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Rapid ultra-performance liquid chromatography-tandem mass spectrometry method for the simultaneous determination of three characteristic urinary saccharide metabolites in patients with glycogen storage diseases (type Ib and II)

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ABSTRACT

Urinary 1,5-anhydroglucitol (1, 5-AG), 6-α-D-glucopyranosyl-maltotriose (Glc_4) and maltotetraose (M_4) are important biomarkers for glycogen storage disease (types Ib and II). This study aimed to develop and validate an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to detect these three urinary saccharide metabolites. Urine samples were diluted and then analyzed. Chromatographic separation was performed on an Acquity™ UPLC Amide column (2.1 × 100 mm, 1.7 μm) with gradient elution. The quantitation of analytes was achieved on a 5500 Qtrap mass spectrometer using negative multiple reaction monitoring (MRM) mode. The calibration curves for all analytes were linear over the range of 0.500 to 100 μg/mL with a correlation coefficient, $R^2 \geq 0.999$. The percent relative standard deviations (RSD%) were ≤12.8%, and the percent relative errors (RE%) were in the range of −11.7%–11.0%. The relative matrix effects of all analytes were between 87.2% and 104% with RSD% < 3.10% across three concentrations. The developed analytical method was simple, accurate, and reliable for rapid and simultaneous analysis of these three urinary saccharide metabolites. It was applied to healthy volunteers and patients. To our knowledge, it was the first validated assay for urinary maltotetraose quantification. This work provides support for exploring the potential of maltotetraose as a biomarker for Pompe disease.

1. Introduction

Glycogen storage diseases (GSDs) are a group of glycogen metabolism disorders caused by inborn enzyme defects, of which type I and type II are the most prevalent [1]. Glycogen storage disease type I (GSD I), also known as Von Gierke disease, is an autosomal recessive genetic disease and includes two main subtypes, GSD Ia and GSD Ib [2]. GSD Ib arises from a defect in the activity of glucose-6-phosphate translocase (G6PT), which plays an important role in energy metabolism [3]. G6PT transfers 1, 5-anhydroglucitol-6-phosphate (1, 5-AG6P), a metabolite derived from 1, 5-anhydroglucitol (1, 5-AG) phosphorylation, into the endoplasmic reticulum for hydrolysis. In tissues and cells deficient in G6PT activity, the excessive accumulation of 1, 5-AG6P inhibits hexokinase activity, resulting in abnormal energy metabolism and subsequent various clinical symptoms [3].

Glycogen storage disease type II (GSD II), also known as Pompe disease, results from the activity deficiency in lysosomal acid α-glucosidase (GAA), leading to irreversible damage to cardiac muscle, skeletal muscle and liver [4]. The clinical symptoms range from rapidly progressive, classic infantile-onset disease (IOPD) to a highly variable, later-onset form (LOPD). IOPD includes the classic infantile type and non-classic infantile type based on prognosis. It is characterized by generalized muscle weakness and motor developmental delay, and death typically occurs due to cardiorespiratory failure in the first year after birth [5]. LOPD progresses slowly, involves muscle weakness, decreased pulmonary function, and other systemic manifestations, and respiratory

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Table 1
Optimized transitions parameters for analytes and their ISs.

Compound	Retention time (minutes)	Mode	Transitions (*m/z*)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell exit potential (V)
1, 5-AG	1.56	Negative	162.9 > 112.7	−83	−12	−25	−6
$^{13}C_6$-1, 5-AG	1.57	Negative	168.9 > 118.0	−92	−10	−20	−6
Glc_4	6.07	Negative	665.2 > 178.9	−115	−10	−38	−9
M_4	5.90	Negative	665.2 > 178.9	−114	−10	−30	−9
$^{13}C_6$-Glc_4	6.07	Negative	671.2 > 185.0	−200	−7	−35	−10

failure is the leading cause of death [6].

Treatment for GSDs was palliative until the approval of enzyme replacement therapy (ERT) [7]. However, there remains a substantial unmet medical need, mainly due to the inability of ERT to penetrate the blood–brain barrier and its immunogenicity. In recent years, gene therapy products based on adeno-associated virus (AAV) are developing rapidly, and many AAV products have entered the clinical trial stage around the world [8]. With the development of new treatments, more specific and sensitive methods are needed to detect biomarkers for monitoring disease progression and determining treatment response.

