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A novel method for the determination of 1,5-anhydroglucitol, a glycemic marker, in human urine utilizing hydrophilic interaction liquid chromatography/MS^3

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ABSTRACT

Plasma levels of 1,5-anhydroglucitol (1-deoxyglucose), a short-term marker of glycemic control, have been measured and used clinically in Japan since the early 1990s. Plasma levels of 1,5-anhydroglucitol are typically measured using either a commercially available enzymatic kit or GC/MS. A more sensitive method is needed for the analysis of 1,5-anhydroglucitol in urine, where levels are significantly lower than in plasma. We have developed a sensitive and selective LC/MS^3 assay utilizing hydrophilic interaction liquid chromatography and ion trap mass spectrometry for the quantitative determination of 1,5-anhydroglucitol in human urine. Diluted human urine samples were analyzed by LC/MS^3 using an APCI source operated in the negative ionization mode. Use of an ion trap allowed monitoring of MS^3 transitions for both 1,5-anhydroglucitol and the internal standard which provided sufficient selectivity and sensitivity for analysis from 50 μL of human urine. Quantitation of 1,5-anhydroglucitol levels in urine was accomplished using a calibration curve generated in water (calibration range 50 ng/mL to 10 μg/mL). Method ruggedness and reproducibility were evaluated by determining the intra- and inter-day accuracies and precision of the assay, as well as the bench–top and freeze–thaw stability. For both inter- and intra-assay evaluations, the accuracy of the assay was found to be acceptable, with the concentrations of all QCs tested not deviating more than 8% from theoretical. Four-hour bench–top and freeze–thaw stabilities were also evaluated; 1,5-anhydroglucitol was found to be stable at room temperature (<18% deviation from theoretical) and during 3 freeze–thaw cycles (<1% deviation from theoretical, except at the lowest QC level). The LC/MS^3 assay was then used to successfully determine the concentration of 1,5-AG in more than 200 urine samples from diabetic patients enrolled in a clinical study.

1. Introduction

1,5-Anhydroglucitol (1,5-AG) is one of the major polyols in the human body and is derived primarily from the diet. Levels of 1,5-AG are particularly high in foods such as soy beans, rice and beef [1]. Plasma concentrations of 1,5-AG are known to track plasma glucose levels and, therefore, monitoring of 1,5-AG has been of interest in the diabetes field, most notably in Japan [1–9]. Monitoring of glycemic control in diabetic patients is traditionally performed by measuring glycated serum proteins, most notably hemoglobin A_{1C} (HbA_{1C}) and fructosamine, both of which provide an average index of glycemic control over a period of several weeks [10–13]. Plasma levels of 1,5-anhydroglucitol (1,5-AG) (Fig. 1) have been shown to decrease in patients with diabetes [2,3,9]. The decrease of 1,5-AG in plasma is caused by competitive inhibition of 1,5-AG reabsorption in the kidney tubule by glucose during hyperglycemia [14]. This inhibition of 1,5-AG plasma reabsorption results in increased 1,5-AG urinary excretion. When blood glucose levels return to baseline, plasma reabsorption of 1,5-AG is also restored, thereby returning plasma levels of 1,5-AG to baseline levels. The response of 1,5-AG to changing blood glucose levels is extremely sensitive (1,5-AG levels reflect virtually real-time changes in blood glucose) making 1,5-AG a useful marker of short-term glycemic control. In contrast, HbA_{1C} and fructosamine both require 2–6 weeks or longer to reflect changes in glycemia [2,3]. Additionally, 1,5-AG plasma levels are extremely stable in euglycemic individuals and are not affected by prandial state, body weight or age [2,7]. 1,5-AG is metabolically inert and plasma levels remain fairly constant over 24 h, allowing flexibility in time of daily sampling [1], an advantage over blood glucose which is subject to diurnal variation and therefore requires

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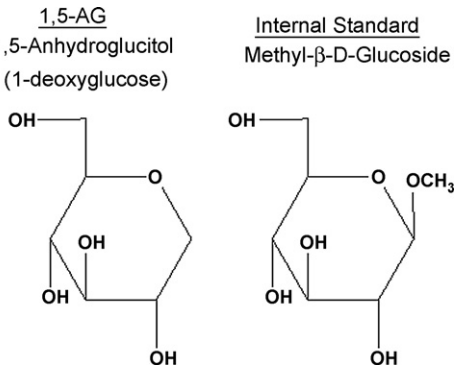

Fig. 1. Structure of 1,5-anhydroglucitol (1,5-AG) and the internal standard methyl-β-D-glucoside.

total 24-hour urine collection. In Japan 1,5-AG has been used as a marker of glycemic control since the early 1990s [5–7]. Although 1,5-AG monitoring is not yet used routinely in US clinics, its potential as a glycemic marker has been documented [15].

