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A Fast and Simple Quantitative Profiling Method of Endogenous Saccharides in Rat Urine Using HILIC-MS/MS

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Abstract: To explore the metabolism of endogenous saccharides in rat, we developed a simple, fast, simultaneously quantitative method for the endogenous saccharides in rat urine, using the hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry. In order to quantify 15 endogenous saccharides in rat urine, various conditions, including columns, chromatographic conditions, MS conditions, and urine preparation methods, were investigated and optimized. The reproducibility, precision, recovery, and stability of the method were verified. The results indicated that 5 times volume of cold organic solvents (methanol/acetonitrile, v/v, 50/50) coupled with vortex for 1 min and incubated at -20°C for 20 min were the most optimal conditions for extraction. The results, according to the linearity, precision, recovery, matrix effect and stability, showed that the method was satisfactory in the quantification of endogenous saccharides in rat urine. Meeting the requirement of quantification in specific expanded metabolomic studies, the quantified analysis of endogenous saccharides in rat urine performed excellently in the sensitivity, high throughput and simple sample preparation.

Keywords: Endogenous Saccharides, Hydrophilic Interaction Chromatography, HILIC-UPLC-MS/MS, Rat Urine

1. Introduction

Saccharides are the most abundant natural products, mainly including monosaccharides, disaccharides and polysaccharides. In organisms saccharides are generally regarded as food sources and structurally fundamental blocks. Recently, many metabonomics studies on cancers and other diseases exhibited that saccharides have attracted more attention for their diverse bioactivities and biological functions, even as potential biomarkers [1-3]. Saccharides have also been described to be useful for improving vibriosis resistance and enhancing immune activity [4]. Yet, many of recent epidemiological studies, clinical trials, and animal studies continue to point to the contribution of excess dietary carbohydrate, especially fructose, to the risk factors for nonalcoholic fatty liver disease [5, 6]. Chen reported that acute myeloid leukemia (AML) cells are prone to fructose utilization with an upregulated fructose transporter GLUT5, which compensates for glucose deficiency. Notably, AML patients with upregulated transcription of the GLUT5-encoding gene SLC2A5 or increased fructose utilization have poor outcomes [7-9].

Analysis of complex carbohydrate mixture has been challengeable because of reasons as follows: many compounds of saccharides with similar property and high hydrophilicity are isomeric, monosaccharides could have different structures (ring opened or closed, different ring size and conformations), structures of oligosaccharides are often branched and most saccharides lack chromophores or fluorophores, a property that makes detection difficult [10].

Chromatographic techniques are commonly used for the analysis of saccharides. Gas chromatography (GC) [11] and gas chromatography-mass spectrometric detection (GS-MS) [12, 13] have been used for the separation of complex mixtures of saccharides. However, derivatization is the necessary step for the GC or GC/MS analysis of saccharides and is not applicable for a high throughput measurement. On

term of high performance liquid chromatography (HPLC), the lack of an effective chromophore or fluorophore group in saccharide structures prevents their direct detection from universal spectrophotometric detectors such as ultraviolet (UV) and fluorescent detection. A tedious derivatization must be performed for the detection of saccharides by UV absorption and fluorescent detectors [14]. Therefore, evaporative light-scattering detection (ELSD) is used as a semi-universal detector for saccharides during the last decade [15-17], coupled with HPLC. Yet, quantification by ELSD is less straightforward, since the response factor is generally not linear. Although The detection of compounds using mass spectrometry (MS) allows the distinction between homologues of different masses, isobars such as glucose, fructose cannot be differentiated by mass alone. Hydrophilic interaction chromatography (HILIC) blooms to appear as an alternative to reversed-phase liquid chromatography (RPLC) for separation of the polar compounds and high compatibility with mass spectrometry [Added references 18-20]. In recent years, such HILIC approaches are increasingly being chosen for various polar compounds analysis [21-23].

To explore the metabolism of endogenous saccharides in rat, in this work, we aimed at developing a simple, fast, simultaneous quantitative method for the endogenous saccharides in rat urine, using the hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry (HILIC-UPLC-ESI -MS/MS).

In order to quantify the endogenous chemicals in urine, the preparation of the endogenous-metabolite-released matrix is another critical problem for the quantitative analysis. Different from the plasma, urine carries much more endogenous chemicals. We developed a multi-step charcoal treated method to prepare the endogenous- metabolite-released urine matrix.

