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Analysis of Selected Sugars and Sugar Phosphates in Mouse Heart Tissue by Reductive Amination and Liquid Chromatography-Electrospray Ionization Mass Spectrometry

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Abstract

Sensitive and reliable analysis of sugars and sugar phosphates in tissues and cells is essential for many biological and cell engineering studies. However, the successful analysis of these endogenous compounds in biological samples by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) is often difficult because of their poor chromatographic retention properties in reversed-phase LC, the complex biological matrices, and the ionization suppression in ESI. This situation is further complicated by the existence of their multiple structural isomers in vivo. This work describes the combination of reductive amination using 3-amino-9-ethylcarbazole, with a new LC approach using a pentafluorophenyl core–shell ultrahigh performance (UP) LC column and methylphosphonic acid as an efficient tail-sweeping reagent for improved chromatographic separation. This new method was used for selected detection and accurate quantitation of the major free and phosphorylated reducing sugars in mouse heart tissue. Among the detected compounds, accurate quantitation of glyceraldehyde, ribose, glucose, glycerylaldehyde-3-phosphate, ribose-5-phosphate, glucose-6-phosphate, and mannose-6-phosphate was achieved by UPLC/multiple-reaction monitoring (MRM)-MS, with analytical accuracies ranging from 87.4% to 109.4% and CVs of ≤8.5% (n = 6). To demonstrate isotope-resolved metabolic profiling, we used UPLC/quadrupole time-of-flight (QTOF)-MS to analyze the isotope distribution patterns of C3 to C6 free and phosphorylated reducing sugars in heart tissues from ^{13}C-labeled wild type and knockout mice. In conclusion, the preanalytical derivatization-LC/ESI-MS method has resulted in selective determination of free and phosphorylated reducing

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Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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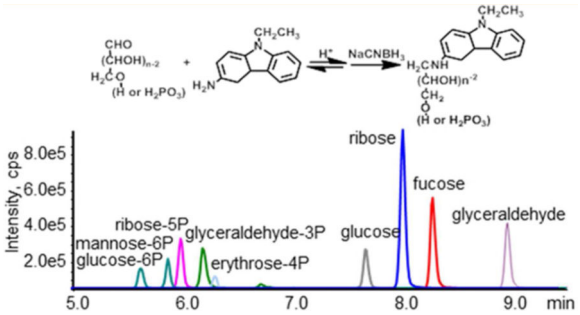
sugars without the interferences from their nonreducing structural isomers in mouse heart tissue, with analytical sensitivities in the femtomole to low picomole range.



As the central hub of energy metabolism in humans and many other living systems, central carbon metabolism (CCM) is a complex enzyme-mediated metabolic network, which is composed of several subpathways such as glycolysis, gluconeogenesis, the pentose phosphate pathway (PPP), and the Krebs cycle (also known as the citric acid cycle or the tricarboxylic acid cycle).[1] Among the endogenous metabolites, low molecular weight sugars are the metabolic precursors or substrates of CCM. They enter the central carbon metabolism pathway via either glycolysis or the pentose phosphate pathway, by reacting with adenosine-5-triphosphate (ATP) and sugar kinases to form sugar phosphates. Sugar phosphates are the important metabolic intermediates of glycolysis, gluconeogenesis, and/or the pentose phosphate pathway.[1] Examination of the small molecule metabolites involved in CCM has been one of the most important approaches for monitoring the anabolic and catabolic status of a biological system. In this regard, the use of one or more isotopic tracers (e.g., ^{13}C-glucose) for metabolic flux analysis represents a commonly used technology in numerous biological and cell engineering studies.[2,3]

Currently, measurements of the metabolites involved in CCM, such as carboxylates, sugar phosphates, phosphocarboxylates, nucleotides, and energy cofactors, are usually carried out using mass spectrometry (MS) with an online front-end chromatographic separation, for example, by gas chromatography/mass spectrometry (GC/MS),[4,5] liquid chromatography/mass spectrometry (LC/MS),[6–9] and capillary electrophoresis/mass spectrometry (CE/MS).[10,11] Among these techniques, LC/MS with electrospray ionization (ESI) has been very attractive for analysis of these anionic compounds because the separation and the ionization are very compatible with the aqueous cell environment where endogenous metabolites are synthesized and metabolized within a biological system. In addition, if ESI is used, the transfer of a metabolite extract from an aqueous phase to a nonaqueous phase, which could induce loss or chemical degradation of some metabolites, is not needed during sample preparation.

Even with these analytical techniques, however, sensitive and reliable measurement of some of the CCM metabolites in biological samples still remains an analytical challenge. For example, when LC/MS is used for analysis of the CCM metabolites, the extremely polar nature of most CCM metabolites leads to little or no chromatographic retention in reversed-phase LC. Ion pairing reagents, such as protonated tertiary amines such as tributylamine, are

often used as the counterion to enhance chromatographic retention.[6,9] Hydrophilic interaction liquid chromatography (HILIC) methods have also been developed for ESI-MS determination of the CCM metabolites.[12,13] CE/ESI-MS is another analytical technique that has shown high sensitivity for the detection of many CCM metabolites including sugar phosphates and a few high-abundance sugar phosphates could be quantified in mouse liver using CE/MS.[11] However, the detection of low-concentration sugar phosphates such as erythrose-4-phosphate (erythrose-4P) and glyceraldehyde-3-phosphate (glyceralde-hyde-3P) in the mouse liver was not achieved by CE/MS.[11]

Successful determination of the low-concentration sugar phosphates, as well as separation of the in vivo structural isomers of sugar phosphates in biological samples using various LC/MS/MS methods, has proven to be difficult,[6,9,14,15] even with HILIC-MS.[12,13] In addition to the complex biological matrices, ionization suppression during ESI from the ion pairing reagents in LC separation or the use of volatile salts in HILIC has contributed to the low detection sensitivity of these compounds by LC/MS.