Plasma levels of 1,5-AG have been measured using several different analytical techniques. The GlycoMark™ assay was developed for the analysis of 1,5-AG in serum [15,16]. The basis of the assay is conversion of 1,5-AG to 1,5-anhydrofructose via pyranose oxidase. Because pyranose oxidase will also oxidize glucose, serum glucose must first be removed or converted to another form that is not reactive. The GlycoMark™ assay converts interfering glucose to glucose-6-phosphate. 1,5-AG is then oxidized by pyranose oxidase and the resulting hydrogen peroxide by-product is measured using a standard peroxidase assay. Detection limits of the assay are approximately 0.3 mg/L based on 0.5 mL of serum. 1,5-AG in serum has also been measured by gas chromatography-mass spectrometry (GC/MS) [1,8,17]. Analysis of 1,5-AG by GC/MS requires labor intensive sample preparation (typically protein precipitation or protein precipitation coupled with ion-exchange chromatography) followed by derivatization, typically via acetylation. High-performance liquid chromatography (HPLC) analysis of 1,5-AG in serum and urine has also been reported. Serum levels of 1,5-AG have been determined by first passing the samples through a 2- or 3-layer ion exchange column and then analyzing by HPLC with pulsed amperometric detection or an enzyme sensor [18]. Detection of 1,5-AG indirectly through oxidation by pyranose oxidase producing hydrogen peroxide (the basis of the GlycoMark™ assay) was also applied to an HPLC platform for determination of 1,5-AG in urine [19]. Typical detection limits for 1,5-AG in serum or urine by HPLC were reported between 100 and 200 ng/mL. Niwa and colleagues analyzed 1,5-AG in serum using cation-exchange chromatography and liquid-chromatography mass spectrometry (LC/MS). In this method 1,5-AG was monitored as its chloride adduct using an atmospheric pressure chemical ionization source (APCI) in the negative ionization mode [20]. Monitoring in single-ion monitoring (SIM) mode and beginning with 0.5 mL of serum, a detection limit of 200 ng/mL for 1,5-AG was reported.

Urinary levels of 1,5-AG can vary greatly among individuals. This, combined with the limited sample volume of patient urine available to us, required a more sensitive and selective method for 1,5-AG analysis than those described above. The literature documents detection limits of 1,5-AG in urine as low as 0.06 μg/mL based on 0.1–0.2 mL of urine which was treated with a 3-layer ion-exchange column prior to derivatization [21,22].

We were interested in simplifying the sample preparation method in order to make the throughput more amenable to a drug discovery setting. Our objective was to develop a sensitive and selective method for the determination of 1,5-AG in human urine using LC/MS that would allow use of sample volumes as low as 50 μL. It was not expected that 1,5-AG would be amenable to traditional reversed phase HPLC because of its hydrophilic nature, and initial method development showed this to be the case; 1,5-AG did not retain on traditional reversed-phased columns. As an alternative to RP-HPLC, hydrophilic interaction liquid chromatography (HILIC) conditions were tested as a more logical approach to analyzing 1,5-AG. HILIC is characterized by use of a hydrophilic stationary phase and a high concentration of organic modifier (typically >60%) in the mobile phase, making it very useful for the analysis of polar, hydrophilic compounds such as carbohydrates, amino acids, polar metabolites and peptides [23–25]. Use of traditional reversed-phase solvents makes HILIC a natural match for coupling to mass spectrometry. The nature of HILIC has made it an extremely useful technique in such fields as metabolite profiling where it has been used to measure polar metabolites of drugs such as morphine and cocaine [26,27] as well as many other polar analytes and/or impurities [28–35]. In our hands HILIC, using a polar amino stationary phase and solvent system containing a high percentage of acetonitrile, proved to be a very robust and reproducible approach to analyzing an extremely polar analyte in a complex biological matrix.

