less reactive to DNA and proteins. Esterification, amidation, and decarboxylation are the common metabolic reactions on this functional group. Carboxylic acid metabolites encompass a large number of metabolites originating from carbohydrate, lipid, and amino acid metabolism. For example, oxaloacetate, citrate, malate, fumarate, succinate, and α-ketoglutarate are indispensable components of tricarboxylic acid cycle (TCA cycle); pyruvate and lactate are the end products of glycolysis; α-keto acids (as described in **1.1.2**), such as α-ketoglutarate, α-ketoisovalerate, α-ketoisocaporate, are important intermediates in amino acid metabolism; short-chain fatty acids, including acetic acid,

propionic acid, and butyric acid, are a source of energy and precursors of other intermediary metabolites. Therefore, the levels of specific carboxylic acids in biofluids, including urine and blood, are commonly used as the indicators or biomarkers of normal metabolic events or metabolic disorders in carbohydrate, lipid and amino acid metabolism (*17-20*). In clinical practice, many inherited metabolic disorders caused by the genetic mutations in specific metabolizing enzymes are diagnosed as organic acidurias, such as methyl malonic acidurias, propionic aciduria, isovaleric aciduria, maple syrup urine disease, and glutaric aciduria Type 1 (*21*). In addition, the levels of carboxylic acids can be used to determine the disease development. For instance, plasma HBA and lactate levels are highly elevated in diabetes (*17, 18*).

1.2 METABOLIC EVENTS AFECTING THE LEVELS OF SHORT-CHAIN KETONE, ALDEHYDE, CARBOXYLIC ACID METABOLITES

Short-chain ketone, aldehyde, and carboxylic acid metabolites in the body come from dietary sources and various endogenous metabolic processes. The driving force in the biosynthesis of these metabolites is oxidative metabolism, which is not only responsible for energy transfer and release in carbohydrate, fatty acid, and amino acid metabolism, but also contributes to the production of reactive species through non-enzymatic processes, like lipid peroxidation. During these metabolic processes, the metabolites in more reduced forms, such as alkanes, alkenes, alcohols, are converted to more oxidized

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forms through diverse biotransformation reactions, including hydration, dehydrogenation, and oxidation. Aldehydes and ketones are formed as metabolic intermediates, while carboxylic acids are commonly produced as the end products of oxidative pathways. The levels of aldehyde, ketone, and carboxylic acid metabolites in cells, tissues, and biofluids are highly regulated. Dysfunction and dysregulation of metabolic processes can lead to abnormal levels of these metabolites. Therefore, monitoring the levels and distribution pattern of these metabolites can reflect the metabolic status and homeostasis of a metabolic system.

1.2.1 Ketones, aldehydes, and carboxylic acids from carbohydrate and energy metabolism

Based on the degree of polymerization, carbohydrates can be divided into monosaccharides, oligosaccharides, and polysaccharides (*22*). The basic structural units of carbohydrates are polyhydroxy aldehydes, such as glucose, or polyhydroxy ketones, such as fructose (*23*). Besides functioning as structural components of glycoproteins, glycolipids, and nucleotides, carbohydrates, especially glucose, play an important role in energy metabolism. Aerobic and anaerobic metabolisms of glucose, including glycolysis, gluconeogenesis, glycogenesis, glycogenolysis, pentose phosphate pathway, and TCA cycle, are indispensable components of energy metabolism in cells, responsible for releasing and storing energy as well as synthesizing the cofactors required for these

activities (*24-27*). During these metabolic processes, diverse ketones, aldehydes, and carboxylic acids are generated. For instance, in glycolysis, glyceraldehyde 3-phosphate, phosphoglycerates, and phosphoenolpyruvate are the intermediate metabolites, while pyruvate and lactate are the end products. Under aerobic conditions, pyruvate is further oxidized to generate acetyl-CoA, which can enter TCA cycle. In TCA cycle, citric acid functions as the recipient of acetyl-CoA from glycolysis and other metabolic pathways. The energy from acetyl-CoA was gradually transferred into high-energy electron carriers, NADH and $FADH_2$, through the formation of carboxylic acid intermediates, including isocitrate, α-ketoglutarate, succinate, fumarate, malate, and oxaloacetate (*28*).

1.2.2 Ketones, aldehydes, and carboxylic acids from fatty acid metabolism

Dependent on the length of aliphatic chain, fatty acids can be classified as short-chain (less than 6 carbons), medium-chain, long-chain fatty acids, which can be saturated or contain single or multiple double bonds (*29*). Long-chain fatty acids, including palmitic acid, stearic acid, oleic acid, linoleic acid, and arachidonic acid, mainly function as energy storage and structural components of complex lipid species. Besides dietary intake, the levels of long-chain fatty acids are greatly affected by fatty acid oxidation and fatty acid biosynthesis. In these metabolic processes, a series of 3-hydroxyacyl-CoA and 3-ketoacyl-CoA metabolites are formed as the intermediates, which can be further hydrolyzed to become 3-hydroxy fatty acids or 3-keto fatty acids, such as ketone bodies

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(acetoacetate and HBA). Short-chain fatty acids (SCFAs), including acetic acid, propionic acid, and butyric acid, belongs to short-chain carboxylic acids. Besides produced by nutrient (fatty acid and glucose) metabolism, SCFAs can also come from microfloral metabolism in the digestive tract. The microbial production of SCFAs is a minor source of energy in non-ruminants, but it is the major source of energy in ruminants (*30*). Therefore, the levels and composition of SCFAs and their metabolites can reflect the metabolic activities and composition of microbiota in gut.

Polyunsaturated fatty acids (PUFAs) are an important source of reactive aldehydes and ketones due to their susceptibility to lipid peroxidation. PUFAs with at least three double bonds, such as arachidonic acid and docosahexaenoic acid (DHA), could be cleaved to form MDA (*31*). 4-Hydroxynonenal (HNE), one of the most bioactive lipid peroxidation product, is generated from the peroxidation of ω-6 PUFAs, such as linoleic acid (*32*). Many other aldehydes are also formed during lipid peroxidation, including saturated, α, β-unsaturated alkenals such as acrolein (*33*), 4-hydroxy-alkenals, 4-hydroperoxy-alkenals, 4-oxo-alkenals, and epoxy-α, β-unsaturated (*34, 35*). These lipid peroxidation products can lead to the changes in membrane permeability and fluidity, as well as the formation of DNA and protein adducts, resulting in the loss of cellular functions and integrity (*36*).

1.2.3 Ketones, aldehydes, and carboxylic acids from amino acid metabolism

Owing to their structural diversity and complex metabolic pathways, amino acids are the precursors of diverse ketones, aldehydes, and carboxylic acids. Transamination and deamination, two common reactions in amino acid metabolism, readily convert specific amino acid to its corresponding α-keto acid and deaminated carboxylic acid, respectively. In addition, based on their metabolic fates, amino acids can be classified as ketogenic, glucogenic, or both. Along with ketogenic or glucogenic biotransformation of amino acids into ketone bodies or glucose, various ketone, aldehyde, carboxylic acid intermediates and end products are generated. Many of these metabolites have important metabolic functions. For example, amino acids are the major sources of organic acids in TCA cycle through anaplerotic metabolism (*37*). Therefore, the disruption of amino acid metabolism commonly results in severe metabolic consequences, such as maple syrup urine disease (MSUD). The deficiency of branched-chain α-keto acid dehydrogenase in MSUD prevents the effective utilization of branched chain amino acids (BCAAs), leading to the toxic accumulation of leucine, isoleucine, valine and their corresponding α-keto acids (*38*).

1.2.4 Aldehydes and carboxylic acids from ethanol metabolism

The main route of ethanol metabolism is a three-step process (ethanol → acetaldehyde → acetic acid → acetyl-CoA), representing the typical oxidative biotransformation from an alcohol to a corresponding carboxylic acid. The formation of acetaldehyde from ethanol

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are catalyzed by alcohol dehydrogenase (ADH), cytochrome P-450 2E1 (CYP2E1), and catalase, while acetaldehyde is then oxidized to acetic acid by aldehyde dehydrogenase (ALDH) (*39*). Acetaldehyde is a highly reactive and unstable metabolite. By forming free radicals or reacting with DNA and proteins, acetaldehyde can cause genotoxicity and disrupt normal protein function (*40*). In addition, high level of acetic acid after ethanol overdose can also cause severe complications such as alcoholic myopathy (*41*).

The formation of acetaldehyde and acetic acid is the direct influence of ethanol metabolism on the levels of aldehydes and carboxylic acids in the body. Indirect influences of ethanol on ketones, aldehydes, and carboxylic acids are achieved through ethanol's negative effects on fatty acid, amino acid, and carbohydrate metabolism (*42-44*). Acute alcohol intake can impair hepatic amino acids uptake, decrease leucine oxidation, increase serum BCAAs, and decrease lipoprotein and albumin biosynthesis, while chronic alcohol consumption correlates with impaired liver protein secretion and heart and gastrointestinal tract protein catabolism (*42*). Ketoacidosis can be induced by long-term alcohol abuse. For example, alcoholic lactic acidosis is contributed by the elevation of $NADH/NAD^+$ ratio, which promotes the formation of lactate from pyruvate, and decreases gluconeogenesis and lactate uptake in the liver (*45*).

1.2.5 Carboxylic acids from microfloral metabolism

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Gut microbiota affects energy balance in the host through their own metabolic activities, including energy harvesting, storage and expenditure (*30*), and can contribute to metabolic disorders such as obesity and diabetes (*46, 47*). It has been reported that obese people have higher fecal concentration of SCFAs than overweight and normal people (*48*). Besides producing SCFAs, (discussed in **1.2.2**), gut microbiota can also generate various distinctive organic acids, among them, valerate, caporate, isobutyrate, 2-methylbutyrate, and isovalerate are produced by the microfloral metabolism of BCAAs (*49*). Hippuric acid is formed through the glycine conjugation of benzoic acid generated from microfloral metabolism (*50*). Phenylpropionic acid can be produced by anaerobic gut flora (*51*). P-cresol is generated by microfloral metabolism of dietary polyphenols or tyrosine (*52*). Hydroxyphenylpropionate is originated from dietary catechins and caffeic acid while 3,4-dihydroxyphenylpropionate is produced from dietary quinolones by various clostridial species (*53, 54*). Therefore, the levels of these microfloral metabolites can be used to monitor the status of gut microbiome. For example, benzoic acid, along with phenylacetate, *p*-hydroxybenzoate, and *p*-hydroxyphenylacetate found to be elevated in the patients who had intestinal bacterial overgrowth (*55*) while the excretion of hippuric acid was reduced in Crohn's disease patients who have diminished microfloral colonies (*56*).