6-α-D-glucopyranosyl-maltotriose (Glc_4) is derived from the degradation of glycogen by GAA, then secreted into plasma and excreted in the urine. The urinary secretion of Glc_4 is an important biomarker for measuring the progress of GSD type II, III, and VI [9–12]. In a cohort study, elevated urinary Glc_4 excretions were observed in 66.7% (10/15) GSD Ia and 90% (9/10) GSD Ib patients urine samples, suggesting that Glc_4 may also be a biomarker for these diseases [12]. Maltotetraose (M_4), an isomer of Glc_4, is also released into plasma and urine during the degradation of glycogen by GAA [13]. In previous studies, M_4 was detected in urine samples from patients with Pompe disease, but it did not receive enough attention due to its low detection rate, and was generally considered as an interference of Glc_4 [4,11]. However, in this study, we found that M_4 was rapidly degraded in urine, and it can be prevented by adjusting the pH of the urine samples to around 9.50. Therefore, urinary M_4 excretion may be a potential biomarker for Pompe disease progression that has not been widely investigated due to its instability.

The renal sodium-glucose co-transporter 2 (SGLT2) inhibitor reduces renal 1, 5-AG reabsorption, resulting in decreased levels of 1, 5-AG6P in blood and cellular [14,15]. In addition, the SGLT2 inhibitors can restore normal neutrophil count and function in patients with GSD Ib, and some studies have confirmed its efficacy against GSD Ib [15–17]. Therefore, the urinary excretion of 1, 5-AG is an important biomarker for evaluating the therapeutic response of SGLT2 inhibitors to patients with GSD Ib [15].

In this study, an efficient assay was needed to quantify these three urinary saccharide metabolites for comprehensive assessment of treatment response in patients with GSD (type Ib and type II). Over the past few decades, several assays for Glc_4, M_4, and 1, 5-AG have been developed, including thin-layer chromatography (TLC) [18], high-performance liquid chromatography (HPLC) [4,19], and liquid chromatography-mass spectrometry (LC-MS) [11,20–22]. However, a method for the rapid simultaneous determination of urinary Glc_4, M_4, and 1, 5-AG has not been reported. Among these methods, only the HPLC method developed by An Y et al [4] and the LC-MS method developed by Sluiter et al [11] were reported to discriminate Glc_4 and M_4. However, the HPLC method [4] required a long chromatographic run time and butyl-p-aminobenzoate derivatization, a time-consuming preparation procedure. Urine samples were analyzed after simple dilution in the LC-MS method [11]. However, this assay cannot quantify M_4, which may cause M_4 to lose its potential as a biomarker.

Therefore, we aimed to develop a simple and rapid ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the simultaneous determination of these three urinary saccharide metabolites. 1, 5-AG, Glc_4 and M_4 can be accurately separated and quantified in the developed method without complicated preparations. Ultimately, the method was successfully to healthy volunteers and patients with GSDs (type Ib and type II).

2. Materials and methods

2.1. Chemicals and reagents

6-α-D-glucopyranosyl-maltotriose (Glc_4, Purity, 97.0%) and its internal standard (IS) $^{13}C_6$-6-α-D-glucopyranosyl-maltotriose ($^{13}C_6$-Glc_4, Purity, 96.0%) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Maltotetraose (M_4, Purity, 97.0%) was bought from ANPEL-TRACE Standard Technical Services (Shanghai) Co., Ltd (Shanghai, China). 1, 5-anhydroglucitol (1, 5-AG, Purity, ≥98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and its IS 1, 5-anhydro-D-$^{13}C_6$-glucitol ($^{13}C_6$-1, 5-AG, Purity, 99.9%) was bought from SHANGHAI ZZBIO CO,. LTD (Shanghai, China). HPLC-grade acetonitrile was purchased from Honeywell (Morris Plains, NJ, USA). Ammonia solution (A.R. grade) was bought from Xilong Scientific Co., Ltd (Shantou, Guangdong, China). Deionized water was purified with a Milli-Q® Ultrapure water system (Millipore Corporation, Bedford, MA, USA). Artificial urine was obtained from Dongguan Chuangfeng Automation Technology Co., Ltd. (DongGuan, Guangdong, China).

2.2. UPLC–MS/MS conditions

A LC-30A UPLC instrument (Shimadzu, Japan) with two solvent delivery units (LC-30AD XR), communication bus module (CBM-30A), autosampler (SIL-30AC XR), degasser (DGU-30A3R), and column oven (CTO-30AC) was utilized for chromatographic analysis. And a 5500 Qtrap mass spectrometer (Applied Biosystems, USA) was used for the detection of targeted analytes. Data acquisition and processing were performed on Analyst software (version 1.7.1, Applied Biosystems, USA).

An ACQUITY™ UPLC BEH amide column (2.1 × 100 mm, 1.7 μm, Waters Corp., Milford, MA, USA) was employed to separate urinary saccharide metabolites. The mobile phase comprised of 0.1% ammonia solution (A) and ACN containing 0.1% ammonia solution (B). The gradient elution program was optimized as follows: 0.0–7.0 min, from 80% (B) to 59% (B); 7.0–7.1 min, from 59% (B) to 80%; 7.1–10.0 min, 80% (B). The flow rate was 0.4 mL/minute, and the column temperature was set to 40 °C with an injection volume of 1 μL.