2. Experimental Method

2.1. Chemicals

Acetonitrile (LC-MS grade), methanol (HPLC grade), formic acid (99.5%, LC/MS), ammonium acetate and ammonium hydroxide solution (≥25% NH_3 in H_2O) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Melibiose, sucrose, maltose, maltotriose, maltotetraose, glucose, fructose, xylose, ribose, raffinose, inosine, adenosine, mannitol, erythritol, arabitol and lamivudine were also purchased from Sigma-Aldrich. L-methionine-$^{13}C_5$, ^{15}N (99%) was from Cambridge Isotope Laboratories (Andover, MA, USA). Pure water was prepared from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Endogenous-Metabolite-Released Urine Matrix

We developed a multi-step charcoal-stripping technique to prepare the endogenous-metabolite-released urine matrix. Pooled rat urine was prepared and 4 g charcoal was added into 100 mL urine. The mixture was vortexed for 30 min, and then centrifuged at 4°C, 13000 rpm for 20 min. Repeated the previous charcoal-stripping process two times with the supernatant in each step, and then the last supernatant was transferred to 96-well plate for LC-MS/MS analysis to check if all endogenous analytes were removed. Usually after three times of charcoal-stripping steps, the last supernatant will be the endogenous-metabolite-released urine matrix, with no endogenous analytes detected.

2.3. Standard Solutions

Stock solutions of the compounds were prepared by dissolving individual compound in pure water with the concentration of 10 - 100 mM and stored at -80°C until use. The compounds were divided into three groups: A, B and C, depending on their concentration in rat urine, and standard working solutions were prepared by dilution with 70% acetonitrile at different concentration levels. Calibration solutions were prepared by spiking aliquots of working solutions to endogenous-metabolite- released urine matrix. The first calibration curve was in the range of 0.001-10 μM, the second within 0.01-100 μM and the third within 0.1-1000 μM. Details on the composition of mixtures A, B, C and the concentrations were given in the Supporting Information (Table 1). The Quality Control (QC) samples were prepared in endogenous-metabolite- released urine matrix spiked with known amounts of analytes and internal standard at three concentration levels. The QC samples were also divided into three groups, including 0.5 μM (LQC), 2 μM (MQC), 10 μM (HQC) in the A group, 5 μM (LQC), 20 μM (MQC), 100 μM (HQC) in the B group, and 50 μM (LQC), 200 μM (MQC), 1000 μM (HQC) in the C group. Lamivudine and L-methionine-$^{13}C_5$, ^{15}N were used as internal standards.

Table 1. *Analytical performance of the method.*

Compound	Linearity range (nM)	LOD (nM)	LOQ (nM)	Correlation Coefficient, r
Melibiose	60-10000	60.00	18.00	0.9966
Sucrose	60-100000	60.00	18.00	0.9977
Maltose	100-100000	100.00	30.00	0.9998
Maltotriose	60-30000	60.00	18.00	0.9977
Maltotetraose	30-30000	30.00	9.00	0.9992
Glucose	600-300000	600.00	180.00	0.9953
Fructose	60-100000	60.00	18.00	0.999
Xylose	300-100000	300.00	90.00	0.9989
Ribose	3000-100000	3000.00	900.00	0.9986
Raffinose	10-100000	30.00	9.00	0.9992
Inosine	1-1000	1.00	0.30	0.9984
Adenosine	6-10000	6.00	1.80	0.9981
Mannitol	60-100000	60.00	18.00	0.9993
Erythritol	100-1000000	100.00	30.00	0.9984
Arabitol	10-100000	10.00	3	0.999

2.4. Sample Preparation

Before analysis, the urine samples were thawed to room temperature, then, an aliquot of 100 μL urine sample was precipitated with 500 μL cold ACN/MeOH (v/v, 50/50) containing 2 μM lamivudine and 10 μM L-methionine-$^{13}C_5$, ^{15}N. The mixture was vortexed for 1 min, placed at -20°C for 20 min, and then centrifuged at 4°C, 13000 rpm for 15 min. 300 μL supernatant was transferred to 96-well plate for LC-MS/MS analysis.

2.5. UPLC-MS/MS Conditions

The UPLC-MS/MS system consisted of an ACQUITY ultra performance liquid chromatographic system (Waters, Milford, MA, USA) coupled with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a Turbo Ion Source (ESI) operating with negative mode. Chromatographic separation was conducted on an ACQUITY UPLC BEH Amide Column (2.1mm ×100 mm, 1.7 μm particle size) equipped with an ACQUITY UPLC BEH Amide 1.7 μm Van-Guard Pre-column.