High-performance anion-exchange chromatography (HPAEC) at high pH coupled with pulsed amperometric detection (PAD) is a sensitive technique for sugar determination[16] and for the analysis of sugar phosphates.[17] Though HPAEC-PAD provides highly sensitive detection of sugars and sugar phosphates, it does not provide the isotopic resolution necessary for isotope-resolved metabolic flux analysis. Chemical derivatization for the analysis of sugars by HPLC with UV, fluorometric, or MS detection has also received a lot of interest.[18–20] Chemical derivatization enhances the detection of sugars by introducing chromophores or fluorophores for separations designed for UV or fluorometric detection or by introducing a charge for MS detection. At the same time, the derivatization also leads to stronger reversed-phase LC retention of the sugars by covalently attaching an aromatic or heterocyclic moiety. Among the various chemical derivatization methods, reductive amination, which adds an amine to the aldehyde group of a reducing sugar (i.e., an aldose), has often been used. Reagents such as 2-aminopyridine,[21] *p*-amino benzoic ethyl ester,[22] 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid,[23] 8-aminonaphthalene-1,3,6-trisulfonate,[24] and 1-aminopyrene-3,6,8-trisulfonate,[25] among others, have been used to derivatize sugars prior to LC or capillary electrophoresis. Some of these approaches, however, suffer from unreliable quantitative analyses due to incomplete derivatization and/or tedious derivatization protocols.

The reagent 3-amino-9-ethylcarbazole (AEC) has been used for derivatizating monosaccharides via reductive amination according to the reaction mechanism shown in Figure 1A. This derivatization approach was used first for the LC/UV analysis of glucose, xylose, mannose, arabinose, and rhamnose in polysaccharide hydrolates[26] and later for analysis of monosaccharides by matrix-assisted laser desorption/ionization mass spectrometry, without LC separation.[27] AEC derivatives of sugars have good chemical stabilities in solution, and most of the excess AEC in the reaction mixture can be easily removed in a simple liquid–liquid extraction (LLE) step.[26] To our knowledge, this derivatization method had not been utilized for biological analysis until recently, when we used it in conjunction with an online HILIC/ESI-MS method for measuring the U-^{13}C-labeled-to-natural glucose ratio in a wild type and a knockout strain mice related to a

myocardial metabolism study.[28] The AEC derivatization approach has not previously been evaluated for the analysis of any sugar phosphates. In fact, there have been very few studies where chemical derivatization was used for the LC/MS determination of sugar phosphates in biological samples.[29]

We present here a new ultrahigh performance (UP)LC/ESI-MS method which provides a robust LC/MS-based method for the selective and sensitive analysis of reducing sugars and their phosphates, including all four of the reducing sugar phosphate intermediates involved in the PPP, without the interferences from their nonreducing isomers. We also demonstrate that AEC derivatization, performed under optimal reaction conditions, can be used for isotope-resolved metabolic profiling and for accurate assay of these metabolites in mouse heart tissues without the need of their individual stable isotope-labeling standard compounds as internal standard for LC/MS/MS quantitation.

EXPERIMENTAL SECTION

Chemicals and Reagents

Acetonitrile, methanol, hexane, water, acetic acid, and formic acid were LC/MS grade. Dichloromethane was HPLC grade. These reagents, together with 3-amino-9-ethylcarbazole (AEC), tributylamine, and sodium cyanoborohydride ($NaCNBH_3$), which were analytical reagent grade, were obtained from Sigma-Aldrich (St. Louis, MO). D-(+)-Glucose (≥99.5%), D-(+)-mannose (≥99.5%), D-(+)-galactose (≥99%), D-(–)-ribose (≥99%), D-(+)-fucose (≥98%), L-(6-^{13}C)-fucose, D-(+)-glyceraldehyde (≥98%), D-glucose 6-phosphate (glucose-6P) sodium (≥98%), D-mannose 6-phosphate (mannose-6P) sodium (≥95%), D-galactose-6-phosphate (galactose-6P) lithium (≥90%), D-ribose 5-phosphate (ribose-5P), D-erythrose 4-phosphate (erythrose-4P) sodium (no less than 60%), and DL-glyceraldehyde 3-phosphate (glyceraldehyde-3P) solution (46.9 mg/mL in water) were purchased from Sigma-Aldrich (St. Louis, MO) or Santa Cruz Biotechnology (Santa Cruz, CA). Before use, these authentic compounds, with the exception of glyceraldehyde-3P, were vacuum-dried in a desiccator with anhydrous phosphorus pentoxide under a vacuum of 30 mmHg, at 4 °C for 72 h.

Mouse Heart Specimens

Mouse hearts used for assay development and validation were obtained from Pel-Frez Biologicals (Rogers, AR). These specimens were freshly harvested and snap-frozen in liquid nitrogen (according to the accompanying sample information sheet) and were shipped on dry ice. Once received, they were stored at –80 °C until used.

U-^{13}C-glucose-labeled mouse heart specimens from *Per2*$^{-/-}$ knockout mice and wild type littermate controls were received from the Department of Anesthesiology, School of Medicine, University of Colorado, Denver, Colorado. *Per2*$^{-/-}$ mice and the controls were matched in age, weight, and gender. 50 mg of U-^{13}C-glucose was administered to each animal intraperitoneally. After 90 min, the hearts were collected using clamps which were precooled to the temperature of liquid nitrogen and snap-frozen in liquid nitrogen. All of the samples were then stored at –80 °C.