The negative electrospray ionization and multiple reaction monitoring (MRM) mode were utilized to detect all analytes. The mass spectrometry conditions were as follows: gas1, 55 psi; gas2, 55 psi; curtain gas (CUR), nitrogen, 35 psi; collision gas, Medium; temperature, 550 °C; the Ionspray Voltage, −4500 V. The ion transition and ionization conditions were optimized for maximum response and the optimized transition parameters were listed in Table 1.

2.3. Stock solutions, calibration standards and quality controls (QC)

The stock solutions (1.00 mg/mL) of Glc_4, M_4, 1, 5-AG and their ISs were separately prepared in methanol–water (v/v, 50/50). The calibration standards were prepared in artificial urine at the concentrations

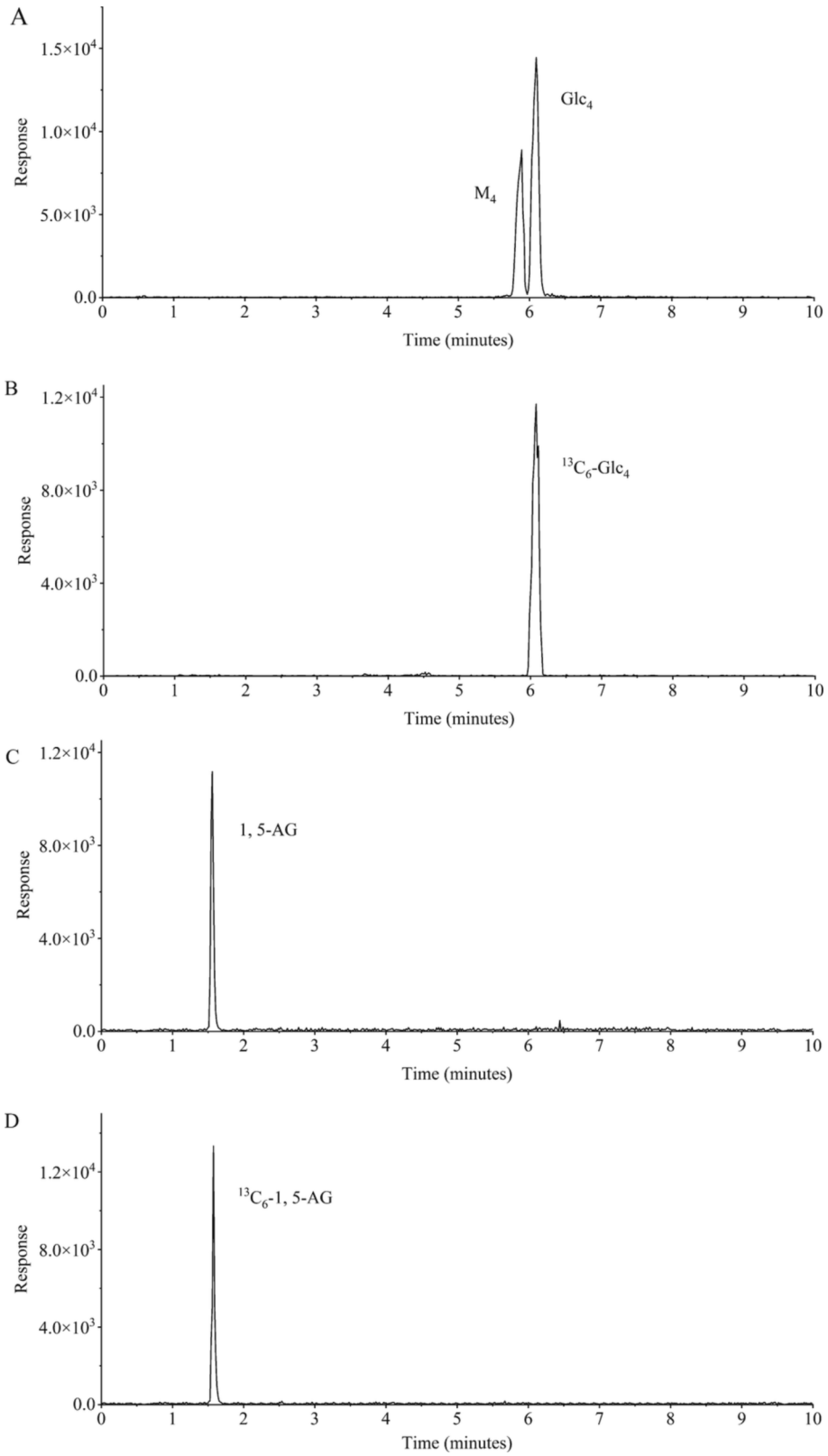

Fig. 1. The representative chromatograms of Glc_4 and M_4 (A), $^{13}C_6$-Glc_4 (B), 1, 5-AG (C) and $^{13}C_6$-1, 5-AG (D) in processed sample.

of 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 μg/mL for all analytes. The lower limit of quantitation (LLOQ) samples were prepared at the concentrations of 0.500 μg/mL in artificial urine for all analytes. The low-concentration quality control (LQC), medium-concentration quality control (MQC) and high-concentration quality control (HQC) samples of all analytes were spiked in pooled urine at the concentrations of 1.00, 10.0, and 50.0 μg/mL, respectively. The IS was diluted to 5.00 μg/mL in acetonitrile–water (v/v, 50/50). All stock solutions, calibration standards, QC, and IS were stored at −80 ℃ until analysis.