1.3. ANALYSIS OF SHORT-CHAIN KETONES, ALDEHYDES, CARBOXYLIC ACIDS

Analyzing short-chain ketone, aldehyde, and carboxylic acid metabolites in biological samples is essential for understanding the mechanisms behind the homeostasis or disruption of biological system, but is often challenging due to several facts: (1) inherent low concentrations of many metabolites, (2) complex biological matrices causing interference and (3) limited availability of adequate analytical methods. To overcome these challenges, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have been widely used to analyze these metabolites.

1.3.1 GC-MS analysis of ketones, aldehydes, and carboxylic acids

GC-MS analysis of ketones, aldehydes and carboxylic acids has been broadly applied for investigating metabolic events in humans and animals (*57*), examining food components (*58*), evaluating environment pollutants (*59*), and identifying bacteria metabolites (*60*). For example, as a metabolite biomarker of alcoholic ketoacidosis, HBA in blood and urine has been analyzed by GC-MS following liquid-liquid extraction and silyl derivatization (*61*). Similarly, acetoin catabolism in *Bacillus pumilus* was also determined by GC-MS after the bacterial extract is purified by extraction and condensation (*60*).

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Furthermore, GC-MS has been widely used to detect the products from BCAA degradation and from ketogenesis, such as hydroxycarboxylic and oxocarboxylic acids in urine, after chemical derivatization to methyl esters and pre-fractionation by thin-layer chromatography (*62*). Compared to other analytical platforms, such as HPLC-UV, GC-MS provides higher resolution in chromatography, shorter analyzing time, and lower detection limit.

A prerequisite for conducting GC-based analysis is that the interested compounds or their derivatives have to be volatile and also thermally stable under the temperature gradient in GC runs. Because ketone, aldehyde, and carboxylic acid metabolites in biological samples exist in aqueous matrix and are often nonvolatile, extensive processing is usually required to make them compatible with GC-MS analysis. The sample preparation often includes pH adjustment, extraction, evaporation, and derivatization. Within these processes, evaporation aims to remove water from samples since GC columns are incompatible with water, while derivatization yields the products that can be effectively separated by GC gradient and sufficiently ionized for MS analysis. For instance, a common two-step procedure in derivatizing urinary metabolites for GC-MS analysis includes oximation and silylation (*63*). Oximation derivatizes carbonyl group in ketones and aldehydes through the reaction with hydroxylamine, while silylation derivatizes carboxylic acids, alcohols, and amines.

1.3.2 LC-MS analysis of ketones, aldehydes, and carboxylic acids

LC-MS has been adopted for analyzing aldehydes, ketones, and carboxylic acids in many scientific fields, including environment evaluation (*64*), disease research (*65*), and nutrient metabolism (*66, 67*). The advantages of LC-MS analysis include high sensitivity, high selectivity and simple sample preparation procedures. However, LC-MS analysis of ketones, aldehydes, and carboxylic acids also faces some obstacles, mainly in chromatographic separation and ionization efficiency. For example, because of their polar chemical structures, many ketone, aldehyde, and carboxylic acid metabolites are highly hydrophilic, leading to poor chromatographic performance in reversed-phase liquid chromatography (RPLC) and co-elution with the mobile phase containing high percent of water. Subsequently, the ionization efficiency is usually suboptimal due to these matrix effects and background interference. To overcome these challenges, chemical derivatization methods are adopted to enhance the detection sensitivity and chromatographic separation of ketones, aldehydes and carboxylic acids in LC-MS analysis (detailed in **2.1**).

1.4. METABOLOMICS

Together with genomics, transcriptomics, and proteomics, metabolomics is an indispensable component of systems biology (*62*). Examination of metabolic phenotypes in metabolomic analysis not only reveals the status of metabolic system under disease, drug treatment, nutrition intervention, and environmental challenge, but also provides a

starting point to explore the changes in proteins (especially metabolizing enzymes) and genes (*68*). Therefore, metabolomics has extensive applications in various scientific fields, including human nutrition (*69*), drug metabolism (*70*), clinical research (*71*) and environmental toxicology (*72*). Metabolomics aims to determine and characterize the global profile of endogenous and exogenous metabolites in the metabolome of a biological system. Since short-chain ketones, aldehydes, and carboxylic acids are important components of small-molecule metabolome, effective analysis of these metabolites is highly desired in metabolomic analysis (*73*).

1.4.1 Analytical platform of Metabolomics

Various analytical methodologies have been adopted for metabolomics. Nuclear magnetic resonance spectroscopy (NMR), GC-MS, and LC-MS are the most widely used instrumental systems for metabolomic analysis of low-molecular weight metabolites in biological samples (*74-78*). Compared to MS, NMR is non-destructive in nature and capable of providing more structural information, but less sensitive for detecting low-abundance metabolites. Another advantage of NMR is that chromatographic separation is not required for NMR analysis. In contrast, majority of MS-based metabolomics requires a separation process prior to sample introduction and mass detection in MS system. GC-MS is an excellent platform for the metabolites that are volatile or could become volatile through derivatization (detailed in **1.3.1**). However, the strength of GC-MS in separating

volatile chemicals also limits its application in analyzing other classes of chemicals in biological samples that are non-volatile or difficult to be converted into volatile derivatives. Therefore, LC is more commonly used in MS-based metabolomics owing to its good compatibility with the metabolites in biological system. High performance liquid chromatography (HPLC) used to be the predominant LC technology used in chemical analysis. However, development of ultra-performance liquid chromatography (UPLC) or ultra-high pressure liquid chromatography (UHPLC) that uses smaller particles, faster flow rate, and higher pressure than HPLC has greatly improved the chromatographic resolution and reduced the running time in the LC system (*79*). After eluting from GC or LC, analytes need to become ionized before they can be detected by mass detectors in MS-based chemical analysis. Electron-impact (EI) ionization, a hard-ionization method, is widely used in GC-MS analysis to establish the fragmentation pattern of derivatized analytes. In contract, soft-ionization methods, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), are commonly used in LC-MS analysis to reduce the fragmentation in the ionization source and facilitate the detection of parent molecules since additional fragmentation of parent molecules can be achieved inside the mass detector. For mass detection, the selection of mass detector is largely based on the nature of MS-based metabolomics. In general, triple-quadrupole and ion-trap mass spectrometers are better platforms for quantitative analysis in targeted metabolomic analysis owing to their sensitivity (*80*), while time-of-flight, Orbitrap, or Fourier transform ion cyclotron resonance mass spectrometers are more suitable for untargeted

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metabolomic analysis because of their high resolution to acquire accurate mass for elemental composition analysis and their high capacity to detect multiple ions simultaneously for comprehensive metabolite profiling (*81-83*).

1.4.2 Chemometrics methods for data analysis

To define the metabolome and the metabolic changes within it, data acquired from NMR, GC-MS or LC-MS measurements need to be processed prior to chemometric analysis. Using LC-MS-based metabolomics as an example, the procedures to process chromatographic and spectral data from LC-MS analysis include data condensation and reduction by centroiding and deisotoping mass spectra; chromatographic alignment to reduce the variation in retention time; filtering to remove noise or background signals; and peak recognition and collection by setting threshold windows for mass (*m/z*) and retention time (RT). Multivariate dataset comprised of sample identities, ion identities (*m/z* and RT), and relative ion intensities can be constructed and used in subsequent multivariate data analysis (MDA) (*84*). MDA includes unsupervised and supervised MDA. Most commonly used unsupervised MDA method is principal components analysis (PCA), which is capable of presenting an indiscriminate overview of the data acquired, the grouping of analyzed samples, as well as identifying outliers (*85*). Supervised MDA includes partial least squares (PLS), orthogonal partial least squares (OPLS), and partial least squares-discriminant analysis (PLS-DA). These analyses are

efficient in identifying metabolites contributing to the separation of sample groups, such as control versus treatment, or normal versus disease, in the multivariate models (*86*).

1.4.3 Application of metabolomics in scientific research

Metabolomics is capable of identifying novel metabolites of xenobiotics, elucidating biotransformation pathways of xenobiotics, monitoring metabolic changes in a biological system, and investigating endogenous metabolism and disease mechanisms. Dependent on experimental design and aims, metabolomics-based research can be categorized as exploratory or hypothesis-driven research.

1.4.3.1 Metabolomics-based exploratory research

Identification of interested metabolites and biomarkers in complex biomatrix is the most common application of metabolomics. For example, to examine the biotransformation of xenobiotics *in vivo*, a straightforward approach is to conduct metabolomics comparison of the control and the treatment groups through analyzing biological samples include urine, serum, feces and tissue extracts. Since xenobiotic and its metabolites only appear in the samples from the xenobiotic treatment, the separation of the untreated controls from the treatment group in the multivariate model is expected to be contributed by

xenobiotic and its metabolites (**Fig. 1.2**). Using this approach, novel metabolites of therapeutic agents and toxicants have been identified (*87-89*). Besides identifying xenobiotic metabolites, similar approach has been used to define the changes in endogenous metabolism through metabolomic comparisons of sample groups under different challenges and health status.