The mass spectrometer used for the studies presented in this paper was a Thermo-Fisher LTQ linear ion trap. Although triple quadrupole mass spectrometers remain the primary choice for small molecule quantitation, ion trap mass spectrometers offer a respectable alternative and have their own unique advantages over traditional selected-reaction monitoring (SRM) experiments. The main advantage of triple quadrupole instruments over ion traps is the use of a continuous ion beam which results in a high duty cycle allowing excellent sensitivity and reproducibility in SRM mode even when monitoring large numbers of analytes simultaneously. Because ion trap mass spectrometers perform tandem mass spectrometry stepwise in-time (collection of ions in the trap, isolation of precursor ions, fragmentation and product ion detection) the duty cycle is slow compared to triple quadrupole instruments, which can be problematic when monitoring MS^n transitions of analytes present at low concentrations or when analyzing several analytes simultaneously. The low duty cycle may prevent collection of an adequate number of data points across the chromatographic peak. Modern ion traps, however, have significantly improved upon this issue with the advent of automatic gain control and are now more routinely used for quantitative SRM experiments [36–39]. Despite the lower duty cycles, ion traps offer unique advantages over triple quadrupoles, perhaps the most important of which is the ability to perform MS^n experiments. This has made ion traps and ion trap hybrid systems invaluable in such areas as metabolite identification [10,11,40–43]. The ability of ion traps to perform MS^n was instrumental in the experiments presented here, as it provided an additional level of selectivity for detecting 1,5-AG in human urine (which contained many matrix interferences) and allowed us to achieve an LOQ of 50 ng/mL from only 50 μL of human urine.

HILIC/MS^3 was applied to determine the concentration of 1,5-AG in human urine from a clinical study in which diabetic patients had received a compound that altered urinary excretion of 1,5-AG.

2. Experimental

2.1. Materials

1,5-Anhydro-D-glucitol was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Methyl-β-D-glucopyranoside hemihydrate (internal standard) was purchased from TCI (Tokyo, Japan). All HPLC solvents (methanol, water, acetonitrile, isopropanol) were HPLC grade and were purchased from VWR

(West Chester, PA). Ammonium acetate was purchased from Sigma–Aldrich (St. Louis, MI). Human control urine was purchased from Bioreclamation, Inc. (Bridgeport, NJ).

2.2. Equipment

The HPLC–MS system used was composed of a Thermo-Finnigan (Waltham, MA) Surveyor MS pump, a Leap Technologies (Carrboro, NC) HTS Pal autosampler with chiller, and a Thermo-Finnigan LTQ ion trap mass spectrometer. Data were acquired using Xcalibur (v. 1.4).

2.3. Chromatographic conditions

HPLC was performed on a Phenomenex (Torrance, CA) 50 mm × 4.6 mm, 3 μm particle size Luna NH_2 column. The mobile phase consisted of solvent A = 98:2, 10 mM ammonium acetate:acetonitrile, and solvent B = 2:98, 10 mM ammonium acetate:acetonitrile; the flow rate was 1 mL/min. 1,5-AG was analyzed using HILIC conditions. The step gradient used was as follows: after injection hold 80% solvent B for 2 min, 80% to 50% solvent B in 0.2 min, hold 50% solvent B for 0.8 min, re-equilibrate to 80% solvent B for 2 min. The total run-time was 5 min. A 10 μl full loop (with 2× loop overfill) sample injection was used. The autosampler wash solvents consisted of wash 1 = water:isopropanol:acetonitrile (1:1:1) and wash 2 = mobile phase solvent B.