Extraction of Sugars and Sugar Phosphates

Mouse heart specimens on dry ice were diced to a fine paste in Eppendorf tubes, using a small hand-held stainless steel tissue homogenizer. A 50 mg aliquot of each individual tissue paste was precisely weighed and transferred to a 2 mL Safe-lock Eppendorf tube. After the addition of 125 μL of water, the tissues were homogenized at 30 Hz for 2 min using two 5 mm stainless steel balls on a Retsch MM400 mixer mill (Haan, Germany). After a short centrifugation, 375 μL of methanol was added. The samples were homogenized again for 2 min, followed by centrifugation at 12 500 rpm and 4 °C for 10 min in a microcentrifuge. The clear supernatants were transferred to 5 mL volumetric flasks. Five hundred μL of 75% methanol in water was added to each tissue residue, and the samples were homogenized for 2 min, followed by clarifying centrifugation. The clear supernatants were pooled in volumetric flasks and brought to 5 mL with 75% methanol in water.

Standard Solution and Calibration Curve

Stock solutions of glyceraldehyde, fucose, ribose, glucose, glyceraldehyde-3P, erythrose-4P, ribose-5P, glucose-6P, and man-nose-6P were individually prepared by dissolving or diluting each standard substance in 75% methanol in water to give a final concentration of 2 mg/mL. A standard solution was then made by mixing a 100 μL aliquot of each of the stock solutions with 100 μL of 75% methanol in water. Working standard solutions were prepared by stepwise dilution of this standard solution with 75% methanol in water, to produce final concentrations for each analyte in the range of 20 μg/mL to 2 ng/mL. L-(6-^{13}C)-Fucose was used as the internal standard (IS), and an IS solution was prepared with 75% methanol in water to a concentration of 2 μg/mL.

Chemical Derivatization

Reductive amination with AEC was performed according to a previously published protocol,[26] with a few modifications. Briefly, a 50 μL aliquot of each individual working standard solution or of each sample solution was mixed with 50 μL of the IS solution in a 1.5 mL Eppendorf tube, followed by the sequential addition of 100 μL of 25 mM AEC in methanol, 50 μL of 50 mM $NaCNBH_3$ solution, and 20 μL of acetic acid as the catalyst. The reaction mixtures from the standard solutions were incubated at 40, 50, 60, and 70 °C in an Eppendorf thermomixer at a shaking frequency of 600 rpm for 20, 40, 60, 80, and 100 min, respectively, to determine the optimal reaction temperature and time. The reaction mixtures from the tissue samples were reacted using the optimized reaction conditions. After reaction, each tube was cooled on ice for 1 min, and then, 300 μL of water and 300 μL of a dichloromethane–hexane (2:1, v/v) solvent were added. Each tube was vortexed and was then centrifuged at 10 000 rpm for 5 min in a microcentrifuge. A 150 μL aliquot of the upper aqueous phase was transferred to an LC autosampler vial and mixed with 850 μL of water. A 10 μL aliquot was used for the LC/MS or LC/multiple-reaction monitoring (MRM)-MS analysis.

UPLC/MRM-MS

LC separations were carried out using an Ultimate 3000 RSLC system (Dionex Inc., Amsterdam, Netherlands). Two reversed-phase UPLC columns, a Waters BEH C_{18} (2.1 ×

100 mm, 1.7 μm) column and a Phenomenex Kinex core–shell pentafluorophenyl (PFP) column (2.1 × 150 mm, 2.6 μm), were compared, using two different mobile phases for gradient elution. The first mobile phase was composed of water/0.1% formic acid (A) and acetonitrile/ 0.1% formic acid (B). The second mobile phase was composed of 1 mM methylphosphonic acid in water (A) and acetonitrile/0.1% formic acid (B). The column flow rate was set at 0.3 mL/min, and the column temperature was maintained at 40 °C. For the C_{18} UPLC column, the optimal LC elution gradient was determined to be 20% to 28% B in 8 min and then held at 100% B for 2.5 min. For the PFP UPLC column, the optimal binary gradient was determined to be 20% to 42% B in 7.5 min and then held at 100% B for 2.5 min. Each column was equilibrated for 3 min with the corresponding initial solvent composition between injections.

A 4000 QTRAP triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada), equipped with a Turbo Ionspray source, was operated in the positive-ion MRM mode for assay development. Typical ESI-MS parameters were: capillary voltage, 5200 V; nebulizer gas (N_2), 30 (arbitrary units); curtain gas (N_2), 20 (arbitrary units); collision gas (N_2), 3 (arbitrary units); entrance potential, 10 V; collision cell exit potential, 23 V; and drying gas (N_2), 400 °C. The Q1 and Q3 operations were set to unit resolution.

The MRM parameters for each of the targeted analytes were optimized using flow injection analysis, by delivering the standard solutions of the individual derivatives at a constant flow rate of 10 μL/min into the ion source of the mass spectrometer. Full-scan data acquisition was performed using the first mass analyzer (Q1) by scanning from *m/z* 50 to 600 in the profile mode, with a cycle time of 2 s with a step size of 0.1 Da and a pause time of 5 ms between scans. For the production scan experiments, the selected precursor ions were fragmented by collision-induced dissociation (CID) in the collision cell by ramping the collision energy voltages from 0 to 120 V. The product ions were detected using the second mass analyzer (Q3). In this way, at least two MRM transitions (i.e., the Q1/Q3 pairs) per analyte were obtained, and the two most sensitive transitions were used in the MRM scan mode to optimize the collision energy for each Q1/Q3 pair. The dwell time was 5 ms per transition, and the pause time between scans was 5 ms. Among the two MRM transitions per analyte, the Q1/Q3 pairs that showed the highest sensitivity for each of the analytes were used as the MRM transitions for quantitative monitoring. These “quantifier” MRM transitions for assay are listed in Table 1. The additional transitions acted as qualifiers for the purpose of verifying the identity of the compounds. The nebulizer, curtain, collision, and auxiliary gas (N_2) flow rates, as well as the temperature of the auxiliary gas, were optimized for the LC/MRM-MS runs, and the optimized values were those that resulted in the maximum signal-to-noise ratios for all of the Q1/Q3 pairs while maintaining stable electrospray throughout the LC/MRM-MS analysis. MRM data was acquired using an Analyst 1.5 software package and processed using a MultiQuant 1.2 software package (AB Sciex, Concord, ON, Canada).