1.4.3.2 Metabolomics-based hypothesis-driven research

Since the metabolic changes observed by metabolomics, including the formation of xenobiotic metabolites and the changes in endogenous metabolites, are contributed and regulated by enzymatic activities, protein and gene expression, metabolomics-guided investigation could provide mechanistic information on which biochemical pathways are potentially involved in observed metabolic changes (**Fig. 1.2).** The roles of metabolizing enzymes in biotransformation can be examined using this hypothesis-driven approach when appropriate experiment models are used, such as transgenic mice containing modified metabolizing enzymes (*75*). For instance, the role of CYP1A2 enzyme in the biotransformation of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP), a common procarcinogen in the human diet, was examined by a metabolomics comparison of urine samples from the wild-type, Cyp1a2-null, and CYP1A2-humanized mice treated with PhIP (*76*). Genotype-dependent separation of three groups of PhIP-treated animals demonstrated the importance of CYP1A2 in PhIP metabolism, and also revealed the

interspecies differences between human and mouse as well as the potential role of other cytochrome P450 enzymes in PhIP metabolism. Besides characterizing the roles of metabolizing enzymes in metabolic events, metabolomics analysis could also guide the protein and gene analysis. In a recent LC-MS-based lipidomics study of cocaine-induced liver injury, the progression of hepatotoxicity in a 3-day cocaine treatment was closely associated with the disruption of serum lipidome since the time-dependent separation of serum samples in a MDA model reflected the contribution of different lipid species on each day of cocaine exposure (*90*). Guided by this lipidomic model and MS-based structural elucidation, the accumulation of long-chain acylcarnitines was defined as a prominent cocaine-induced metabolic change. Because of the importance of long-chain acylcarnitines in mitochondrial fatty acid catabolism, this observation led to the identification of cocaine-induced inhibition of fatty acid oxidation in the liver. The relevance of this observation to cocaine-induced hepatotoxicity was further validated by cotreatment with fenofibrate, which activated peroxisome proliferator-activated receptor α (PPARα), a central regulator of fatty acid oxidation, and protected the mice against toxicity. Furthermore, LC-MS-based lipidomics revealed that cotreatment with the PPARα ligand reversed cocaine-induced changes in the lipidome.

Table 1.1. Representative ketone, aldehyde and carboxylic acid metabolites. The metabolites enlisted in this table are discussed in **1.1**.

Ketones	Aldehydes	Carboxylic acids
Acetone	Acetaldehyde	Acetic acid
Acetoacetic acid	Malondialdehyde	Malic acid
α-ketoglutaric acid	4-Hydroxynonenal	Oxaloacetic acid
4-oxononenal	Succinate semialdehyde	Citric acid

Figure 1.1. The work flow of LC-MS-based metabolomics. Biological samples such as urine, blood, tissue, and cell pellet need to be processed appropriately to make them compatible with LC-MS-based metabolomic analysis. Chemical derivatization can be performed to enhance the detection sensitivity. Chromatographic and spectral data are acquired by high-resolution LC-MS. Subsequent data processing, such as centroiding, deisotoping, filtering, peak recognition, yields a data matrix containing information on sample identity, ion identity (RT and *m/z*) and ion abundance. With appropriate data transformation and scaling, a multivariate model can be established through unsupervised or supervised MDA. The scores plot illustrates the principal or latent components of the model and sample classification, while the loadings plot presents the contribution of each ion to each principal component of the MDA model.

| Sample collection | Urine | Blood | Tissue | Cell pellet |

| Sample preparation | *Derivatization* |

| LC-MS analysis | LC column | MS system |

| Data acquisition | Chromatogram | Spectrum |

| Data analysis | |

| Metabolomics-guided mechanistic investigation |
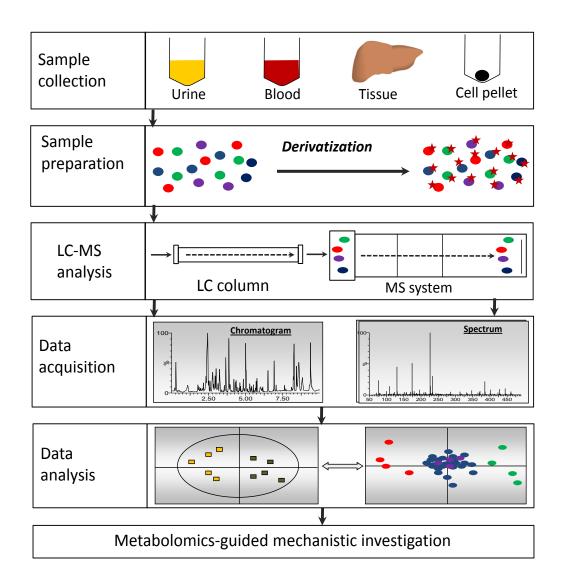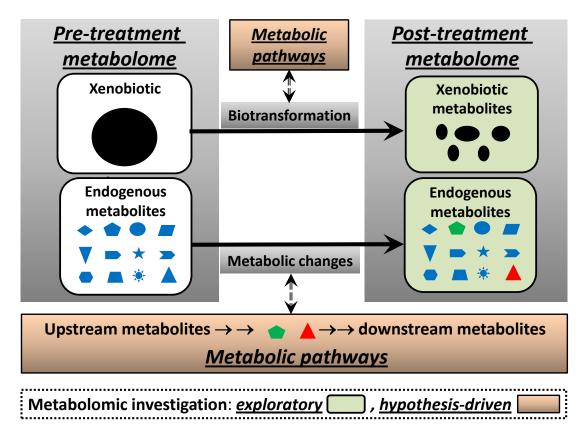
Figure 1.2. Exploratory and hypothesis-driven metabolomics. Utilization of metabolomics in exploratory and hypothesis-driven investigation is exemplified by the studies on xenobiotic-related metabolic events. Metabolic events in xenobiotic treatment include xenobiotic biotransformation and xenobiotic-induced metabolic changes. Metabolomics is not only able to identify the metabolites generated or affected by these events (exploratory investigation), but also capable of revealing the mechanisms underlying these events when combined with other experimental models and biochemical analyses on upstream/ downstream metabolic reactions and regulatory pathways (hypothesis-driven investigation)

Chapter 2

DEVELOPMENT OF 2-HYDRAZINOQUINOLINE AS A DERIVATIZATION AGENT FOR LC-MS ANALYSIS OF SHORT-CHAIN KETONES, ALDEHYDES, AND CARBOXYLIC ACIDS

2.1 INTRODUCTION

Short-chain polar ketones, aldehydes, and carboxylic acids, are important biochemicals inside the body and cells, especially in nutrient and energy metabolism (detailed in **1.1** and **1.2**). Monitoring their levels *in vivo* or *in situ* is essential for understanding the mechanisms behind the homeostasis or disruption of biological system (*63, 91, 92*). However, analyzing these metabolites can be challenging due to various factors, including the interferences of complex biological matrices, inherent low concentrations in samples, and limitation of analytical methods.

Gas chromatography-mass spectrometry (GC-MS) has been a major analytical platform for detecting polar ketones, aldehydes, carboxylic acids, and many other endogenous metabolites in the past four decades because of its high resolution and sensitivity as well as the availability of comprehensive compound library for structural identification (*93*). Since most metabolites in biological samples are nonvolatile or semi-volatile, chemical derivatization is generally required for making targeted metabolites volatile and compatible with the GC system. Among many derivatization methods for GC analysis, a tandem oximation-silylation procedure has been widely adopted in practice, in which oximation of aldehydes and ketones is commonly achieved by the reactions between carbonyl group and methoxyamine while the reactions with silylation agents replaces the active hydrogen in alcohols, carboxylic acids, amines, and thiols (*94, 95*). In general, derivatization yields more stable products that have better chromatographic performance

than precursor metabolites. However, one disadvantage of GC-based analysis is the general incompatibility of GC system with water (*96*), which nevertheless is the basic solvent of unprocessed biological samples. Therefore, dehydration process through solvent extraction, evaporation, or lyophilization is commonly performed before derivatizing biological samples (*97-99*), making sample preparation in GC-MS analysis more time-consuming and less efficient, and also potentially leading to the loss of volatile metabolites, such as acetone, in samples.

Compared to GC-MS, liquid chromatography-mass spectrometry (LC-MS) is more compatible with aqueous matrix of biological samples. However, direct LC-MS analysis of short-chain polar carboxylic acids, ketones, and aldehydes without derivatization is still hindered by poor chromatographic performance and low ionization efficiency. Reverse phase LC column, as the most commonly used LC column, is not capable of retaining and separating these hydrophilic metabolites effectively, leading to poor or no signal in mass detector. Recent development of hydrophilic interaction liquid chromatography (HILIC) has improved the chromatographic separation of polar compounds for MS analysis (*100*). However, the instability of reactive carbonyl metabolites, such as acetoacetate and oxaloacetate, in the LC system and ion source, still prevents reliable detection and measurement of these metabolites (*101*). To overcome the abovementioned challenges, utilization of chemical derivatization agents offers an alternative solution to enhance chromatographic performance, stability, and detectability of carboxylic acids, ketones, and aldehydes in LC-MS system (*102*). The considerations

in the selection of derivatizing agent for LC-MS analysis include the reactivity of functional groups in targeted metabolites, the chromatographic and ionization performance of derivatizing agent, and the technical properties of LC-MS system (*103*). Esterification and amidation are two common types of derivatization reactions for carboxylic acids. For example, 2-picolylamine (PA) and 2-hydrazinopyridine (HP) have been used to improve the ionization efficiency of poorly ionized carboxylic acids in biological samples by poor ionization by forming amide and acyl hydrazide derivatives, respectively (*104*). Other derivatization reagents for carboxylic acids include 2-chloro-1-methylpyridinium (*105*), triethylamine (*106*), and heptadecafluoroundecylamine (*107*). As for aldehydes and ketones, the reactions with hydroxylamine (*108*) and hydrazines, such as 2,4-dinitrophenylhydrazine (DNPH) (*109*) and dansyl hydrazine (*110*), have been used extensively to produce the derivatives that are more stable and prone to ionization than the parent compounds. Other derivatization agents for aldehydes and ketones include (4-hydrazino-4-oxobutyl) [tris(2,4,6-trimethoxyphenyl] phosphonium bromide (*111*), Girard reagent P (*112*), Girard reagent T (*113*), 1-phenyl-3-methyl-5-pyrazolone (*114*), and o-phenylenediamine (*115*). Even though a large selection of LC-MS derivatization agents are available for specific reactions with carbonyl group in ketones and aldehydes or specific reactions with carboxylic acids, few of them is capable of effectively reacting with aldehydes, ketones, and carboxylic acids for simultaneous analysis of these three classes of metabolites. In this study, 2-hydrazinoquinoline (HQ) was developed as an

efficient derivatization agent for simultaneous LC-MS analysis of aldehydes, ketones and carboxylic acids in biological samples.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

HQ, 3-hydroxylbutyric acid (HBA), butyric acid, fumaric acid, propionic acid, mannose, acetoin, α-ketoglutaric acid, and triphenylphosphine (TPP) were purchased from Alfa Aesar (Ward Hill, MA). Acetone, acetaldehyde, 2-picolylamine (PA), acetic acid, succinic acid, formaldehyde, 4-hydroxyphenylpyruvic acid, and deuterated acetic acid (2H4-acetic acid) were purchased from Sigma-Aldrich (St. Louis, MO). 2, 2'-Dipyridyl disulfide (DPDS), dansyl hydrazine (DH), pyruvic acid, and acetoacetic acid were purchased from MP Biomedicals (Santa Ana, CA). 2-Hydrazinopyridine (HP), malic acid, 4-hydroxyphenylacetic acid, sodium citrate, LC-MS-grade water and acetonitrile were purchased from Fisher Scientific (Houston, TX).