2.4. Mass spectrometry

Mass spectrometry was performed in the negative ionization mode using an APCI source. Mass spectrometry parameters were optimized by infusing a 10 μg/mL solution of 1,5-AG into mobile phase flow via a tee union and manually adjusting MS settings to achieve maximum response. Optimized voltages and gas settings were as follows: sheath gas flow 72, auxillary gas flow 44, sweep gas flow 3, spray voltage 3 kV, capillary voltage −25 V, tube lens −170 V and capillary temperature 400 °C. The HPLC eluent was introduced directly into the ionization source with no splitting. Data were acquired in MS/MS/MS mode using 2 scan events (for 1,5-AG and the IS, respectively). The first scan event monitored the MS^3 fragmentation of 1,5-AG from m/z 223.0 (acetate adduct) → m/z 163.0 $(M-H)^- \rightarrow m/z$ 101.1 + m/z 113.2 using a collision energy of 37 and an isolation width of 3. The second scan event monitored MS^3 fragmentation of the IS from m/z 253.0 (acetate adduct) → m/z 193.0 $(M-H)^- \rightarrow m/z$ 101.1 + m/z 113.2 using a collision energy of 35 and an isolation width of 3.

2.5. Preparation of stock, standard and QC solutions

Stock solutions of 1,5-AG and the IS were prepared at 1 mg/mL in water and methanol, respectively. The IS working solution was prepared at 100 μg/mL in MeOH. 1,5-AG calibration standard working solutions were prepared in water at concentrations of 50, 100, 500, 1000, 5000, 10,000 and 50,000 ng/mL. Calibration and IS solutions were prepared fresh daily. QC dilution solutions were prepared in water at 50,000 and 15,000 ng/mL. QC working solutions were prepared in water from the dilution solutions at three concentrations: 150 ng/mL (QC1), 2500 ng/mL (QC2) and 25,000 ng/mL (QC3).

2.6. Sample preparation

Human urine samples (control or patient) were thawed, vortexed, then centrifuged for 10 min at 13,000 rpm in an Eppendorf® T6000B microcentrifuge. 50 μL of the supernatant was then removed and transferred to a 96-well plate. The urine was then diluted with 200 μL of IS working solution in cold methanol. The samples were capped tightly with cap-mats then vortexed and centrifuged for 10 min at 5000 rpm in a Sorvall T6000B centrifuge equipped with a 96-well plate capacity rotor. 150 μL of the supernatant was then transferred to a shallow 96-well PCR plate (Axygen Scientific, Inc., Union City, CA) and placed into the chilled (5 °C) autosampler tray for analysis.

2.7. Method evaluation

2.7.1. Quantitation

Due to the endogenous presence of 1,5-AG in urine, quantitation of 1,5-AG in urine was based on calibration curves generated in water. The calibration curves were generated using peak area ratios of 1,5-AG to IS, and were fitted with a linear regression using a $1/x$ weighting.

2.7.2. Endogenous levels of 1,5-AG in human urine

Human control urine from 5 individual female and 5 individual male donors was obtained and analyzed for 1,5-AG by LC/MS/MS/MS as described in this section.

2.7.3. Inter- and intra-day evaluations

Intra-day accuracy and precision were determined by analyzing 6 replicates of QC1, QC2 and QC3 in a 1-day period. Inter-day accuracy and precision were determined by preparing and analyzing 6 replicates of QC1, QC2 and QC3 independently over a 3-day period.

2.7.4. Bench–top and freeze–thaw stabilities

Six sets of QC1, QC2 and QC3 were subjected to either 4 h at room temperature (bench–top stability) or 3 freeze (−40 °C)–thaw cycles. Following completion of the stability tests the samples were diluted with IS in cold MeOH and analyzed for 1,5-AG as described previously.

2.8. Analysis of patient samples

Human urine was collected from a clinical study in which diabetic patients received either placebo or increasing doses of a proprietary BMS compound that was known to alter urinary levels of 1,5-AG. The urine samples (220) were analyzed in random order over a 3-day period. The analyst was blinded to the patient group identification until all samples were analyzed and all data had been processed.

3. Results and discussion

3.1. Optimization of LC/MS conditions

1,5-AG was not well retained on traditional reversed phase (C18, C8, phenyl-hexyl) HPLC columns because of its highly water soluble and polar character. HILIC chromatography conditions were much more successful in retaining 1,5-AG and the IS. Several HILIC columns were tested (Waters Hilic, Phenomenex Luna CN), and the best peak shape for both compounds was obtained on a Phenomenex amino column using gradient conditions and a mobile phase of ammonium acetate and methanol (Fig. 2).