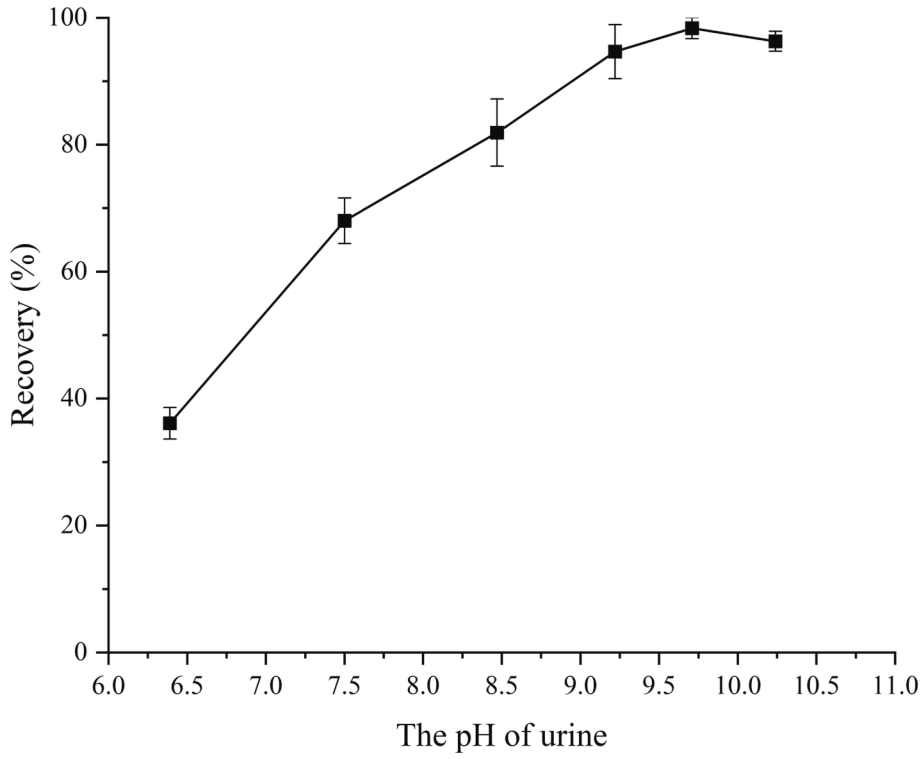

Fig. 2. The effect of ammonia solution on the stability of M4 in urine.

2.4. Sample preparation

Urine samples were thawed at 37 °C, and 10 μL aliquots were transferred to 1.5 mL Eppendorf tubes, then 10 μL IS and 380 μL 0.1% ammonia solution were added. After vortex mixing and centrifugation at 17000g for 10 min, 1 μL of the supernatant was injected into UPLC-MS/MS system for analysis.

2.5. Method validation

The method was validated in accordance with the Clinical and Laboratory Standards Institute guideline (CLSI 62-A)[23] and the US Food and Drug Administration guideline[24].

2.5.1. Linearity

The peak area ratios of the analytes to their ISs at eight levels were plotted against the nominal concentration (x) by least squares linear regression with a weighting factor of $1/X^2$ to evaluate the linearity. The percent relative error (RE%) should be within ± 15% of the nominal concentrations (±20% for LLOQ), and the correlation coefficients (R^2) was required to be ≥ 0.990.

2.5.2. Selectivity

Six copies of artificial urine from different batches were analyzed and compared with their corresponding spiked LLOQ samples to evaluate the selectivity. Responses attributable to interfering components should be within 20% of the analytes' response in the LLOQ sample (≤5% for IS).

2.5.3. Precision, accuracy and LLOQ

Six replicates of LLOQ, LQC, MQC, and HQC samples were analyzed to assess the precision, accuracy and LLOQ in three different days. The precision was calculated as the percent relative standard deviation (RSD %), while the accuracy was calculated as RE%.