2.2.2 Animal treatment and sample collection

Male C57BL/6 mice (10 to 12-week old) from Charles River Laboratories (Wilmington, MA) were used in this study. All animals were maintained in a University of Minnesota (UMN) animal facility under a standard 12 h light/12 h dark cycle with food and water *ad libitum*. Handling and treatment procedures were in accordance with animal study

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protocols approved by the UMN Institutional Animal Care and Use Committee. Serum samples were collected by submandibular bleeding. Urine samples were collected by housing mice in the metabolic cages for 24 hours. Liver and other tissue samples were harvested after animals were euthanized by carbon dioxide. All urine and tissue samples were stored at -80°C before further analysis.

2.2.3 Preparation of aqueous liver extraction

Aqueous fraction of liver tissue was prepared using Bligh's method (*116*). Liver tissue was homogenized in methanol. After adding chloroform and water, the extraction solution was vortexed and then centrifuged at 18,000 × g for 10 min to separate lipid and aqueous phase. The aqueous phase of liver extract was transferred to a 1.5-mL tube and stored at -80oC before further analysis.

2.2.4 Derivatization of test solution and biological samples using PA, HP, HQ, and DH as derivatizing agents

The test solution contains 500 µM acetic acid, 50 µM HBA, 2 mM malic acid, 200 µM acetaldehyde, 1 mM acetone, and 400 µM pyruvic acid. Biological samples include urine, serum and liver extracts. Acetonitrile was used as the solvent for the stock solutions of PA, HP, HQ, DH, DPDS, and TPP. To compare the performance of four derivatization agents, 5 µL of test solution was added into a 100 µL of freshly-prepared acetonitrile solution containing 1 mM DPDS, 1 mM TPP and 1 mM derivatization agent (PA, HP, HQ, or DH) , and 10 µM 2H4-acetic acid (internal standard). The reaction mixture was

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incubated at 60°C for 15 min, chilled on ice, and then mixed with 100 µL of H2O. After centrifugation at 18,000 × g for 10 min, the supernatant was transferred into a HPLC vial for LC-MS analysis.

2.2.5 Optimization of HQ derivatization reaction conditions

To determine the kinetics of HQ derivatization reactions, 5 µL of six-compound test solution was added into a 100 µL of freshly-prepared acetonitrile solution containing 1 mM DPDS, 1 mM TPP and 1 mM HQ. The reaction mixture was incubated at 60°C for 5, 15, 30, 45, 60, or 75 min. The optimal reaction temperature was defined by conducting the same HQ derivatization reactions at 25, 37, 50, 60, or 75°C for 60 min. The HQ derivatives in the reaction mixture were analyzed by LC-MS.

2.2.6 Liquid chromatography-mass spectrometry (LC-MS) analysis of HQ derivatization products

The processed reaction mixture from chemical derivatization of standards or biological samples was injected into a Waters Acquity^{TM} ultra-performance liquid chromatography (UPLC) system (Milford, MA) and separated by a gradient of mobile phase ranging from 0.05% aqueous acetic acid containing 2 mM ammonium acetate to 95% aqueous acetonitrile containing 0.05% acetic acid and 2 mM ammonium acetate over a 10-min run. LC elute was introduced into a Waters SYNAPT QTOF mass spectrometer (QTOF-MS) for MS analysis. Capillary voltage and cone voltage were maintained at 3.2 kV and 30 V, respectively, in positive electrospray ionization (ESI). Source temperature and

desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (700 L/h), and argon as the collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ([M+H]$^+$ = 556.2771 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroid format. Additional structural information was obtained by tandem MS (MS/MS) fragmentation with collision energies ranging from 15 to 40 eV.

2.2.7 Chemometric analysis of LC-MS data from urine, serum, and liver extracts

Chromatographic and spectral data from HQ derivatization of urine, serum and liver extract samples were deconvoluted by MarkerLynxTM software (Waters). A multivariate data matrix containing information on sample identity, ion identity (RT and m/z) and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition and integration. To avoid the influences of unreacted derivatization agents on chemometric analysis, the ions from these chemicals were excluded from the data matrix. The intensity of each ion was calculated by normalizing the single ion counts (SIC) versus the total ion counts (TIC) in the whole chromatogram. The data matrix was further exported into SIMCA-P+^{TM} software (Umetrics, Kinnelon, NJ), and transformed by mean-centering and Pareto scaling, a technique that increases the importance of low abundance ions without significant amplification of noise. Principal components analysis (PCA) was adopted to model the data from LC-MS analysis of HQ-derivatized biological

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samples (*117*). Major latent variables in the data matrix were described in a scores scatter plot of multivariate model. Metabolites were identified by analyzing ions contributing to the principal components and to the separation of sample groups in the loadings scatter plot. The chemical identities of interested metabolites were determined by accurate mass measurement, elemental composition analysis, MS/MS fragmentation, and comparisons with authentic standards if available.

2.3 RESULTS

2.3.1 Identification of 2-hydroxyquinoline as an effective derivatizing agent for LC-MS detection of short-chain carboxylic acids, aldehydes, and ketones

In an effort to derivatize short-chain carboxylic acids in urine for LC-MS analysis, we used 2-hydrazinopyridine (HP), an established derivatization reagent (*104*), to react with carboxylic acids, but observed poor retention of derivatization products in regular reversed-phase C18 column (data not shown). Subsequently, HQ (**Fig. 2.1A**), an analog of HP with greater hydrophobicity, was chosen in order to prolong the retention of derivatized carboxylic acids in reversed-phase LC (RPLC) system. The initial observations from LC-MS analysis of HQ-derived urine samples indicated that HQ derivatization not only improved both chromatographic performance and ionization efficiency of short-chain carboxylic acid derivatives, but also generated various derivatives from aldehydes and ketones (data not shown). To further confirm this

observation and explore the application of HQ in LC-MS-based metabolite profiling and metabolomic analysis, the reactivities of HQ, HP, 2-picolylamine (PA), and dansyl hydrazine (DH) with a mixture of test compounds were compared under a common reaction condition (*104, 118*). The selection of PA, HP, and DH were based on their known applications in derivatizing carboxylic acids (PA and HP), aldehydes and ketones (DH) (*104, 118*). Six test compounds, including acetic acid, 3-hydroxybutyrate, malic acid, acetaldehyde, acetone, and pyruvic acid, represent monocarboxylic acids, dicarboxylic acids, hydroxyl acids, keto acids, aldehydes, and ketones, which are commonly produced in energy and nutrient metabolism. The performances of these derivatization agents were evaluated based on: 1) their capacity to effectively react with six test compounds in the mixture; 2) chromatographic separation of their derivatives by reversed-phase C18 LC column; 3) signal intensity of their derivatives in mass spectrometer. The results showed that HQ derivatization facilitated the separation and detection of all six test compounds under a common condition for LC-MS analysis, including aqueous acetonitrile mobile phases, reverse-phase C18 column, and positive ESI mode (**Fig. 2.1B** and **Table 2.1**). In contrast, HP and PA only reacted with carboxylic acids, including acetic acid, HBA, malate, and pyruvic acid, while DH with carbonyl compounds in this mixture, including acetaldehyde, acetone, and pyruvic acid (**Table 2.1 and Fig. 2.2**). Owing to the presence of both carbonyl and carboxylic acid moieties in its structure, pyruvic acid is the only compound that has reacted with all four derivatization agents. Overall, the results from LC-MS analysis clearly indicated that HQ had a better

performance than three other agents in detecting all six test compounds under defined experimental conditions.

2.3.2 Reaction mechanisms of HQ-mediated derivatization of carboxylic acids, aldehydes, and ketones

Analysis of MS/MS fragmentograms of these HQ derivatives (**Fig. 2.1C-G**) suggested that the derivatization of carboxylic acids was through the formation of hydrazines, while the derivatization of aldehyde and ketones is through forming hydrazone, the hydrazine Schiff base. In the reaction mixture, DPDS and TPP function as the activation agent to convert carboxylic acid to acyloxyphosphonium ion before it can react with HQ to form hydrazide bond (**Scheme 2.1**). As for HQ derivatization of aldehydes and ketones, DPDS and TPP are not needed since the terminal hydrazinyl nitrogen in HQ, a strong nucleophile, directly attack the carbonyl carbon in aldehydes and ketones, leading to dehydration and the formation of C=N bond in hydrazone (**Scheme 2.2**).

2.3.3 Optimization of HQ-mediated derivatization reaction conditions

The influences of solvent, temperature, and reaction time on HQ derivatization were examined to optimize the reaction conditions. Acetonitrile was selected as the solvent of derivatizing agents and reactions after observing much more effective derivatization

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reactions in acetonitrile than the ones in methanol, ethanol, and water (data not shown). The optimal reaction time was determined by monitoring the kinetics of HQ derivatization reactions ranging from 5 min to 90 min. Among six test compounds, the derivatization of acetic acid, HBA, acetone, and pyruvate was almost complete within the first 15 min of the reactions, while the derivatization of malic acid and acetaldehyde continued within the first 60 min (**Fig. 2.3A**). Therefore, 60 min was selected as the reaction time for the following HQ derivatization reactions. Furthermore, $60^{o}C$ was chosen as the optimal reaction temperature for six test compounds after comparing the reaction rate at 25, 37, 50, 60, and $75^{o}C$ (**Fig. 2.3B**).