The best sensitivity for detecting 1,5-AG was achieved using APCI in the negative ionization mode. In MS mode the most intense ion formed for both 1,5-AG and the IS was the acetate adduct. MS/MS of the acetate adduct resulted in loss of acetate to form the deprotonated molecule. Analysis of a diluted human urine sample by LC/MS^2 (monitoring m/z 223 → 163 for 1,5-AG and m/z 253 → 193 for the internal standard) showed the presence of a

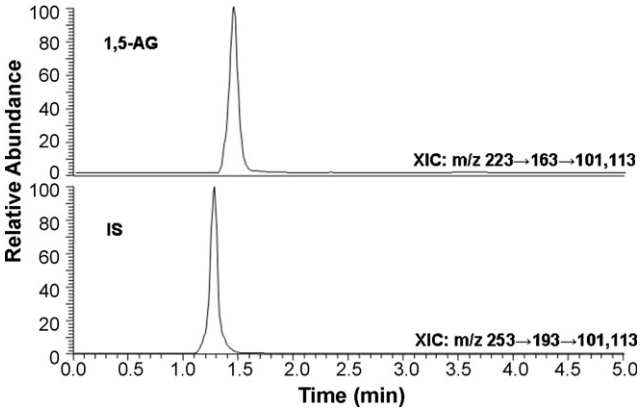

Fig. 2. Representative LC/MS^3 chromatograms of a human urine sample. The top panel shows the extracted ion chromatogram for 1,5-AG in urine (m/z 223 → 163 → 101, 113); the bottom panel shows the extracted ion chromatogram for the internal standard methyl-β-D-glucoside in urine (m/z 253 → 193 → 101, 113). Pooled human control urine was diluted 1:4 with IS in cold methanol.

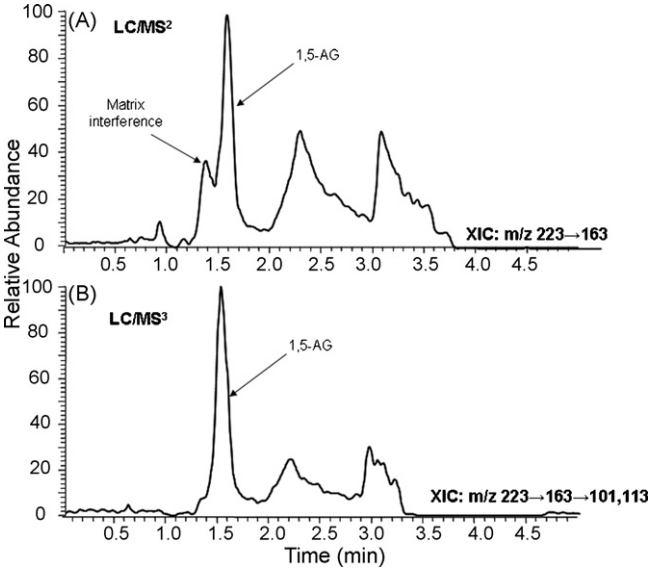

Fig. 3. Improved selectivity of LC/MS^3 vs. LC/MS^2. Panel A: LC/MS^2 extracted ion chromatogram (m/z 223 → 163) of a diluted human urine sample. Panel B: LC/MS^3 extracted ion chromatogram (m/z 223 → 163 → 101, 113) of the same diluted human urine sample. The human urine sample was diluted 1:4 with IS in cold methanol.

matrix peak that interfered with accurate peak integration of 1,5-AG (Fig. 3A). This was not unexpected, since monitoring acetate loss does not always provide adequate selectivity when analyzing complex matrices such as urine. Further fragmentation of the deprotonated molecule of 1,5-AG resulted in formation of multiple fragments of m/z 131.0, 113.2 and 101.1. Further fragmentation of the IS deprotonated molecule resulted in fragments of m/z 101.1, 113.2 and 161.1. Analysis of the same diluted human urine sample in LC/MS^3 mode resulted in improved selectivity compared to LC/MS^2 and eliminated the interfering matrix peak (Fig. 3B). To determine the MS^3 transition that produced the best sensitivity for detecting 1,5-AG, urine samples were analyzed using several LC/MS^3 transitions or using a combination of multiple transitions. Monitoring the different transitions produced varying degrees of selectivity and sensitivity (Fig. 4A). Ultimately, the optimal selectivity and sensitivity was obtained by processing the data using a combination of the MS^3 transitions m/z 101 and m/z 113 (Fig. 4B).