2.5.4. Matrix effect

1, 5-AG, Glc_4 and M_4 were endogenous substances, and it is difficult to find a blank urine matrix without them. Therefore, the relative matrix effect was evaluated using a mixing experiment. The solution matrix was prepared at the concentration of low (1.50 μg/mL), medium (7.50 μg/mL) and high (75.0 μg/mL) in artificial urine. Six different urine matrices were separately mixed with the solution matrix at a ratio of 1:1 to make the corresponding mixed matrix, and each in triplicate. The concentration of the mixed matrix samples should deviate less than 20% from the mean concentration of the urine matrix samples and the solution matrix samples. And the RSD% of matrix effect factor should be less than 15%.

2.5.5. Carryover

In each batch, a double blank sample after the highest calibration standard sample was analyzed to assess the method carryover. Carryover in the double blank samples should not be greater than 20% of the response of analyte in the LLOQ and 5% of the response of the IS.

2.5.6. Stability

The stability was assessed by analyzing six replicates of spiked LQC and HQC samples. The short-term stability was assessed after the QC samples were kept at room temperature for 12 h. The long-term stability was assessed after the QC samples were kept at −80 °C for 53 days. The autosampler stability was tested after the processed QC samples were placed in an autosampler (10 °C) for 24 h. The freeze–thaw stability was evaluated after three cycles of the freeze (-80 °C) and thaw (room temperature) before QC samples preparation. The stock solution stability was assessed after being kept at room temperature for 8 h.

2.6. Method application

A total of 11 random urine samples were collected from anonymous healthy volunteers (n = 6), GSD Ib (n = 3) and Pompe disease (n = 2) patients in Peking Union Medical College Hospital for the assessment of treatment response. The study was in accordance with the Declaration of Helsinki and was approved by the Peking Union Medical College Hospital Ethics Committee (I-22PJ394). After sample collection, the pH of

Table 2
Linearity for all analytes.

Compound	Range	Calibration Curves	R^2
Glc_4	0.500 ~ 100 μg/mL	Y = 0.234X + 0.0265	0.999
M_4	0.500 ~ 100 μg/mL	Y = 0.107X + 0.0120	0.999
1,5-AG	0.500 ~ 100 μg/mL	Y = 0.0341X + 0.00325	0.999

the urine was adjusted to around 9.50, then the samples were stored at −80 °C until analysis. Due to high intra- and inter-individual variation in random urine collected, the saccharide metabolites concentration was corrected by urinary creatinine, which was determined based on the LC-MS/MS method reported by Dziadosz et al[25].

3. Results and discussion

3.1. Method development

3.1.1. UPLC-MS/MS

The stock solutions (1.00 mg/mL) of the analytes and their ISs were diluted separately to 100 ng/mL with methanol–water (v/v, 50/50), then injected into the mass spectrometer for optimized ionization parameters (Table 1). Different chromatographic columns (Acquity™ UPLC BEH C_{18}, Acquity™ UPLC HSS T3, Acquity™ UPLC BEH Amide, 2.1 × 100 mm 1.7 μm, Waters, Eschborn, Germany), mobile phases (methanol and acetonitrile) and mobile phase additives (acetic acid, ammonium acetate, and ammonia solution) were tested, and the Acquity™ UPLC BEH Amide column provided the optimal peak shape and separation under gradient elution using 0.1% ammonia solution (A) and acetonitrile containing 0.1% ammonia solution (B) as mobile phases. The representative chromatograms of analytes and their ISs in prepared sample (5.00 μg/mL) were shown in Fig. 1.

3.1.2. Sample preparation

It was found that M_4 was unstable in real urine samples, and the pH may be the cause. Therefore, a certain volume of ammonia solution was added to the urine sample spiked with 10.0 μg/mL M_4 to evaluate the effect of pH on the stability of M_4 in urine, which was described by the recovery. As shown in Fig. 2, **M4** was stable when the pH of the urine was adjusted to 9.00 ~ 10.0. Therefore, the pH of urine samples was adjusted to around 9.50 immediately after collection, and then stored at −80 °C until analysis.

Different methods of sample preparation (direct dilution, liquid–liquid extraction and solid-phase extraction) were explored to reduce matrix interference, and direct dilution was adopted due to its simplicity and expediency. Initially, the acetonitrile–water (v/v, 80/20) containing 0.1% ammonia solution was used as the diluent solution. However, the response of Glc_4 and its IS fluctuated considerably under this