2.3.4 Application of HQ derivatization in examining carboxylic acids, aldehydes and ketones in biological samples.

Results from the proof-of-concept experiments revealed that HQ can derivatize a mixture of carboxylic acids, aldehydes, and ketones dissolved in water (**Fig. 2.1** and **Fig. 2.3**). To further test whether HQ could function as an effective derivatization agent for simultaneous detection of carboxylic acids, aldehydes, and ketones in biological samples, urine, serum, and liver extract samples from the wild-type mice were derivatized by HQ and then analyzed by LC-MS-based metabolomics. Comparable signals from spiked deuterated acetic acid (internal standard) were observed after LC-MS analysis of HQ-derivatized urine, serum and liver samples, indicating that different matrices of these

biological samples did not significantly affect HQ derivatization. Through the principal components analysis (PCA) of LC-MS, HQ-derivatized urine, serum, and liver extract samples were distinctively separated in a two-component model (**Fig. 2.4A**). Through accurate mass measurement, elemental composition analysis, MS/MS fragmentation analysis, and chromatographic and spectroscopic comparisons with standards, diverse ketones, aldehydes and carboxylic acid metabolites were identified, and their different distribution in urine, serum, and liver, determined the classification of three types of biological samples in the metabolomic analysis (**Fig. 2.4B**). Major ketone, aldehyde, and carboxylic acid metabolites in urine, serum, and liver extracts, are listed in **Table 2.2**, covering diverse intermediates and end products in energy and nutrient metabolism. Since urine, serum, and liver are widely used for examining the metabolic status of human and animals, the result from this metabolomic analysis suggested that HQ derivatization method is well suitable for analyzing short-chain ketones, aldehydes, and carboxylic acids in biological samples.

2.4 DISCUSSION

To meet the needs for targeted analysis, majority of chemical derivatization agents for LC-MS analysis only aims to react with one functional group in interested molecules, such as carbonyl group in ketones and aldehydes, or carboxyl group in carboxylic acids, since the specific reactivity of these derivatization agents is well suited for targeted

analysis of individual metabolites. However, for comprehensive metabolomic analysis of biological system or exploratory investigation of metabolic disorder, the derivatization agents that can facilitate simultaneous measurement of diverse metabolites are advantageous. In this study, HQ was identified as an effective reagent that can react with aldehydes, ketones and carboxylic acids in biological samples simultaneously and thus facilitate their detection in LC-MS analysis (**Fig. 2.1-2.4**). Considering the metabolites detected by HQ-based LC-MS analysis are important intermediates or end products in nutrient and energy metabolism, HQ derivatization method has the potential to find more applications in examining the homeostasis and disorder of metabolic system.

The conditions of HQ derivatization have been examined in this study. Aldehydes and ketones directly react with the hydrazine group in HQ to form hydrazones, while TPP and DPDS-mediated activation of carboxylic acid is required for the formation of hydrazides with HQ (**Scheme 1** and **2**). Compared to the nitrogen atom in the amine group of PA, the terminal nitrogen in the hydrazine group of HQ is much more nucleophilic for the reaction with positively-charged carbon in the carbonyl group of aldehydes and ketones (**Table 1**). However, the versatility of HQ in the derivatization of carboxylic acids, aldehydes and ketones cannot be solely attributed to its hydrazine moiety since HP and DH also contain hydrazine moiety in their structures. Therefore, besides hydrazine, other structural features of HQ, HP, and DH also contribute to their reactivity (**Table 1**). Compared to the pyridine moiety of HP, the quinoline moiety of HQ could function as a better electron donor for the nucleophilic activity of hydrazine group, which may explain

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why HQ can react with ketones and aldehydes but not HP. As for the differences between DH and HQ, two possible mechanisms might contribute to the incapability of DH to derivatize carboxylic acids: 1) the sulfonyl group in DH can withdraw electrons from its hydrazine group, making it less reactive for the reactions with carboxylic acid; 2) water in the six-compound test solution and biological samples may negatively affect the reactions between DH and carboxylic acid. In fact, the formation of DH derivatives of carboxylic acids (acetic acid and propionic acid) was observed in a preliminary experiment when the reactions were conducted in an anhydrous condition (data not shown). The exact mechanism behind this observation is not determined, but could be contributed by the altered reactivity of DH in the presence of water, as reported previously (*119*). In contrast, HQ derivatization reactions can proceed with about 5% water content in the reaction mixture, suggesting that HQ derivatization is compatible with the water-based biological samples.

In summary, the identification of HQ as an effective derivatization agent for simultaneous LC-MS analysis of ketones, aldehydes, and carboxylic acids provides a new technical platform to conduct metabolomic analysis of these compounds in the metabolic system. The utilization of this technical platform in chemical-induced metabolic disorder was further explored in the LC-MS-based metabolomic analysis of streptozotocin-induced type 1 diabetes (**Chapter 3**).

Table 2.1 Reactivity of derivatization agents with endogenous carboxylic acid, aldehyde, and ketone metabolites. Four derivatization agents (PA: 2-picolylamine, HP: 2-hydrazinopyridine, HQ: 2-hydrozinoquinoline, DH: dansyl hydrazine) reacted individually with a mixture of acetic acid, 3-hydroxybutyrate, malic acid, acetaldehyde, acetone, and pyruvate. The derivatization reactions and the molecular formula of derivatization products are summarized in this table. The structures and MS/MS spectra of enlisted HQ derivatives are presented in **Fig. 2.1.** The structures of enlisted PA, HP, and DH derivatives are presented in **Fig. 2.2 A-K**. N.D. indicates that the derivative is not detected.

Derivatization reactions	Acetate	HBA	Malate	Acetaldehyde	Acetone	Pyruvate
PA	Forming amide $C_8H_{10}N_2O$	Forming amide $C_{10}H_{14}N_2O_2$	Forming di-amide $C_{16}H_{18}N_4O_3$	N.D.	N.D.	Forming amide $C_9H_{10}N_2O_2$
HP	Forming hydrazide $C_7H_9N_3O$	Forming hydrazide $C_9H_{13}N_3O_2$	Forming di-hydrazide $C_{14}H_{16}N_6O_3$	N.D.	N.D.	Forming hydrazide $C_8H_9N_3O_2$
HQ	Forming hydrazide $C_{11}H_{11}N_3O$	Forming hydrazide $C_{13}H_{15}N_3O_2$	Forming di-hydrazide $C_{22}H_{20}N_6O_3$	Forming hydrazone $C_{11}H_{11}N_3$	Forming hydrazone $C_{12}H_{13}N_3$	Forming hydrazone $C_{12}H_{11}N_3O_2$
DH	N.D.	N.D.	N.D.	Forming hydrazone $C_{14}H_{17}N_3O_2S$	Forming hydrazone $C_{15}H_{19}N_3O_2S$	Forming hydrazone $C_{15}H_{17}N_3O_4S$

Table 2.2 A list of confirmed HQ derivatives of carboxylic acid, aldehyde, and ketone metabolites. Information on each metabolite include its molecular formula, the formula of its HQ derivative, and the exact mass of protonated HQ derivative ($[M+H]^+$). The metabolites contributing to the separation of urine, serum and liver extracts in **Fig. 2.3A** were presented with their identities (ID) labeled in **Fig. 2.3B**. U, S, and L indicate the significant presence of specific metabolites in urine, serum, and liver, respectively, based on the signals detected by mass spectrometer.

Compounds	ID	Formula	Derivative Formula (HQ moiety)	Exact mass of $[M+H]^+$	Distribution
Formaldehyde	XIV	CH_2O	$C_{10}H_9N_3$ (1)	172.0875	L
Acetaldehyde	XVII	C_2H_4O	$C_{11}H_{11}N_3$ (1)	186.1031	L
Acetic acid	VIII	$C_2H_4O_2$	$C_{11}H_{11}N_3O$ (1)	202.0980	S
Acetone	XIII	C_3H_6O	$C_{12}H_{13}N_3$ (1)	200.1188	U/S/L
Propionic acid	XV	$C_3H_6O_2$	$C_{12}H_{13}N_3O$ (1)	216.1137	U/L
Pyruvic acid	VII	$C_3H_4O_3$	$C_{12}H_{11}N_3O_2$ (1)	230.0930	S/L
Lactic acid	XII	$C_3H_6O_3$	$C_{12}H_{13}N_3O_2$ (1)	232.1086	S/L
Acetoin	V	$C_4H_8O_2$	$C_{13}H_{15}N_3O$ (1)	230.1288	U
Butyric acid		$C_4H_8O_2$	$C_{13}H_{15}N_3O$ (1)	230.1288	
Acetoacetic acid		$C_4H_6O_3$	$C_{13}H_{13}N_3O_2$ (1)	244.1086	U
HBA	XI	$C_4H_8O_3$	$C_{13}H_{15}N_3O_2$ (1)	246.1243	S
Fumaric acid		$C_4H_4O_4$	$C_{13}H_{11}N_3O_3$ (1)	258.0873	