3.2. Optimization of sample preparation conditions

A simple and fast sample preparation procedure was desired which would provide adequate sensitivity from a small volume of urine while producing sufficiently clean extracts to ensure method ruggedness and long-term performance. A simple 4-fold dilution of urine with cold acetonitrile was tried initially. (It has been observed

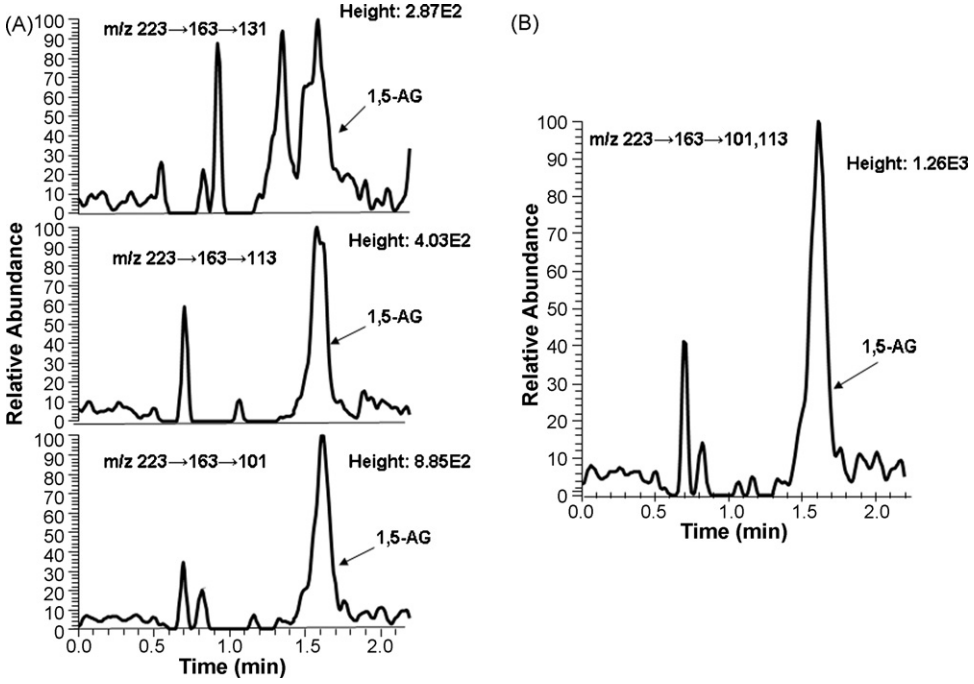

Fig. 4. Extracted ion chromatograms of human urine samples depicting the effect of MS^3 transition selection on selectivity. Panel A: Extracted ion chromatogram of a human urine sample using the MS^3 transitions of m/z 223 → 163 → 131, 113, or 101. Panel B: Extracted ion chromatogram of a human urine sample using the optimal combined MS^3 transition of m/z 223 → 163 → 101,113. Human urine was diluted 1:4 with IS in cold methanol.