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Standard stock solutions of the sugars and sugar phosphates were diluted stepwise with 75% methanol in water, with a dilution factor of 3. These standard solutions were subjected to

AEC derivatization, followed by LC/MRM-MS. The signal-to-noise ratios were used to determine the LODs and LOQs. The lower LODs and lower LOQs were defined as the on-column amounts that led to peaks with signal-to-noise ratios (S/N) of 3 and 10, respectively, according to the US FDA Guideline for Bioanalytical Method Validation.[30]

Assay Precision and Accuracy

The precision of the quantitation was measured as the intraday coefficient of variation (CV), determined by injecting eight analytical replicates prepared from 50 μL aliquots of a pooled mouse heart tissue extract every 3 h, within a single day. The accuracy of quantitation was measured as the analytical recovery and was determined by adding known amounts of each of the sugar and sugar phosphate standard compounds to different aliquots of a pooled mouse heart tissue extract, followed by AEC derivatization, LLE, and LC/MRM-MS analysis. The spike-in amounts corresponded to 100%, 200%, and 300% of the measured sample concentrations. The resulting samples, with the IS added, were subjected to AEC derivatization and LC/MRM-MS. The percent recovery was calculated as [(mean observed concentration)/(spiked concentration)] × 100%.

UPLC/Quadrupole Time-of-Flight MS

Stable isotope-resolved metabolic profiling required higher mass resolution and was performed using an Acquity UPLC system (Waters Corp., Milford, MA) coupled to a Synapt HDMS quadrupole time-of-flight (QTOF) mass spectrometer (Waters Corp.) which was operated in the positive-ion mode. Chromatography was done using the same PFP column, using 1 mM methylphosphonic acid in water (A) and acetonitrile/0.1% formic acid in water (B) for gradient elution, with the same gradient as for LC/MRM-MS assay. Sample injections were performed in the partial-loop injection mode, with an overfill factor of 3. The mass spectrometer was tuned to give highest sensitivity of a lock-mass spray solution (50 pg/μL leucine enkephalin in 60% aqueous methanol, 10 μL/min) and was calibrated using the sodiated cluster ions from spraying a 2 mM sodium acetate solution in 50% isopropanol in water. The ESI-MS operating parameters were: spray voltage, 3.0 kV; desolvation gas (N_2) flow and temperature, 750 L/h and 350 °C; drying gas (N_2) flow and temperature, 50 L/h and 125 °C; sampling cone voltage, 35 V; extraction cone voltage, 4 V; and data acquisition rate, 0.25 s. Full-mass detection was performed over the mass range of *m/z* 100–1000. The background gas (Ar) in the collision cell was held at 0.5 mL/min. The lock-mass spray was used to ensure mass accuracy throughout the UPLC/MS run, by switching between the sample spray and the lock-mass spray every 15 s. The UPLC/QTOF MS data were processed with the MassLynx software suite (Waters Corp., Milford, MA).

RESULTS AND DISCUSION

Optimization of Derivatization

To determine which reducing sugars and sugar phosphates were present in mouse heart tissue, we performed AEC derivatization, followed by UPLC/QTOF MS. Data analysis and interpretation showed that glyceraldehyde, ribose, glucose, glyceraldehyde-3P, erythrose-4P, ribose-5P, glucose-6P, and mannose-6P were the major eight C3 to C6 reducing sugars (i.e., aldoses) and reducing sugar phosphates in mouse heart tissue. The identities of

these sugars and sugar phosphates were confirmed with the retention times and mass-to-charge ratios of their authentic compounds. Fucose, a deoxy C6 aldose, was also detected, but it was at very low abundance in all the samples tested. In addition, four reducing oligosaccharides with molecular formulas of $C_{6n}H_{12n-2}O_{6n-1}$ (n = 2 to 5) were also detected. Most of the detected C3 to C6 reducing sugars and sugar phosphates are important precursors or substrates for, or intermediates of, gluconeogenesis, glycolysis, or the PPP. To our knowledge, this is the first time that AEC derivatization was utilized for qualitative analysis of sugar phosphates in biological samples using LC/MS.

Although the optimized reaction conditions for glucose had been determined previously,[26] the optimal derivatization conditions had not been determined for other sugars or for any of the sugar phosphates targeted in this study. In order to develop an LC/MS-based method for precise and accurate quantitation of all of the C3 to C6 reducing sugars and sugar phosphates in biological samples, we optimized the AEC derivatization conditions for these compounds that were detected in the mouse heart tissue. Figure 1B,C shows the influence of reaction temperatures (60 and 70 °C) and reaction duration (from 20 to 120 min) on the completeness of derivatization. On the basis of these plots, the optimal reaction temperature and time combination was 60 °C for 80 min or 70 °C for 60 min. For the subsequent work on this project, 70 °C and 60 min were used. As shown in Figure 1B,C, neutral sugars (ribose, fucose, and glucose) exhibited slower reaction rates that required longer reaction times, while most sugar phosphates reached complete derivatization within a shorter reaction time. This may be because the phosphoryl groups of sugar phosphates provide an intramolecular acidic environment that favors formation of the activated imide intermediate with AEC, prior to reduction with $NaCNBH_3$. The use of dichloromethane–hexane (2:1, v/v), instead of chloroform,[26] as the organic solvent was found to be better than either chloroform or dichloromethane alone at removing as much excess AEC as possible from the reaction mixture prior to LC/MRM-MS analysis. The removal of AEC from the aqueous phase helped minimize the “memory effect” during LC/MRM-MS analysis. This was verified by operating the instrument in selected ion monitoring mode (i.e., by using the *m/z* 211.1 ion (the $M + H^+$ of AEC) as both the precursor and product ion monitored during the LC/MRM-MS analyses).