The column temperature was maintained at 60°C. Mobile phase A consisted of 10 mM ammonium acetate in H_2O/acetonitrile (v/v, 95/5), and mobile phase B consisted of 10 mM ammonium acetate in acetonitrile/H_2O (v/v, 95/5). The separation of saccharides was achieved using the following gradient program at a flow rate of 250 μL/min over a course of 10 min: started with a linear increase from 2% A to 40% A in 6.0 min, then maintained at 40% A for 2 min. The mobile phase was allowed to return to the initial condition within 0.1 min, followed by column re-equilibration for 1.9 min. The injection volume was 10 μL.

The compounds of interest were detected by MS/MS in negative ion multiple reaction monitoring (MRM) mode. The $[M-H]^-$ precursor ions were used for the saccharides and their internal standards. Turbo V ion source parameters were common to all analytes: The capillary voltage was operated at -4500 V, and source temperature was at 550°C. The curtain gas (N_2) and collision gas (N_2) setting were 30 psi and 10 psi, respectively. The pressure for nebulization gas and vaporization gas setting were 60 psi. The entrance potential (EP) and collision cell exit (CXP) were -50 V and -10 V, respectively. The declustering potential (DP) and collision energy were optimized for each analyte. The specific mass transition of each carbohydrate and IS were presented in Table 1. Analyst 1.5 software (AB Sciex, Foster City, CA, USA) was used for data acquisition and processing.

2.6. Method Validation

The method was validated for linearity, Limit of detection (LOD), limit of quantification (LOQ), precision, recovery and stability. The analytical curves were examined by using internal standard spiked calibration solutions, ranging from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ). The injection volume was 10 μL. Integrated peak areas of the selected quantification MRM transitions were used to build the curves, and the curves were fitted by a weighted (1/X) least squares regression analysis using the Analyst 1.5 software. LOD and LOQ were calculated based on the signal-to-noise ratio of 3:1 and 10:1, respectively. The method precision was evaluated using the standard working solution spiked to the treated matrix. Six replicates were analyzed to determine the precision. Precision was expressed as the coefficient of variation (CV) of the six replicates. The precision of retention time for each analyte was also evaluated. Recovery was calculated by dividing the corrected mean peak area of each analyte spiked before extraction by that of each analyte spiked after extraction, comparing the peak area ratio of the same concentration of analytes and IS in spiked sample [24, 25].

Matrix effect was evaluated at high QC concentration in 6 replicates. As the analytes were endogenous and the endogenous-metabolite- released surrogate matrix of charcoal-stripped rat urine was used, the matrix effect was evaluated at two levels: the endogenous urine sample compared to the endogenous-metabolite-released surrogate matrix, and the endogenous-metabolite-released surrogate matrix compared to the neat standard solution. In details, HQC work solution was spiked in the surrogate matrix, precipitated and got the peak area ratio of the analytes and IS (a); HQC work solution was spiked in the real urine sample, precipitated and got the peak area ratio of the analytes and IS (b); HQC work solution was spiked in the neat standard solution (standard in 50% acetonitrile/water, v/v), precipitated and got the peak area ratio of the analytes and IS (c); The real urine sample was precipitated directly, and got the peak area ratio of the analytes and IS, which was the endogenous value of each real urine (d); The matrix effects were calculated using a modified version of the equation described by Matuszewski et al. [26]:

$$ME^1\% = (b-d) / a \times 100\%,$$

$$ME^2\% = a / c \times 100\%$$

3. Result and Discussion

3.1. Tandem Mass Spectrometry and Chromatographic Condition Optimization

Mass spectrometry and chromatographic condition were modified, evaluated and optimized. The 15 saccharides were detected in the negative ESI mode. The declustering potential (DP) and collision energy were summarized as in the Table 2.

Table 2. *Recovery, matrix effect, precision and Retention time reproducibility of the method.*

Compound	Recovery (%)	Precision (n=6)	Retention time reproducibility (CV%)	Matrix effect[1]%	Matrix effect[2]%
Melibiose	101%	3.81%	0.24%	115.0	108.7
Sucrose	100%	0.81%	0.09%	116.0	109.2
Maltose	100%	2.48%	0.07%	117.1	110.4
Maltotriose	99%	1.56%	0.30%	109.3	119.5
Maltotetraose	95%	2.65%	0.08%	117.9	115.9
Glucose	102%	1.93%	0.14%	116.1	103.4
Fructose	104%	2.43%	0.14%	115.9	105.8
Xylose	98%	2.29%	0.31%	98.9	94.1
Ribose	100%	4.20%	0.18%	106.0	96.8
Raffinose	101%	1.16%	0.06%	119.7	109.2