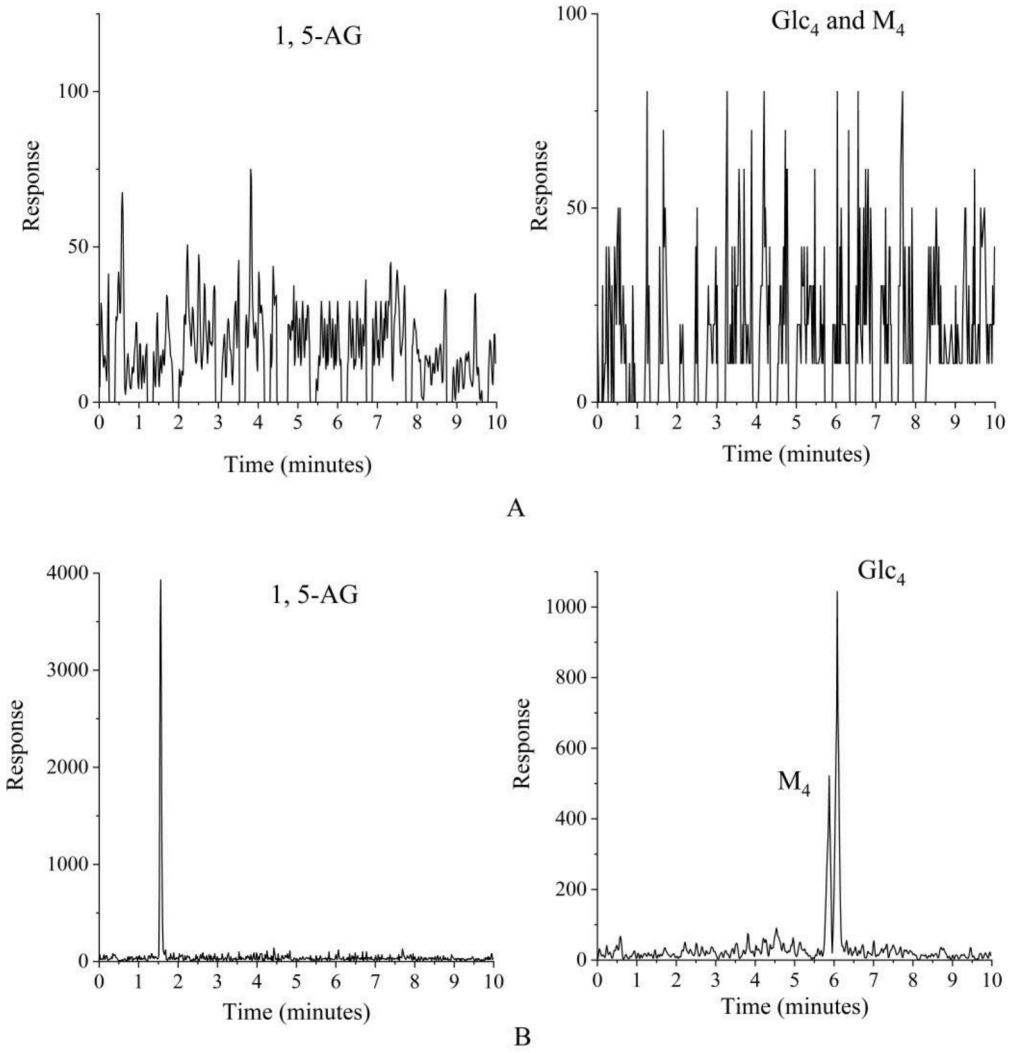

Fig. 3. The UPLC/MS/MS chromatograms of extracted blank plasmas (A) and spiked with the lower limit of quantitation of 0.500 μg/mL in blank plasmas (B) of all analytes.

Table 3
Precision and accuracy of all analytes.

Run Batch	Analytes	LLOQ		LQC		MQC		HQC	
		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
Intra-day 1 (n = 6)	Glc_4	10.6	−1.01	2.79	3.02	1.71	3.01	1.61	0.00
	M_4	12.8	−2.30	3.41	−10.9	3.51	−9.60	2.8	−9.60
	1,5-AG	9.51	7.01	2.02	9.11	2.52	11.0	2.31	8.00
Intra-day 2 (n = 6)	Glc_4	5.71	2.10	2.78	−2.12	1.31	−2.51	1.56	−4.91
	M_4	1.71	−3.01	3.42	−9.12	2.5	−7.01	1.2	−9.01
	1,5-AG	0.701	−2.09	3.11	−14.7	1.67	−5.91	0.901	−6.11
Intra-day 3 (n = 6)	Glc_4	0.401	6.11	3.52	−5.51	2.11	−1.21	2.21	−3.70
	M_4	7.62	−6.81	3.76	−3.09	2.6	0.00	1.78	−1.81
	1,5-AG	2.41	−1.51	3.01	−0.800	2.01	0.00	1.11	0.00
Inter-day (n = 18)	Glc_4	6.12	2.01	4.38	−1.61	2.78	−0.301	2.78	−2.80
	M_4	7.11	−4.02	5.21	−5.20	4.78	−5.21	4.01	−6.70
	1,5-AG	6.61	1.14	6.78	7.00	7.1	2.00	6.12	1.10

Table 4
Relative matrix effects of all analytes.