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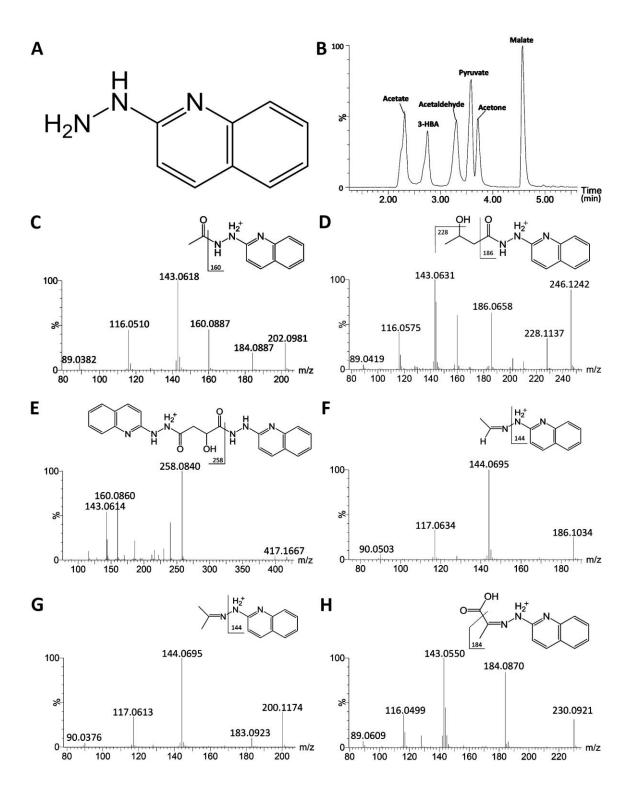
Succinic acid		$C_4H_6O_4$	$C_{13}H_{13}N_3O_3$ (1)	260.1030	U
Malic acid		$C_4H_6O_5$	$C_{22}H_{20}N_6O_3$ (2)	417.1675	
α-Ketoisovaleric acid	I	$C_5H_8O_3$	$C_{14}H_{15}N_3O_2$ (1)	258.1243	U
α-Ketoglutaric acid	IV	$C_5H_6O_5$	$C_{14}H_{13}N_3O_4$ (1)	288.0984	U
Dehydroascorbic acid	XVI	$C_6H_6O_6$	$C_{15}H_{13}N_3O_5$ (1)	316.0928	U/L
α-Ketoisocaproic acid	III	$C_6H_{10}O_3$	$C_{15}H_{17}N_3O_2$ (1)	272.1399	U
Glucose	IX	$C_6H_{12}O_6$	$C_{15}H_{19}N_3O_5$ (1)	322.1397	S/L
Mannose	X	$C_6H_{12}O_6$	$C_{15}H_{19}N_3O_5$ (1)	322.1397	S/L
Citric acid		$C_6H_8O_7$	$C_{13}H_{11}N_3O_3$ (1)	258.0873	U
Formiminoglutamic acid	II	$C_6H_{10}N_2O_4$	$C_{15}H_{17}N_5O_3$ (1)	316.1404	U
4-Hydroxyphenylacetic acid	VI	$C_8H_8O_3$	$C_{17}H_{15}N_3O_2$ (1)	294.1237	U
4-Hydroxyphenylpyruvic acid		$C_9H_8O_4$	$C_{18}H_{15}N_3O_3$ (1)	322.1192	U

Scheme 2.1. HQ derivatization of carboxylic acids. Carboxylic acids are activated by DPDS and TPP to form acyloxyphosphonium ions, which then react with HQ to form hydrazides.

Scheme 2.2. HQ derivatization of aldehydes and ketones. HQ reacts with aldehydes and ketones to form hydrazone.

Figure 2.1. Chromatogram and MSMS spectra of HQ derivatives of carboxylic acids, aldehydes, and ketones (acetic acid, pyruvate, acetaldehyde, acetone, HBA, malate). *A*. Structure of HQ. *B*. Representatives of chromatograph of HQ derivatives. *C*. MSMS spectrum of acetate-HQ derivatives. *D*. MSMS spectrum of HBA-HQ derivatives. *E*. MSMS spectrum of malate-HQ derivatives. *F*. MSMS spectrum of acetaldehyde-HQ derivatives. *G*. MSMS spectrum of acetone-HQ derivatives. *H*. MSMS spectrum of pyruvate-HQ derivatives.

A

B

C

D

E

F

G

H

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Fig 2.2. PA, HP, and DH derivatives of carboxylic acids, aldehydes, and ketones. Molecular formula and chemical structures of these derivatives were determined by accurate mass measurement, elemental composition analysis, and MS/MS fragmentation. The value of $[M+H]^+$ is the mass-to-charge ratio (*m/z*) of protonated derivative. *A*. Acetate-PA. *B*. HBA-PA. *C*. Malate-PA. *D*. Pyruvate-PA. *E*. Acetate-HP. *F*. HBA-HP. *G*. Malate-HP. *H*. Pyruvate-HP. *I*. Acetaldehyde-DH. *J*. Acetone-DH. *K*. Pyruvate-DH.

A

$C_8H_{10}N_2O$
$[M+H]^+ = 151.0866$

B

$C_{10}H_{14}N_2O_2$
$[M+H]^+ = 195.1128$

C

$C_{16}H_{18}N_4O_3$
$[M+H]^+ = 315.1452$

D

$C_9H_{10}N_2O_2$
$[M+H]^+ = 179.0815$

E

$C_7H_9N_3O$
$[M+H]^+ = 152.0818$

F

$C_9H_{13}N_3O_2$
$[M+H]^+ = 196.1081$

G

$C_{14}H_{16}N_6O_3$
$[M+H]^+ = 317.1357$

H

$C_8H_9N_3O_2$
$[M+H]^+ = 180.0768$

I

$C_{14}H_{17}N_3O_2S$
$[M+H]^+ = 292.1114$

J

$C_{15}H_{19}N_3O_2S$
$[M+H]^+ = 306.1271$

K

$C_{15}H_{17}N_3O_4S$
$[M+H]^+ = 336.1013$

Figure 2.3. Optimization of HQ derivatization reactions. *A*. Effect of reaction time on HQ derivatization. *B*. Effect of reaction temperature on HQ derivatization. The amounts of the derivatives formed by incubations at 60°C for 60 min were arbitrarily set as 1. Experimental values are expressed as mean ± standard deviation (SD).
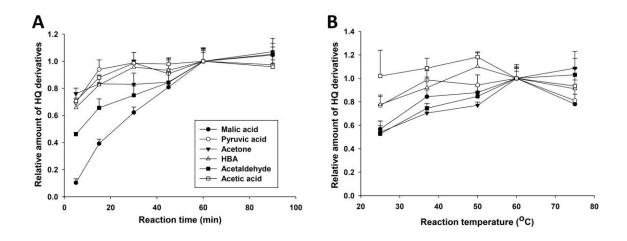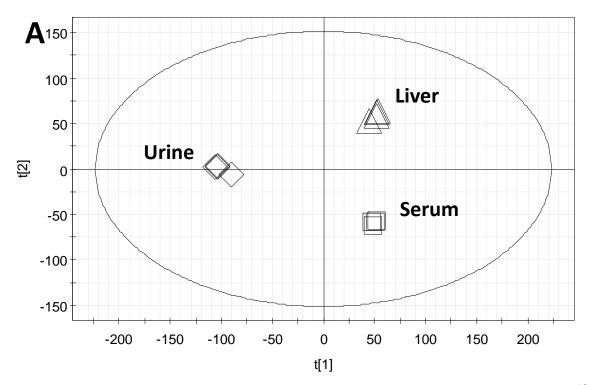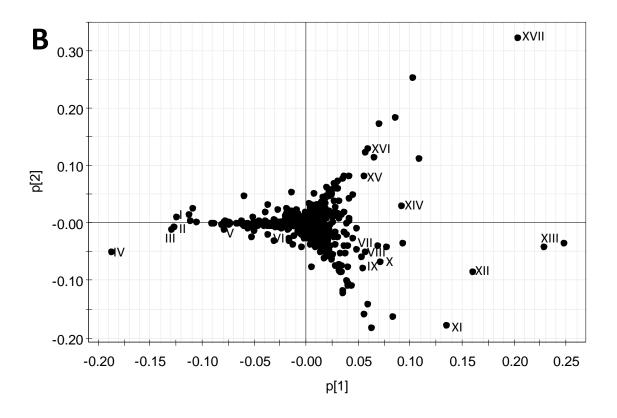
Figure 2.4 LC-MS-based metabolomic analysis of HQ-derivatized of serum, urine and liver extract samples from the wild-type mice. *A.* The scores plot of a PCA model on three groups of biological samples (n= 4). The *t*[1] and *t*[2] values represent the scores of each sample in the principal component 1 and 2, respectively. *B*. The loadings plot of ions detected by LC-MS analysis. The *p*[1] and *p*[2] values represent the contributing weights of each ion to the principal components 1 and 2 of the PCA model, respectively. Major contributing ions in each sample group are labeled. **I**: α-ketoisovaleric acid; **II**: formiminoglutamic acid; **III**: α-ketoisocaproic acid; **IV**: α-ketoglutaric acid; **V**: acetoin; **VI**: 4-hydroxyphenylacetic acid; **VII**: pyruvic acid; **VIII**: acetic acid; **IX**: glucose; **X**: mannose; **XI**: HBA; **XII**: lactic acid; **XIII**: acetone; **XIV**: formaldehyde; **XV**: propionic acid; **XVI**: dehydroascorbic acid; **XVII**: acetaldehyde.

Chapter 3

APPLICATION OF 2-HYDRAZINOQUINOLINE DERIVATIZATION IN LC-MS-BASED METABOLOMIC INVESTIGATION OF KETOACIDOSIS IN STREPTOZOTOCIN-ELICITED DIABETES

[3] **Key Words:** HQ, chemical derivatization, metabolomics, type 1 diabetes

Abbreviations: NAC, N-acetylcysteine; STZ, streptozotocin; TPP, triphenylphosphine; DPDS, 2, 2'-dipyridyl disulfide; hematoxylin and eosin (H&E); UPLC, ultra-performance liquid chromatography; QTOF-MS, QTOF mass spectrometer; ESI, electrospray ionization; SIC, single ion counts; TIC, total ion counts; i.p., intraperitoneal; BCAAs, branch chain amino acids; SD, standard deviation.

3.1 INTRODUCTION

Diabetes mellitus is a metabolic disorder represented by chronic hyperglycemia and disturbances of carbohydrate, fat and protein metabolism (*120*). According to the American Diabetes Association, in 2011, 8.3% of the population in the US has diabetes. Pathogenesis of diabetes is attributed to the defects in insulin secretion, insulin action, or both (*110*). More specifically, destruction of pancreatic islets of β-cells through autoimmunity-dependent or -independent mechanisms leads to type 1 diabetes, while insulin resistance due to the lack of response to insulin signaling results in type 2 diabetes (*121*).