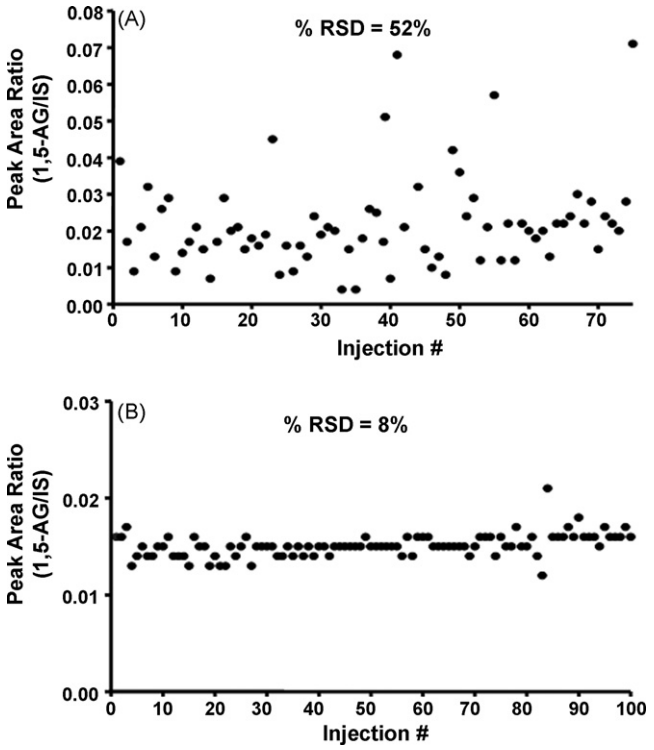

Fig. 5. Effect of dilution solvent on injection-to-injection reproducibility of human urine samples. Panel A: Peak area ratio (1,5-AG/IS) vs. injection # resulting from more than 70 injections from the same human urine sample diluted with cold acetonitrile. Panel B: Peak area ratio (1,5-AG/IS) vs. injection # resulting from more than 100 injections from the same human urine sample diluted with cold methanol. Pooled human urine was diluted 1:4 with either IS in cold acetonitrile or IS in cold methanol; a large enough volume was generated to allow 70 or more injections from the same vial.

in previous studies in our lab that use of cold organic solvents vs. room temperature solvents results in more efficient precipitation of proteins and salts from plasma, urine and cell-based assay samples.) This approach, however, resulted in very poor (% RSD > 50%) injection-to-injection reproducibility (Fig. 5A). Dilution of human urine with cold acetonitrile on a larger scale (0.5 mL of urine with 2 mL of cold acetonitrile) revealed the formation of two immiscible layers. Presumably, the high ionic strength of urine caused phase separation of acetonitrile and the aqueous portion which in turn produced erratic results from sample to sample. Because salts are more soluble in methanol, human urine was diluted 4-fold with cold (−20 °C) methanol and then analyzed by LC/MS/MS/MS to determine the effect of methanol on reproducibility. Changing the dilution solvent from acetonitrile to methanol greatly improved the reproducibility, consistently producing injection-to-injection % RSDs < 10% (Fig. 5B). Based on these findings, the final sample preparation procedure employed was dilution of 50 μL of human urine with 200 μL of IS in cold methanol.

3.3. Method evaluation

3.3.1. Quantitation of 1,5-AG in human urine

It is well established that 1,5-AG is an endogenous component of human urine. To demonstrate the degree of variation in 1,5-AG levels from individual to individual, control human urine was purchased from 5 female and 5 male donors and analyzed for 1,5-AG. The analysis showed that the concentration of 1,5-AG in the small pool of donors tested varied approximately 4-fold within the male donors, 9-fold within the female donors, and approximately 9-fold between genders (Table 1). The endogenous presence of 1,5-AG in urine complicated the quantitation strategy. Typically, calibration standards are generated by spiking known standard amounts into the same matrix as the unknown samples to be analyzed. In this case, however, known amounts of 1,5-AG would be spiked into control urine already containing endogenous levels of 1,5-AG. Rather than attempt to generate calibration standards in human control urine by subtracting out endogenous levels of 1,5-AG, we opted to generate calibration standards of 1,5-AG in water. Matrix effects of human urine on the ionization of 1,5-AG were minimal. This conclusion was based on experiments in which 4-fold diluted human urine was injected into a continuously infusing sample of 1,5-AG (data not shown) [44]. Injection of the urine matrix did cause suppression of the 1,5-AG ionization signal, but the signal was fully recovered by 1.5 min, which is the chromatographic retention time of 1,5-AG. Therefore, integration of the 1,5-AG peak area should not be affected by matrix, and comparable results should be observed whether analyzing urine or pure solvent. It was thus determined that quantitation of 1,5-AG in human urine based on calibration curves in water was an acceptable approach for our studies.

Table 1
Endogenous levels of 1,5-AG in individual human male or female control urine

Urine donor	1,5-AG (ng/mL) Mean ± S.D.
Female 1	3098 ± 511
Female 2	602 ± 20.5
Female 3	2690 ± 104
Female 4	4610 ± 43.9
Female 5	1670 ± 116
Male 1	4680 ± 650
Male 2	2000 ± 344
Male 3	4660 ± 554
Male 4	1254 ± 332
Male 5	1440 ± 23.1

Data shown is the mean ± S.D. of duplicate determinations.