Compound	Recovery (%)	Precision (n=6)	Retention time reproducibility (CV%)	Matrix effect[1]%	Matrix effect[2]%
Inosine	107%	3.99%	0.11%	99.5	104.8
Adenosine	105%	1.15%	0.00%	94.3	95.5
Mannitol	99%	0.86%	0.00%	89.9	108.7
Erythritol	104%	1.13%	0.00%	96.8	84.5
Arabitol	104%	0.80%	0.00%	110.1	110.8

Matrix effect[1]%, the spiked endogenous urine sample compared to the spiked endogenous-metabolite-released surrogate matrix.
Matrix effect[2]%, the spiked endogenous-metabolite-released surrogate matrix compared to the neat standard solution.

Columns, including Phenomenex Gemini C18 column, ACQUITY UPLC HSS T3 column, XBridge BEH C18 column, were evaluated and optimized. Newly released ACQUITY UPLC BEH Amide Column (2.1 ×100 mm, 1.7 μm particle size), among all the columns, is the one most suitable for the saccharides, with all peaks well retained and separated from each other.

The optimized chromatographic condition was shown in Section 2, with a high throughput of less than 10 min in a run. The typical MRM chromatogram of the analytes spiked in charcoal treated urine was shown in Figure 1.

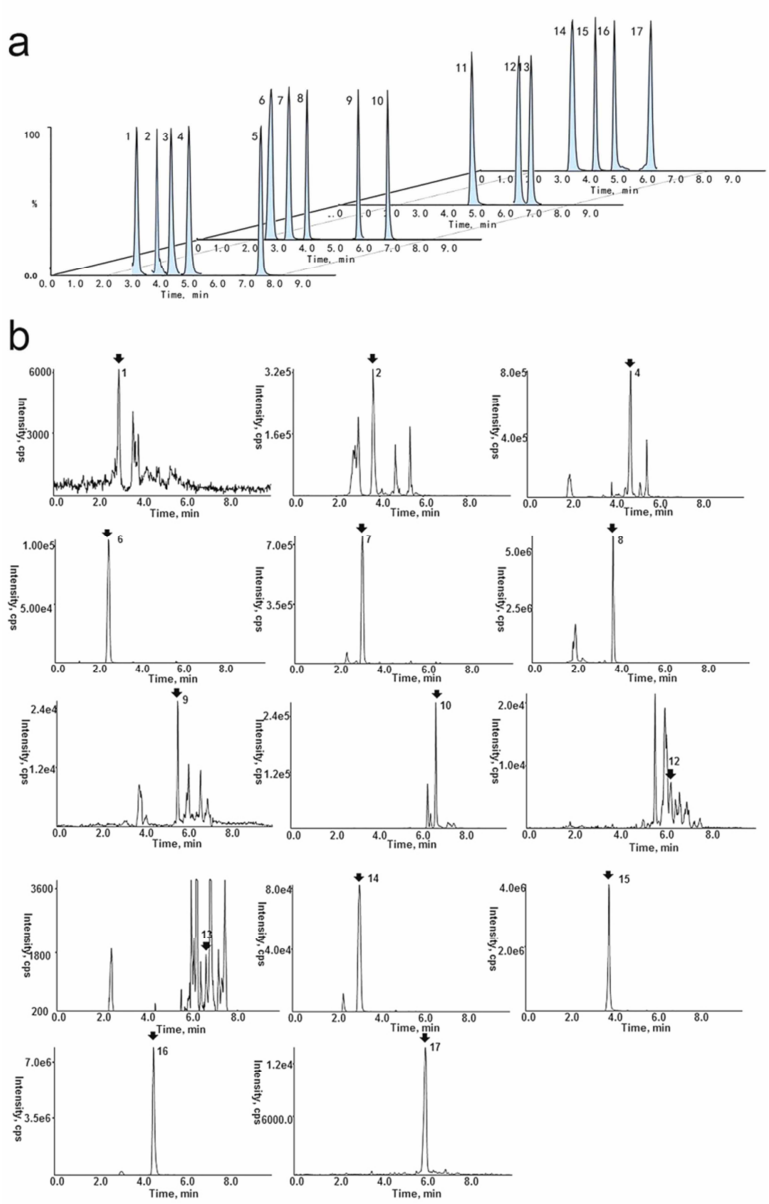

Figure 1. *Typical chromatograms of fifteen carbohydrates and two internal standards: (a) Chromatograms of standards in the neat solution; (b) Chromatograms of endogenous compounds in the rat urine sample. 1). ribose (RT: 3.06 min) 2). xylose (RT: 3.83 min) 3). fructose (RT: 4.22 min) 4). glucose (RT: 4.82 min) 5). maltotetraose (RT: 7.31 min) 6). lamivudine (RT: 2.61 min) 7). adenosine (RT: 3.18 min) 8). inosine (RT: 3.80 min) 9). sucrose (RT: 5.60 min) 10). raffinose (RT: 6.65 min) 11). methionine (RT: 4.63 min) 12). melibiose (RT: 6.30 min) 13). maltotriose (RT: 6.74 min) 14). erythritol (RT: 3.18 min) 15). arabitol (RT: 3.99 min) 16). mannitol (RT: 4.66 min) 17). maitose (RT: 5.95 min).*

3.2. Sample Preparation

Sample preparation of urine is a critical step in an accurate and reliable LC/MS/MS assay. Cold organic solvents were used for extraction. The composition of the organic solvents was evaluated and optimized. Cold solvent of ACN/MeOH (v/v, 50/50) to extract and placed at -20°C for 20min after the vortex of the mixture of solvent and urine performed excellent for the preparation of urine prior to the HILIC-UPLC-MS/MS.

3.3. Method Validation

As the analytes in the urine were endogenous, the removal of the endogenous metabolites was critical for the matrix preparation in the linearity of standards in the quantitative method. In this assay, multi-step charcoal stripped rat urine was selected as the endogenous-metabolite-released matrix for the preparation of calibration standards [23].

3.3.1. Linearity and Sensitivity

After the optimization of analytical conditions, the linearity was studied. The calibration curves showed a good linearity (mostly, correlation coefficient of $r > 0.999$), where y is the peak area ratio of the analyte to the IS and x is the urine concentration of the analyte. All back-calculated standards met the criteria of $\leq \pm 15\%$ deviation from nominal concentration. LODs and LOQs were calculated based on the signal-to-noise ratio of 3:1 and 10:1, respectively. The linear range, coefficients of determination, LODs and LOQs of all analytes were summarized in Table 1.