Dramatic disturbances of metabolic system occur during the development of diabetes, leading to significant changes in many intermediary and energy metabolism pathways. To define these metabolic changes, metabolomics has been adopted in recent years to examine the metabolic profiles of diabetic patients and animal models. Technique platforms, including NMR, GC-MS, and LC-MS, have been used for the metabolomic investigation of diabetes. For example, NMR-based metabolomic analysis of urine samples from type 1 diabetic rats observed decreased levels of acetate, succinate, citrate, α-ketoglutarate, creatine,creatinine, *N*-acetylcysteine (NAC), methionine, alanine, choline, lactate, but increased levels of hippurate and dimethylamine (*122*). GC-MS-based metabolomics confirmed the diabetes-induced perturbation of TCA cycle, including citrate, malate, succinate, and aconitate (*113*) and observed the increases of

glucose, 2-hydroxyisobutyric acid, linoleic acid, palmitic acid and phosphate in the plasma samples of diabetic patients. (*123*). LC-MS based metabolomics revealed that the increased catabolism of BCAAs and elevated concentrations of neutral amino acids is correlated with insulin resistance in obese humans and type 2 diabetes patients (*124, 125*).

Among many metabolic changes induced by diabetes, one prominent metabolic phenotype of type 1 diabetes is the development of ketoacidosis, which is represented by the high-abundance presence of ketone bodies (acetone, acetoacetate, and 3-hydroxybutyrate) in biofluids and the dramatic changes in organic acids. In this study, the kinetics and profiles of diabetic ketoacidosis in a type 1 diabetes mouse model were examined by the LC-MS-based metabolomics using HQ as the derivatization agent of ketones, aldehydes, and carboxylic acids.

3.2 MATERIAL AND METHODS

3.2.1 Reagents

Streptozotocin (STZ) was purchased from Calbiochem (Billerica, MA). D-glucose, acetone, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Sodium citrate, LC-MS-grade water and acetonitrile were purchased from Fisher Scientific (Houston, TX). HQ, 3-hydroxylbutyrate acid, and triphenylphosphine (TPP) were

purchased from Alfa Aesar (Ward Hill, MA), 2, 2'-Dipyridyl disulfide (DPDS) and lactic acid were purchased from MP Biomedicals (Santa Ana, CA).

3.2.2 Animal treatment and sample collection

Male C57BL/6 mice (10 to 12-week old) from Charles River Laboratories (Wilmington, MA) were used in this study. All animals were maintained in a University of Minnesota (UMN) animal facility under a standard 12 h light/12 h dark cycle with food and water *ad libitum*. Handling and treatment procedures were in accordance with animal study protocols approved by the UMN Institutional Animal Care and Use Committee. STZ treatment was performed following the protocol recommended by the Animal Models of Diabetic Complications Consortium (http://www.diacomp.org/). Prior to STZ treatment, mice were fasted for 4 hours. Freshly prepared STZ solution (buffered by sodium citrate, pH 4.5) was administered by a single intraperitoneal (i.p.) injection to mice at the dose of 180 mg/kg. The drinking water containing 1% sucrose was supplied overnight after the injection to prevent the mortality caused by STZ-induced acute hypoglycemia. Serum samples were collected by submandibular bleeding. Urine samples were collected by housing mice in the metabolic cages for 24 hours. Liver and other tissue samples were harvested after animals were euthanized by carbon dioxide. All urine, serum and tissue samples were stored at $-80^{\circ}C$ before further analysis.

3.2.3 Biochemical and histological analysis of STZ-induced diabetes

The glucose concentration in tail vein blood was monitored using an Omnis blood glucose meter (Miramar, FL). Hyperglycemia was defined by the blood glucose level that is higher than 250 mg/dl (*126*). The pancreas tissue was immediately fixed in 10% formalin solution after dissection, and then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for general histology.

3.2.4 HQ derivatization of urine from Type 1 diabetes mice model

Urine samples collected before and after STZ treatment were derivatized by HQ. The HQ derivatization reaction was conducted by mixing 5 µL urine with a 100 µL of freshly-prepared acetonitrile solution containing 1 mM DPDS, 1 mM TPP, 1 mM HQ, and 10 µM $^2H_4$-acetic acid (internal standard). The reaction mixture was incubated at 60°C for 60 min, chilled on ice, and then mixed with 100 µl of H_2O. After centrifugation at 18,000 $\times$ *g* for 10 min, the supernatant was transferred into HPLC vials for LC-MS analysis.

3.2.5 LC-MS-based metabolomic analysis of HQ derivatization product.

HQ-derivatized urine samples were injected into a Waters Acquity^{TM} ultra-performance liquid chromatography (UPLC) system (Milford, MA) and separated by a gradient of

mobile phase ranging from 0.05% aqueous acetic acid containing 2 mM ammonium acetate to 95% aqueous acetonitrile containing 0.05% acetic acid and 2 mM ammonium acetate over a 10-min run. LC elute was introduced into a Waters SYNAPT QTOF mass spectrometer (QTOF-MS) for MS analysis. Capillary voltage and cone voltage were maintained at 3.2 kV and 30 V, respectively, in positive electrospray ionization (ESI). Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (700 L/h), and argon as the collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range m/z 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ([M+H]$^+$ = 556.2771 m/z) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroid format. Additional structural information was obtained by tandem MS (MS/MS) fragmentation with collision energies ranging from 15 to 40 eV. Chromatographic and spectral data of HQ-derivatized urine samples were deconvoluted by MarkerLynxTM software (Waters). A multivariate data matrix containing information on sample identity, ion identity (RT and m/z) and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition and integration. The intensity of each ion was calculated by normalizing the single ion counts (SIC) versus the total ion counts (TIC) in the whole chromatogram. The data matrix was further exported into SIMCA-P+^{TM} software (Umetrics, Kinnelon, NJ), and transformed by mean-centering and *Pareto* scaling, a technique that increases the importance of low

abundance ions without significant amplification of noise. Partial least squares-discriminant analysis (PLS-DA) was adopted to model the data from the control and STZ-treated diabetic mice. Major latent variables in the data matrix were described in a scores scatter plot of multivariate model. Metabolites were identified by analyzing ions contributing to the principal components and to the separation of sample groups in the loadings scatter plot. The chemical identities of urinary metabolites affected by STZ treatment were determined by accurate mass measurement, elemental composition analysis, MS/MS fragmentation, and comparisons with authentic standards if available.

3.2.6 Statistics

Experimental values are expressed as mean ± standard deviation (SD). Statistical analysis was performed with two-tailed Student's t-tests for unpaired data, with a p-value of <0.05 considered as statistically significant.

3.3 RESULTS

3.3.1 Phenotypes of STZ-induced type 1 diabetes in C57BL/6 mice

The influences of STZ treatment on pancreas were examined by histological analysis. H&E staining showed that compared to control mice, the number and the size of β-cell islets in pancreas were greatly reduced in the STZ-treated mice (**Fig. 3.2**). Consistent to

these histological changes, hyperglycemia developed rapidly after STZ treatment and was sustained during 6 days of sample collection (**Fig. 3.1A**). All these observations indicated that the type 1 diabetes was developed in the STZ-treated mice. Body weight of STZ-treated mice decreased dramatically (**Fig. 3.1C**), even though food intake increased rapidly 2 days after STZ treatment (**Fig. 3.1B**). In addition, water consumption (**Fig. 3.1D**) and urine production also increased dramatically (**Fig. 3.1E**). All these results confirmed that STZ-treated mice exhibited typical phenotypes of type 1 diabetes.

3.3.2 Metabolomic analysis of HQ-derivatized urine sample from STZ-treated mice

Considering ketoacidosis is the most prominent metabolic phenotype reported in previous studies on diabetes-induced metabolic changes, newly-established HQ derivatization method (detailed in **Chapter 2**) was adopted to examine the metabolomic profiles of urine samples from STZ-treated mice. After the data from LC-MS analysis of HQ-derivatized urine samples were processed by supervised PLS-DA, time-dependent separation of urine samples was observed in a two-component model (**Fig. 3.3A**). Subsequently, the metabolites contributing to the separation of sample groups were further defined by examining the detected ions in the loadings plot of PLS-DA model (**Fig. 3.3B**). The result suggested that the combination of HQ derivatization and LC-MS analysis is capable of revealing the kinetics of STZ-induced metabolic changes in type 1 diabetic mice.

3.3.3 Characterization of metabolite biomarkers of STZ-induced type 1 diabetes model

Multivariate analysis of LC-MS data revealed time-dependent metabolic changes induced by STZ treatment (**Fig. 3.3**). Chemical identities of urinary metabolites contributing to the shift of urinary metabolome, which are mainly HQ-derivatized ketones, aldehydes and carboxylic acids, were defined by accurate mass measurement, elemental composition analysis, MS/MS fragmentation; chromatographic and spectroscopic comparisons with standards. For example, the levels of glucose (an aldehyde) and ketone bodies (acetone, HBA, and acetoacetate) increased after STZ treatment (**Fig. 3.4A-D**). However, the patterns of these changes in urinary glucose and ketone bodies were different. Glucose was absent in pre-STZ urine and its level steadily increased after STZ treatment (**Fig. 3.4A**). In contrast, the elevation of ketone bodies appeared to be transient as their levels peaked on day 3 and 4 but decreased afterwards (**Fig. 3.4B-D**). Another group of urinary metabolites that were greatly affected by STZ treatment were α-keto acids (**Fig. 3.4E-H**). The levels of α-ketoglutaric acid and 4-hydroxyphenylpyruvic acid, which are α-keto acid metabolites of glutamate and tyrosine, increased at first after STZ treatment, but decreased thereafter and became even lower than the controls (**Fig. 3.4E-F**). In addition, two branched-chain amino acid metabolites, α-ketoisovaleric acid from valine and α-ketoisocaproic acid from leucine, also decreased dramatically by STZ treatment even though a transient increase of α-ketoisovaleric acid was observed on day 1 (**Fig. 3.4G-H**). Pyruvate, lactate, and acetate are three important intermediates in intermediary metabolism. Pyruvate level was largely unaffected after STZ treatment

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except a transient increase on day 1 (**Fig. 3.4I**). Lactate, as the product of NADH-dependent reduction of pyruvate, remained unchanged in the first 4 days of STZ treatment, but its level increased on day 5 and 6 (**Fig. 3.4J**). The profile of acetic acid was similar to the profile of glucose as its level in urine gradually increased after the treatment (**Fig. 3.4K**). Acetoin (3-hydroxy-2-butanone) was another ketone metabolite affected by STZ. Interestingly, its level in urine decreased dramatically after STZ treatment, but started to increase on day 4 and rose to a much higher level than the controls (**Fig. 3.4L**). Overall, the observed metabolic changes in carboxylic acid, aldehyde, and ketone metabolites are largely consistent with the expected metabolic phenotypes of type 1 diabetics, suggesting that HQ derivatization facilitated the LC-MS-based metabolomic analysis of diabetic ketoacidosis.