Analytes	Relative matrix effect, %			RSD, %
	LQC	MQC	HQC	
Glc_4	99.3	99.5	104	2.52
M_4	92.4	87.2	88.1	3.10
1,5-AG	101	99.5	99.4	0.863

condition. Later, acetonitrile–water (v/v, 50/50) containing 0.1% ammonia solution, acetonitrile–water (v/v, 20/80) containing 0.1% ammonia solution and 0.1% ammonia solution were tested, and the 0.1% ammonia solution provided stable response. Dilution multiple was a compromise between the lower limit of quantification and matrix effects, and the 40-fold dilution obtained both the LLOQ of 0.500 μg/mL and negligible matrix interference.

3.2. Method validation

3.2.1. Linearity

The method showed good linearity with correlation coefficients (R^2) of greater than 0.999 between 0.500 ~ 100 μg/mL (Table 2) for all analytes.

3.2.2. Selectivity

No potential interfering substances at retention times of the analytes and their ISs was observed in double blank samples (Fig. 3).

3.2.3. Precision, accuracy and LLOQ

The intra- and inter-day precision RSD% were both less than 12.8%, and the RE% were in the range of −14.7%~11.0% (Table 3). Therefore, the precision and accuracy of the method met the requirements of the international guidelines.

3.2.4. Matrix effects

The relative matrix effects of the analytes were all in the range of

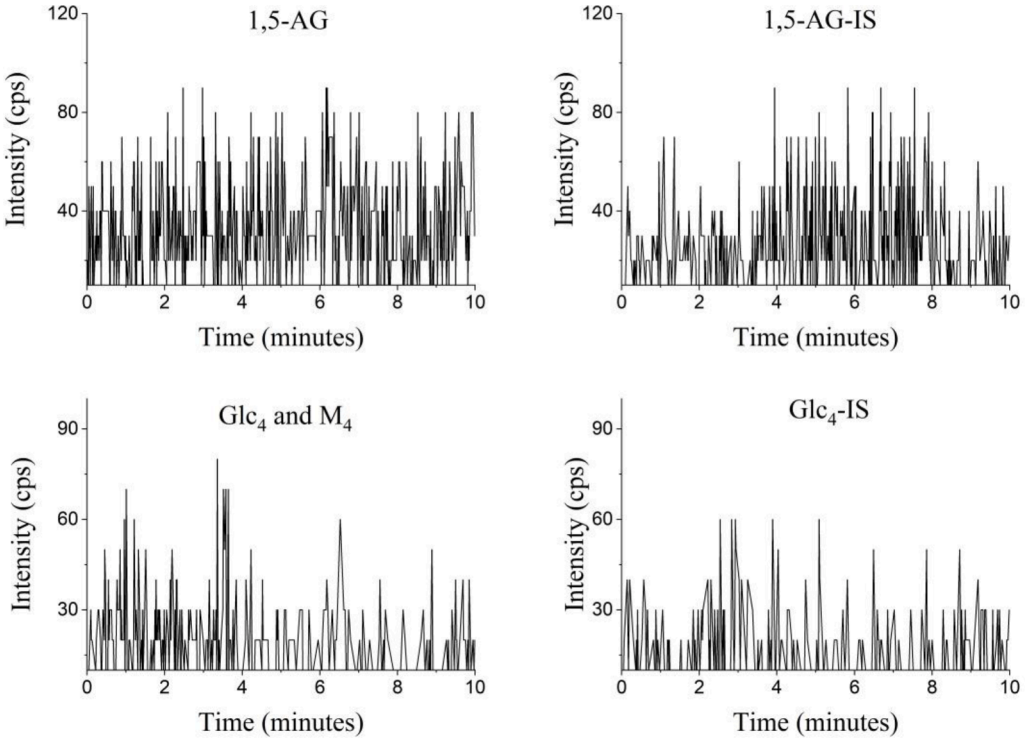

Fig. 4. The chromatograms of the double blank samples after the highest calibration standard samples.

Table 5
Stability of all analytes under different conditions in urine.

Conditions	Analyte	LQC		HQC	
		RE (%)	RSD (%)	RE (%)	RSD (%)
Short-term stability (room temperature for 12 h)	Glc_4	3.42	2.82	−0.517	1.33
	M_4	6.21	3.44	−0.398	3.11
	1,5-AG	0.291	1.56	−0.695	1.59
Autosampler stability (10 ℃ for 24 h)	Glc_4	4.77	1.57	0.00	2.53
	M_4	5.80	1.74	0.752	1.76
	1,5-AG	−0.870	3.28	0.873	2.18
Freeze and thaw stability (−80 ℃ to room temperature, 3 cycles)	Glc_4	7.45	3.17	1.05	1.88
	M_4	8.20	4.64	2.23	0.787
	1,5-AG	0.291	3.85	−0.476	2.77
Long-term stability (−80 ℃ for 53 days)	Glc_4	2.83	4.57	3.24	3.07
	M_4	4.21	6.06	3.63	3.86
	1,5-AG	−0.581	2.15	0.576	1.60

87.2%~104% with RSD% < 3.10%, and met the requirements of the intended use (Table 4).