Optimization of the LC Separation

Initially, the chromatographic behavior of the derivatized sugars and sugar phosphates was tested on several reversed-phase C_{18} UPLC columns using water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) for binary gradient elution. Baseline separations were achieved for the derivatives of the free sugars. It was difficult, however, to separate the sugar derivatives from the sugar phosphate derivatives, even when using the optimal elution gradient conditions. This would render accurate quantitation difficult due to ionization suppression in ESI, particularly since isotopically labeled standards were not used as internal standards for quantitation of the individual sugars and sugar phosphates in this work. In addition, all of the sugar phosphate derivatives showed peak tailing on the reversed-phase C_{18} columns, which has been attributed to interactions between the phosphate group and the metallic surfaces of the UPLC system, column, connecting tubing, and the stainless-steel ESI spray capillary.[31]

To achieve better separation of the derivatized sugars from the derivatized sugar phosphates, a polar pentafluorophenyl (PFP)-bonded core–shell UPLC column was tested, and it was found that the PFP column provided an improved selectivity for separating the derivatives of sugars from those of sugar phosphates. On the other hand, although the separation of the derivatized sugars from the derivatized sugar phosphates was improved using the PFP column, the sugar phosphate derivatives showed even more pronounced peak tailing on this polar reversed-phase column. To improve the chromatographic peak shapes of the phosphate-containing analytes, phosphoric acid or phosphate buffer has been used for LC with UV detection.[31] However, the low volatility of phosphoric acid or inorganic phosphate is not compatible with ESI-MS. To reduce the unfavorable interaction of the phosphate group with the metallic surfaces, while maintaining good chromatographic separation of the analytes, we evaluated methylphosphonic acid, an organic phosphoric acid, as a tail-sweeping reagent. We found that 1 mM methylphosphonic acid in water, when used as solvent A, significantly improved the chromatographic peak shapes. Figure 2A shows an LC/MRM-MS profile of a standard solution of eight sugars and sugar phosphates and the IS on the PFP column under the optimal gradient elution conditions. As can be seen from this figure, all of the sugar phosphate derivatives showed symmetrical peak shapes, and baseline separations were obtained for most of the sugars and sugar phosphates, with the exception of the separation between glyceraldehyde-3P and erythrose-4P, which were the most difficult-to-resolve pair in this study.

The inclusion of 1 mM methylphosphonic acid in the mobile phase for positive-ion ESI did not produce any observable adverse effects on the LC/MS system in our laboratory even after more than 6 months of use, and methylphosphonic acid could be completely removed by flushing the LC/MS system with water or an aqueous organic solvent for as little as 15 min. As described in the Experimental Section, the optimal gradient for LC separation was obtained by using a PFP column for LC separation, with 1 mM methylphosphonic acid in water as solvent A for the gradient elution. To determine if the detection of glucose was affected by its two major aldose isomers, mannose and galactose, which might exist in the mouse heart tissues, separation of glucose from the potential interferences was tested by using authentic standards. As shown in Supporting Information Figure 1A, these two hexoaldoses are completely separated from glucose, although separation of mannose from galactose was not achieved under these UPLC conditions. As shown in the same figure, another reducing hexose phosphate, galactose-6P, could be completely separated from glucose-6P and partially separated from mannose-6P. Supporting Information Figure 1B shows that mannose, galactose, and galactose-6P were not detected in any of the tested mouse hearts; therefore, these reducing structural isomers could not interfere with the detection and quantitation of glucose and glucose-6P in the mouse heart samples.

Structural Isomers of Glyceraldehyde and Glyceraldehyde-3P—The combination of the PFP UPLC column and the use of methylphosphonic acid as a tail-sweeping reagent not only achieved improved separation of the derivatized aldoses and reducing sugar phosphates but also enabled MS detection of two possible structural isomers of glyceraldehyde and glyceraldehyde-3P in the mouse heart tissues. Figure 2B shows an LC/MRM-MS profile of the eight reducing sugars and sugar phosphates detected in mouse heart

tissue. Two unknown compounds, labeled as compounds **6** and **11**, were consistently detected in all of the mouse heart samples tested. Compound **6** is an isomer of glyceraldehyde-3P, whose *m/z* of the $(M + H)^+$ ion was measured by UPLC/QTOF-MS at 365.126, the same *m/z* for derivatized glyceraldehyde-3P. This isomer was tentatively assigned as glycerylaldehyde-2P, as this is the only possible aldehyde structural isomer of glyceraldehyde-3P. Figure 3A shows the separation of glyceraldehyde-3P and this putative isomer, using three different Q1/Q3 pairs, i.e., 365.1/267.2, 365.1/249.2, and 365.1/223.2. The three MRM transitions displayed different sensitivities for the two compounds. For the authentic glyceraldehyde-3P, the relative abundance of the 267.2 ion, which results from the neutral loss of a phosphoric acid molecule from the precursor ion, was 3-fold higher than that of the 249.2 ion and ca. 5-fold higher than that of the 223.2 ion. Because the putative glyceraldehyde-2P isomer showed a much lower relative abundance of the 267.2 ion, the 365.1/267.2 transition was chosen for quantitation of glyceraldehyde-3P in order to minimize interference from the putative glyceraldehyde-2P isomer in the subsequent MRM assay. Separation of the authentic glyceraldehyde-3P from the putative glyceraldehyde-2P isomer was not achieved on any of the C_{18} UPLC columns tested in our experiments, and Figure 3B shows the coelution of these two compounds on a C_{18} UPLC column with 1 mM methylphosphonic acid in solvent A. Compound **11**, detected by both UPLC/QTOF MS and LC/MRM-MS, seems to be a structural isomer of glyceraldehyde. We have tentatively assigned this compound as 1-carbon-ylproprionic acid because this is the only possible aldehyde isomer of glyceraldehyde.