3.4 DISCUSSION

As essential intermediates and end products of nutrient and energy metabolism, short-chain ketones, aldehydes, and carboxylic acids in tissue and biofluids are sensitive to metabolic disturbance, and thus can function as the biomarkers of metabolic events caused by exogenous challenges and disease development. Targeted analyses of these metabolite biomarkers are commonly used in clinical diagnosis of inherited or acquired metabolic diseases (*127*). However, for defining the metabolic profile of a biological system or identifying the mechanism of metabolic disorder, a technical platform capable of conveniently and simultaneously measuring diverse metabolites is preferred. The
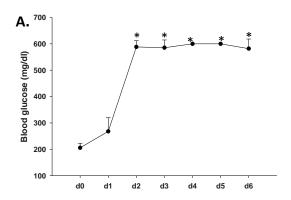
results from this study showed that the multivariate models and metabolite analysis based on the LC-MS analysis of HQ-derivatized urine samples not only confirmed the expected ketoacidotic phenotype of type 1 diabetes, but also revealed the kinetics of metabolic changes and potential correlations among observed metabolite biomarkers (**Fig. 3.3** and **3.4**). Compared to the results from LC-MS-based metabolomic analysis of underivatized urine samples from the same experiment (data not shown), these results are highly relevant to the disruption of carbohydrate, fatty acid, and amino acid metabolism in type 1 diabetes, suggesting that the application of HQ derivatization in LC-MS-based metabolomic analysis provides a robust tool to investigate many important metabolic events involving in ketone, aldehyde, and carboxylic acid metabolites.
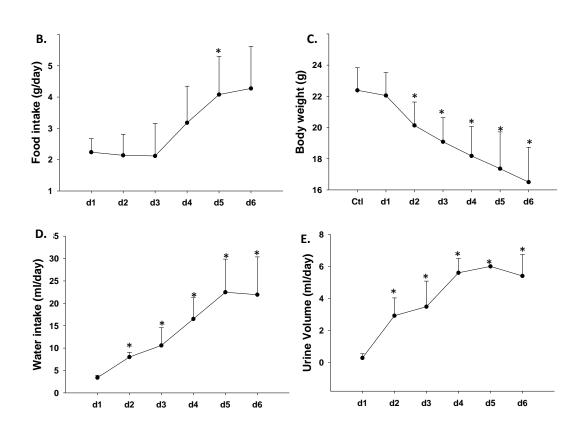
Diabetic ketoacidosis, triggered by insulin deficiency and subsequent excess of insulin counteractive hormones (glucagon, catecholamines, and cortisol), mainly occurs in type 1 diabetic patients, and occasionally in type 2 diabetic patients. Marked hyperglycemia, ketosis, and acidosis have been defined as three elements of diabetic ketoacidosis (*128*). The observation of increased glucose, ketone bodies, and carboxylic acids (lactate and acetate) in STZ-treated mice confirmed these metabolic phenotypes of diabetic ketoacidosis (**Fig. 3.4**). In addition, this observation also correlated with the decrease of multiple α-keto acids from amino acid metabolism (**Fig. 3.4**) since ketogenic and glucogenic amino acids are major sources of glucose and ketone bodies in diabetes. The decreases of 4-hydroxyphenylpyruvic acid, α-ketoisovaleric acid, and α-ketoisocaproic acid were likely caused by the enhanced degradation of tyrosine, leucine, and valine,

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since the upregulation of α-keto acids dehydrogenases that are responsible for their degradation have been observed in diabetic animal models (*129-131*). α-Ketoglutaric acid is a key intermediate in the TCA cycle. The observed decrease in its level reflects the cataplerotic effects of type 1 diabetes (*132*). To the best of our knowledge, the changes in urinary acetoin observed in this study have not been reported previously even though it is known that acetoin can be formed in bacteria, animals, and humans through the metabolism of acetolactate, pyruvate, and ethanol (*133, 134*). Further studies are required to understand the metabolic events behind the diabetes-induced changes in acetoin as well as the significance of these changes.

The capacity of HQ derivatization to characterize the metabolic changes in biological samples was demonstrated by the LC-MS-based metabolomic analysis of type 1 diabetic mice. Because of its simple procedure and its compatibility with water-based samples, HQ derivatization has the potential to become a useful tool for LC-MS-based comprehensive metabolomic profiling, complementary to the existing metabolomic methods for lipids, amino acids, and other classes of metabolites.

Figure 3.1. Phenotypes of STZ-induced Type 1 diabetes *C57Bl6* mice model. *A*. Blood glucose concentration from day 0 to day 6 of STZ treatment, *B*. Body weight from day 0 to day 6 of STZ treatment, *C*. Urine volume from day 1 to day 6 after STZ treatment, *D*. food intake from day 1 to day 6 after STZ treatment, *E*. water intake from day 1 to day 6 after STZ treatment. *: $p<0.05$.

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Figure 3.2. Histology of pancreatic islets. A: control mice normal pancreatic islet, B: STZ-treated Type 1 diabetes B6 mice model shrinking pancreatic islet on day 6 after STZ injection.
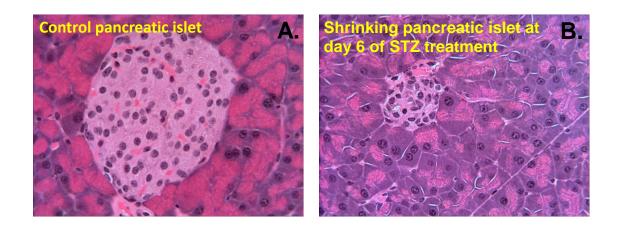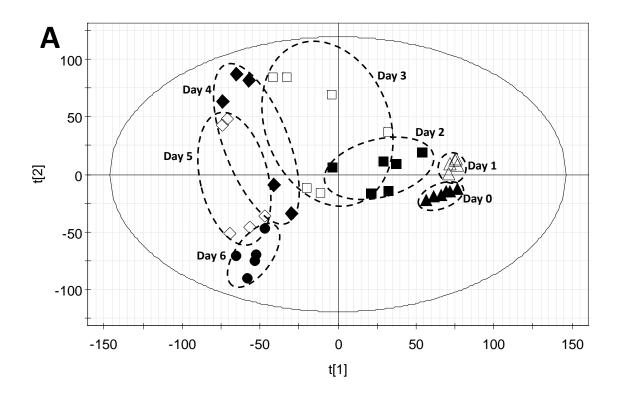
Figure 3.3. LC-MS-based metabolomic analysis of STZ-induced metabolic changes in mice. Urine samples were derivatized by HQ before LC-MS analysis. *A*. The scores plot of a PLS-DA model on urine samples from control and STZ-treated mice. The *t*[1] and *t*[2] values represent the scores of each sample in the principal component 1 and 2, respectively. *B*. The loadings plot of urinary ions contributing to the time-dependent separation of HQ-derivatized urine samples in the PLS-DA model. The *w*c*[1] and *w*c*[2] values represent the contributing weights of each ion to the principal components 1 and 2 of the PLS-DA model, respectively. Major urinary ions were labeled.

B

acetone

acetoacetic acid

α-ketoisovaleric acid

HBA

α-ketoglutaric acid

4-hydroxyphenylpyruvic acid

α-ketoisocaproic acid

glucose

lactic acid
acetic acid

pyruvic acid

acetoin

w*c[2]

w*c[1]
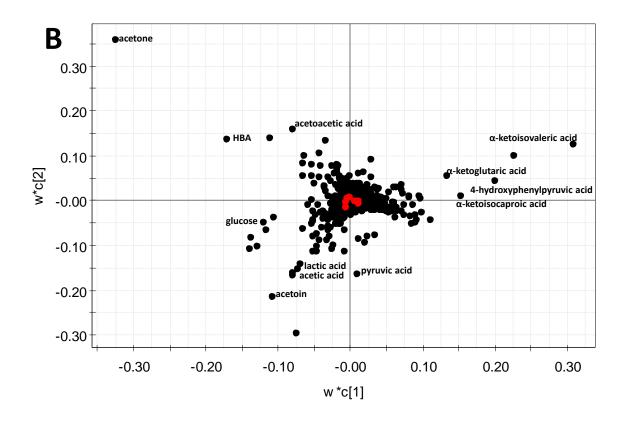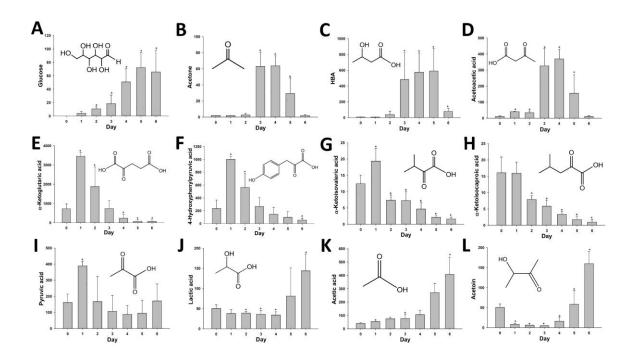
Figure 3.4. Influences of STZ treatment on ketone, aldehyde, and carboxylic acid metabolites in mouse urine. The relative abundance of urinary metabolite was determined by calculating the ratio between the signal ion counts (SIC) of interested metabolite and the total ion counts (TIC) of a sample. *: $p<0.05$. **A**. Glucose. **B**. Acetone. **C**. 3- HBA. **D**. Acetoacetic acid. **E**. α- Ketoglutaric acid. **F**. 4- Hydroxyphenylpyruvic acid. **G**. α- Ketoisovaleric acid. **H**. α- Ketoisocaproic acid. **I**. Pyruvic acid. **J**. Lactic acid. **K**. Acetic acid. **L**. Acetoin.

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