3.3.2. Recovery, Precision and Reproducibility of Retention Time

The precision, reproducibility of retention time and extraction recoveries of all analytes were shown in Table 2. The precision (CV) ranged from 0.799% to 4.20%. The reproducibility of retention time for the analytes, expressed as CV (%), was less than 0.307%. The recovery values ranged from 95% to 107%. Precision and recovery were all within the acceptable range, indicating that the current method was reliable and reproducible. The two levels of matrix effect performed well, with the ME^1% (the endogenous urine sample compared to the endogenous-metabolite-released surrogate matrix) ranged from 89.9 to 117.9, and the ME^2% (the endogenous-metabolite-released surrogate matrix compared to the neat standard solution) ranged from 84.5 to 119.5.

3.3.3. Application in Rat Urine Samples

The proposed method was applied in the determination of 16 Sprague Dawley rat urine samples. Using this analytical method, we were able to measure the concentration of 12 saccharides in all the 16 rat urine samples. Some of the saccharides were previously reported in the quantification of the rat urine and our data was consistent with the results in the literatures. Some of the saccharides were first quantified in the rat urine. The quantified results of saccharides were shown as in Table 3.

Table 3. *Saccharides concentration in SD rat urine.*

Compounds	Concentration (nM, n=16)
Melibiose	4016.25±263.86
Sucrose	1841.87±150.95
Maltose	16115.63±1025.23
Glucose	146442.5±17521.81
Xylose	200530±12051.87
Ribose	67768.13±5534.53
Raffinose	3298.88±288.21
Inosine	1016.15±107.94
Adenosine	14571±1754.59
Mannitol	70310±7276.6
Erythritol	403168.75±25322.17
Arabitol	248800±13550.71

4. Conclusions

In this work, hydrophilic interaction liquid chromatography was coupled with negative ion mode ESI-MS/MS for the simultaneously quantitative analysis of 15 polar metabolites, such as sugars, sugar alcohols and nucleosides in rat urine. This quantification method performed excellently in the sensitivity, high throughput and simple sample preparation, and can be well used in the specific expanded metabolomic studies after the global metabolic profiling research and metabolism research.

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Conflict of Interest

The authors have declared no conflict of interest.

Abbreviations

CE, collision energy; CV, coefficient of variation; DP, declustering potential; ELSD, evaporative light-scattering detection; GS-MS, gas chromatography-mass spectrometric detection; LLOQ, the lower limit of quantification; RPLC, reversed-phase liquid chromatography; ULOQ, the upper limit of quantification; ACN, acetonitrile; MeOH, methanol.

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