Neither the putative glyceraldehyde-2P nor 1-carbonylproprionic acid has been reported as endogenous metabolites in mammals. With the current UPLC/MRM-MS method with preanalytical AEC derivatization, the interference from these putative endogenous compounds on reliable measurements of glyceraldehyde and glyceraldehyde-3P in the mouse heart tissue would be minimized. Glyceraldehyde-2P is a compound that could be produced from glyceraldehyde by the activity of a dihydroxyacetone kinase, DhaM.[32] More work, however, is needed for structural confirmation of these two potentially new endogenous compounds in mouse and other mammalian species. This, however, is beyond the scope of this present work.

Analysis Metrics

Sensitivity and Linearity—The sensitivity of this newly developed LC/MS method was determined using the optimized LC/MRM-MS quantitation method described above. Table 2 lists the resulting on-column lower-limits of detection and quantitation (LLODs and LLOQs), which ranged from femtomoles to low picomoles for the eight sugars and sugar phosphates. As expected, not only did the aromatic and heterocyclic moieties of the derivatives lead to stronger retention of the sugars and sugar phosphates on the reversed-phase PFP UPLC column, but also the tertiary amino group introduced by the derivatization with AEC created a positively charged functional group that resulted in highly sensitive detection of these derivatives in the positive-ion ESI/MS mode. The analytical sensitivity of this UPLC/MRM-MS method was compared with Buescher's ion pairing UPLC/(–)ESI-MRM-MS method which used 10 mM tributylamine as the paired ion reagent.[6] Comparisons were performed on the same MS instrument, using the same mouse heart

tissue extract, and the same four C3 to C6 reducing sugar phosphates were detected. In this comparison, our UPLC/MRM-MS method demonstrated ca. 200 to more than 1000 times higher sensitivity for these four sugar phosphates than the ion pairing LC/MRM-MS method. Supporting Information Figure 2 shows the detection of erythrose-4P using these two methods, where a sensitivity enhancement of ca. 1000 times was observed.

Table 2 also lists the measured linearity of the standard calibration curves for these sugars and sugar phosphates. Since endogenous fucose was detected at very low concentrations in all samples tested, L-(6-^{13}C)-fucose was chosen as an appropriate internal standard for the LC/MRM-MS assay. In this way, the influence of endogenous fucose on accurate quantitation was minimized. Correlation coefficients (R^2) of linear regression were ≥0.9993 for all the sugars and sugar phosphates, indicating a good quantitative relationship between the MS responses and the analyte concentrations. In our experiment, all the analytes showed dynamic ranges of >1000 for the MS responses, which covered the biological concentration ranges of all of the analytes detected in the mouse heart tissues.

Assay Precision and Accuracy—The precision of quantitation was evaluated by LC/MRM-MS as the intraday coefficients of variation (CVs) from eight different analytical replicates prepared and analyzed every 3 h for 24 h. The measured intraday CVs are included in Table 2. All the CVs were within 13% (n = 8). The chemical stabilities of the derivatives for the eight sugars and sugar phosphates were also tested by performing replicate injections of a prepared and derivatized sample solution every 6 h for 3 days. In these experiments, no significant peak area changes were observed for all analytes when the solution was kept at 5 °C in the UPLC autosampler.

To determine the accuracy of quantitation, the analytical recoveries were measured by LC/MRM-MS using the standard compound spiking-in approach for seven of the eight sugars and sugar phosphates. Table 3 lists the measured endogenous concentrations of eight sugars and sugar phosphates from a mouse heart sample, as well as the analytical recoveries at three spiking levels, corresponding to 100%, 200%, and 300% of their measured tissue concentrations, except for those of erythrose-4P (see below). All recoveries determined were within 87.4% to 109.4%, with all the CVs ≤ 8.5% (n = 6).

Erythrose-4P recovery was not tested because the content of erythrose-4P authentic compound was actually unknown, with its purity labeled only as “no less than 60%”. Thus, this authentic compound could not be used as the standard substance for accurate quantitation, and the absolute concentration of erythrose-4P in the mouse heart samples could not be determined.

The precision and recovery results indicated that the AEC derivatization-LC/MRM-MS method allowed accurate quantitation of the sugars and sugar phosphates in the mouse hearts, without the need to use the isotopic-labeling analogues for each of the analytes. In addition, only ca. 1.3 μg of the mouse heart tissue amount was consumed for each LC injection, indicating the high sensitivity of this quantitation method for the biological analysis.

Isotope-Resolved Metabolic Profiling

Stable isotope-resolved metabolite profiling is an important step for metabolic flux analysis, which is the examination of the energy flux of specific metabolic pathways within a biological system. To do this, sensitive and reliable determination of the incorporation levels of a tracing isotope into individual metabolites is required. Following our optimized AEC derivatization protocol, high-resolution UPLC/MS using a QTOF instrument was used to determine the isotopic distribution patterns of the reducing sugars and sugar phosphates detected in the heart tissues from a wild type and a *Per2*$^{-/-}$ mouse, which had been administered at 50 mg of U-^{13}C-glucose as the isotopic precursor. Supporting Information Figure 3 shows the mass spectra obtained from three of the four reducing oligosaccharides which had molecular formulas of $C_{6n}H_{12n-2}O_{6n-1}$ (n = 2 to 4). The incorporation of 6, 12, and 18 ^{13}C into these sugars was clearly observed, indicating that glucose was the substrate, or at least one of the substrates, for biosynthesis of these oligosaccharides.

Glyceraldehyde-3P, erythrose-4P, ribose-5P, and glucose-6P are the four phosphorylated aldoses involved in the PPP (Supporting Information Figure 4A), one of the CCM subpathways. With the high selectivity and the high sensitivity of this analytical method, the isotopic distribution patterns of these four sugar phosphates could be clearly observed and their incorporation levels could be precisely measured (data not shown). The observed isotopic distribution patterns of these compounds, together with that of glucose from a *Per2*$^{-/-}$ mouse heart tissue sample, are displayed in Supporting Information Figure 4B. As can be seen in the figure, erthrose-4P was detected at a very low level in the sample, and although its ion abundance was only ca. 0.02% that of glucose-6P, its isotopic distribution pattern could be clearly observed.