3.2.5. Carryover

No significant peak of all analytes and their ISs was observed in double blank samples after the highest calibration standard samples (Fig. 4).

3.2.6. Stability

After being kept at room temperature for 8 h, the RE% of stock solution were from −7.90% to 3.39%, and the RSD% were less than 7.34%. The samples stability results were shown in Table 5. The RSD% were less than 6.06%, and the RE% ranged from −0.870% to 8.20%, indicating that these analytes were stable during routine storage and preparation.

3.3. Method application

Urinary saccharide metabolites excretion levels of 6 healthy volunteers and 3 patients were summarized in Table 6. M_4 was not detected in the urine of healthy volunteers, but was detected in the urine of patients with GSD Ib and Pompe disease. Urinary 1,5-AG level was elevated in the GSD Ib patient after receiving 10 mg empagliflozin orally once daily for one day, and decreased after empagliflozin treatment for 11 days. It was probably due to the fact that urinary 1,5-AG excretion was increased immediately after receiving empagliflozin treatment, and as treatment duration increases, 1,5-AG levels in the GSD Ib patient gradually decreased and returned to normal human levels. In addition, the urinary Glc_4 and M_4 levels of GSD Ib patients also increased, and the change trends were similar to that of 1,5-AG. Urinary Glc_4 and M_4 levels were elevated in Pompe disease patients, and M_4 accounted for 4.62% ~5.12% of urinary tetrasaccharides, which was consistent with the study reported by An et al[26]. Therefore, the excretion levels of these three urinary characteristic saccharide metabolites have the potential to be used as disease biomarkers. However, due to the small number of samples, it is difficult to determine the specific quantitative judgment criteria, which requires further research.

In the past few decades, several assays have been developed for the detection of Glc_4, M_4, and 1, 5-AG, including TLC[18], HPLC[4,19], and LC-MS[11,20–22]. Compared with these assays, the present method has unique advantages in high analytical efficiency, high detection sensitivity and small sample volume (Table 7). In addition, this method can accurately quantify M_4 in urine, which has not been reported in previous methods, and can provide support for exploring the potential of M_4 as a biomarker.

4. Conclusion

An UPLC-MS/MS method was developed and validated for the simultaneous analysis of three urinary saccharide metabolites in human urine. The method was simple, rapid, efficient, sensitive and robust. Ultimately, the method was successfully applied to patients with GSD Ib and Pompe disease for the assessment of treatment response. To our knowledge, this is the first validated assay for urinary M_4 and can provide support for exploring the potential of M_4 as a biomarker for Pompe disease.

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Ethical approval

The study was approved by the Peking Union Medical College

Table 6
Summary of urinary saccharide metabolites levels in subjects.

Subject number	Conditions	Sample collection	Glc_4 (μg/mg creatinine)	M_4 (μg/mg creatinine)	1,5-AG (μg/mg creatinine)
1	Health	None	4.51	0.00	3.01
2	Health	None	11.3	0.00	8.71
3	Health	None	9.02	0.00	9.01
4	Health	None	3.71	0.00	27.5
5	Health	None	7.22	0.00	5.80
6	Health	None	3.51	0.00	26.8
7	GSD Ib	Before treatment	90.4	4.51	56.8
7	GSD Ib	1 day after empagliflozin treatment	150	7.00	166
7	GSD Ib	11 days after empagliflozin treatment	55.2	1.31	114
8	Pompe disease	Before treatment	102	5.51	115
9	Pompe disease	Before treatment	328	15.9	5.61

Table 7
Comparison of this method with methods reported previously.

Method	Analytes	Distinguishing Glc_4 from M_4	Preparation	Instrument	LLOQ (μg/mL)	Run time (minutes)	Sample volume (μL)
An et al. [4]	Glc_4	Yes	Derivatization	HPLC	1.20	35.0	50.0
Sluiter et al. [11]	Glc_4	Yes	Direct dilution	LC-MS	2.00	10.0	100
Manwaring et al. [19]	Glc_4	Yes	Desalting and centrifugation	HPLC	5.00	40.0	1000
Young et al. [21]	Glc_4	No	Derivatization	LC-MS	2.00	40.0	50.0
This method	Glc_4, M_4 and 1,5-AG	Yes	Direct dilution	LC-MS	0.500	10.0	10.0

Hospital Ethics Committee (I-22PJ394).

CRediT authorship contribution statement

Jianwei Ren: Methodology, Validation, Writing – original draft. **Yufang Ma:** Writing – review & editing. **Mingsheng Ma:** Investigation, Funding acquisition. **Juan Ding:** Investigation. **Jingjing Jiang:** Investigation. **Xin Zheng:** Writing – review & editing, Funding acquisition. **Xiaohong Han:** Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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