This experiment demonstrates the usefulness of this analytical method for isotope-resolved metabolic profiling of these reducing sugars and sugar phosphates and eliminates potential interferences from their ketose and other nonreducing structural isomers. When combined with other analytical techniques, this LC/MS method for AEC-derivatized sugars and sugar phosphates can be used for isotope-labeled metabolic flux analysis of the metabolic pathways in which they are involved.

CONCLUSIONS

In this study, we report the development of a method combining chemical derivatization with AEC and LC/MS for the targeted analysis of the major reducing sugars and sugar phosphates in mouse heart tissue. LC/MRM-MS was used for accurate quantitation, and UPLC/Q-TOF MS with full-scan detection was used for isotope-resolved metabolic profiling. This is the first evaluation of reductive amination with AEC for the selective quantitation of reducing sugar phosphates by LC/MS. Because nonreducing sugars will not react with AEC, this method results in the elimination of interferences from the nonreducing structural isomers of the individual analytes, making these measurements highly selective. The use of a PFP-bonded-phase LC column and the inclusion of methylphosphonic acid in the mobile phase resulted in good separation between the individual derivatized sugars and sugar phosphates and symmetrical chromatographic peak shapes. Methylphosphonic acid was shown to be an efficient tail-sweeping reagent that helped to minimize the unfavorable

interactions between the phosphate-containing analytes and the metallic surfaces of the LC/MS systems.

The AEC derivatization not only enhanced the chromatographic retention of the sugars and sugar phosphates, due to the hydrophobic properties of the two aromatic rings and the one heterocyclic ring of AEC, but also enabled highly sensitive detection of both sugars and sugar phosphates in the positiveion ESI-MS mode, due to the introduction of a tertiary amino group in the derivative. This allowed accurate quantitation using multiple-reaction monitoring, without the need for stable isotope-labeled standard analogues for each of the sugars and sugar phosphates, which are either costly or not commercially available. The high selectivity, high sensitivity, and high reproducibility of this LC/MS-based method demonstrated that it is well-suited for the metabolomic analysis of these free and phosphorylated aldoses in mouse heart tissue. Although only the quantitative analysis of these metabolites in mouse heart tissue is shown here, we are currently using this multiplexed MRM approach for the quantitation of these endogenous metabolites in cells and several other tissue types. The results of these studies will be presented elsewhere. This method can also be applied to the analysis of these endogenous metabolites in cells and other tissues.

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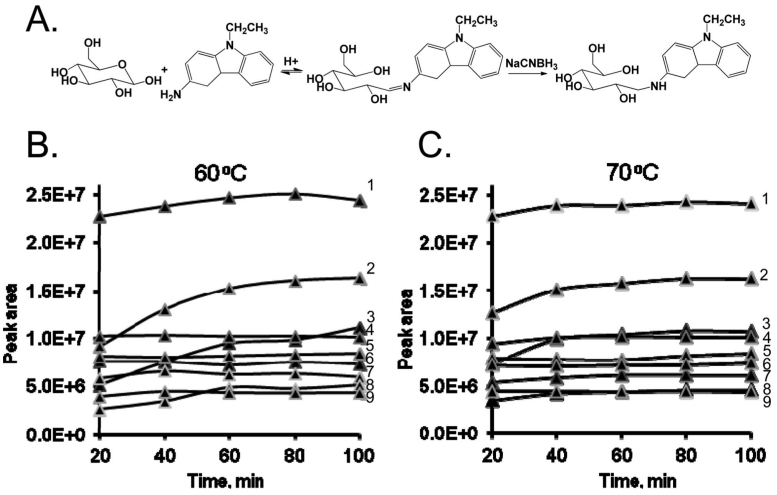

Figure 1.

Influence of reaction temperature and reaction time on AEC derivatization. (A) Reaction mechanism for the AEC derivatization of reducing sugars, using glucose as an example. (B, C) The influence of reaction temperatures (60 and 70 °C, respectively) and reaction duration (from 20 to 120 min) on the completeness of derivatization. The tested sugars and sugar phosphates are (1) ribose, (2) fucose, (3) glucose, (4) glyceraldehyde-3P, (5) ribose-5P, (6) glyceraldehyde, (7) erythrose-4P, (8) glucose-6P, and (9) mannose-6P.

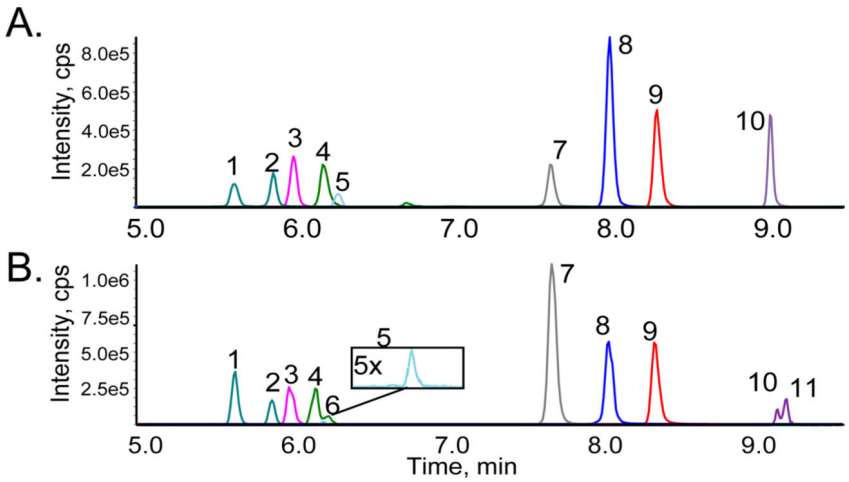

Figure 2.

UPLC/MRM-MS profiles of sugar and sugar phosphate derivatives of a standard solution (A) and a mouse heart tissue (B), on a Phenomenex core–shell PFP (2.1 × 150 mm, 2.6 μm) column with 1 mM methylphosphonic acid in water (solvent A) and acetonitrile containing aqueous 0.1% formic acid (solvent B) as the mobile phases. (1) Glucose-6P, (2) mannose-6P, (3) ribose-5P, (4) glyceraldehyde-3P, (5) erythrose-4P, (6) putative glyceraldhyde-2P, (7) glucose, (8) ribose, (9) L-6-^{13}C-fucose (IS), (10) glyceraldehyde, and (11) putative 1-carbonylpropionic acid.

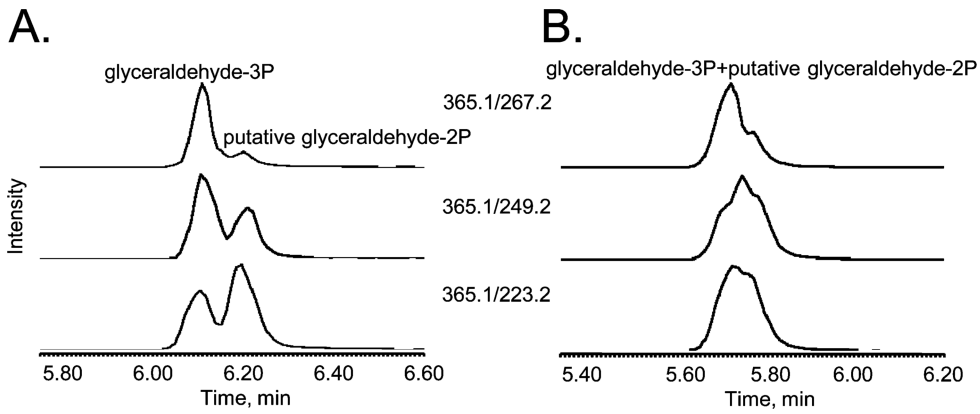

Figure 3.

Comparison of a chromatographic separation of glyceraldehyde-3-phosphate and its putative isomer, glyceraldehyde-2-phosphate, detected in mouse heart tissues. Selective MRM-MS monitoring of three different Q1/Q3 pairs on (A) a PFP core–shell UPLC column and (B) a C_{18} UPLC column.

Table 1

List of MRM Transitions of Selected Sugars and Sugar Phosphates for LC/MS/MS Assay

compound	Q1 (*m/z*)	Q3 (*m/z*)	scan time (ms)	declustering potential (V)	collision energy (V)
glucose	375.2	210.1	20	60	34
ribose	345.2	210.1	20	85	38
fucose	359.1	210.1	20	85	39
^{13}C6-fucose	360.1	210.1	20	85	39
glyceraldehyde	285.2	210.1	20	85	31
glucose-6P	455.2	210.1	20	91	45
mannose-6P	455.2	210.1	20	91	45
ribose-5P	425.2	210.1	20	85	27
erythrose-4P	395.1	297.1	20	90	25
glyceraldehyde-3P	365.1	267.1	20	95	23

Table 2

Lower Limit of Detection (LLOD), Lower Limit of Quantitation (LLOQ), Linearity, and Intraday Variation of Selected Sugars and Sugar Phosphates Analyzed by LC/MRM-MS after AEC Derivatization

compound	LLOD [pmol]	LLOQ [pmol]	linearity	R^2	intraday CV %
glucose	0.08	0.24	As/Ai = 4.9624*C* + 0.0016[a]	0.9995	7.7
ribose	0.09	0.26	As/Ai = 22.314*C* + 0.0020	0.9996	8.4
fucose	0.06	0.20	As/Ai = 15.5486*C* + 0.0007	0.9997	N/A
glyceraldehyde	0.06	0.20	As/Ai = 18.354*C* – 0.0013	0.9996	8.2
glucose-6P	0.44	1.33	As/Ai = 11.661*C* + 0.0002	0.9999	8.0
mannose-6P	0.24	0.71	As/Ai = 17.313*C* – 0.0001	0.9997	8.3
ribose-5P	0.83	2.49	As/Ai = 7.0066*C* + 0.0009	0.9998	10.2
erythrose-4P	1.37	4.12	As/Ai = 6.5420*C* – 0.0015	1.0000	7.4
glyceraldehyde-3P	0.24	0.72	As/Ai = 3.5464*C* + 0.0011	0.9993	10.2

[a] As/Ai: peak area ratio of an analyte to the IS; C: concentration in μg/mL.

Table 3

Reproducibility and Accuracy of LC/MRM-MS Analysis of Selected Sugars and Sugar Phosphates after AEC Derivatization

	concentration (n = 6)		recovery at three spiked-in levels (n = 6)					
compound	**μg/mg**	**CV%**	**100%**	**CV%**	**200%**	**CV%**	**300%**	**CV%**
glucose	0.98	4.6	98.1	5.9	91.4	3.6	94.5	4.6
ribose	0.18	6.7	108.4	6.3	95.3	4.3	97.0	1.9
glyceraldehyde	0.02	6.9	101.8	6.5	105.2	4.5	109.4	7.0
glucose-6P	0.37	7.7	99.1	5.0	95.5	4.1	95.6	4.9
mannose-6P	0.11	9.8	93.4	6.8	103.8	7.5	103.6	8.1
ribose-5P	0.15	9.0	96.7	2.8	94.3	6.6	87.4	3.4
erythrose-4P	<0.01	6.8	N/A	N/A	N/A	N/A	N/A	N/A
glyceraldehyde-3P	0.11	6.9	105.9	6.5	105.7	8.5	104.4	6.1