Stable-labeled 1,5-AG was not commercially available, so a structurally similar compound (methyl-β-D-glucoside, a methoxy derivative of 1,5-AG) which was not an endogenous metabolite of 1,5-AG was chosen as an IS.

3.3.2. Intra-, inter-assay evaluations, bench–top, freeze–thaw stabilities and autosampler stability

Because of the discovery application of this assay, a full, FDA-guideline validation was not deemed necessary. However, demonstration of assay accuracy, reproducibility, and ruggedness was still essential. To evaluate assay reproducibility, 6 replicates of QCs containing 1,5-AG at 3 concentrations (QC1: 150 ng/mL, QC2: 2500 ng/mL and QC3: 25,000 ng/mL) were analyzed within the same day (intra-day evaluation) and on 3 separate days (inter-day evaluation). The QCs at all levels were accurate to within approximately 3% deviation or less for the intra-day evaluation, and within approximately 7% or less for the inter-day evaluation. Precision results for both analyses ranged from 3.4% to 16%. 1,5-AG was stable when exposed to ambient conditions on the bench for 4 h, accuracies were within the ±20% acceptance level (ranging from −6% to −17%). The mid- and high-level QCs were stable through 3 freeze–thaw cycles. The low-level QC (150 ng/mL) fell outside the typical validation acceptance criteria range (accuracy = −27% deviation). It was decided that this was not an immediate concern for our studies, however, as only 3 of the 220 human patient samples analyzed had levels of 1,5-AG below 150 ng/mL (the large majority of urine samples had 1,5-AG levels between QC2 and QC3) and because the patient urine samples were only subjected to one freeze–thaw cycle (Table 2).

Table 2
1,5-AG summary table of method evaluation results

QC	Intra-day evaluation		Inter-day evaluation (3 days)		Bench–top stability (4 h)		Freeze–thaw stability (3 cycles)	
	Accuracy (% deviation)	Precision (% RSD)	Accuracy (% deviation)	Precision (% RSD)	Accuracy (% deviation)	Precision (% RSD)	Accuracy (% deviation)	Precision (% RSD)
QC1 150 ng/mL	3.1	8.8	7.1	16	−6.0	5.7	−27	26
QC2 2500 ng/mL	−0.70	3.4	1.7	9.9	−6.6	14	−0.70	3.4
QC3 25,000 ng/mL	0.73	4.5	2.3	13.6	−17	6.7	0.73	4.5

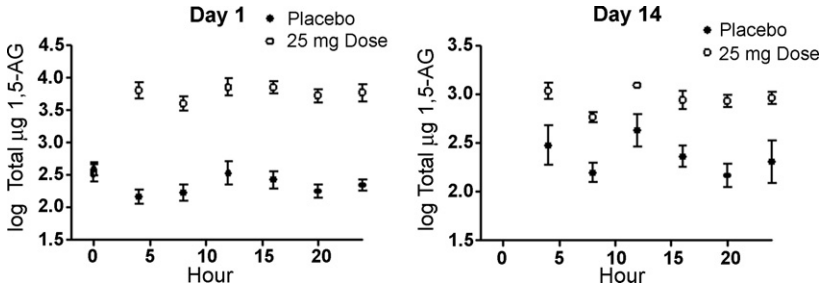

Fig. 6. Determination of 1,5-AG in diabetic patient urine. Human urine was collected from a clinical study in which diabetic patients received either placebo or 25 mg of a compound known to alter urinary levels of 1,5-AG. Urine was collected over a 24-h period on days 1 and 14 of the clinical study.

Autosampler stability was addressed during optimization of sample preparation conditions (see Section 3.2). 1,5-AG was determined to be stable at 5 °C in the autosampler for up to 100 injections.

3.4. Urine normalization

Normal urine is composed of various organic and inorganic solutes including urea, uric acid, creatinine, carbohydrates, enzymes, fatty acids, hormones and ions such as sodium, potassium, chloride, magnesium and calcium. The concentration of these components in urine can vary greatly depending on urinary output. One of the challenges associated with measuring drugs, drug metabolites, or other components excreted in urine is how to quantitatively express concentration data in such a manner as to compensate for differences in factors such as individual urinary output. Creatinine is commonly measured in the clinic to assess proteinuria. Creatinine levels, however, are affected by renal function and therefore would not serve as a reliable normalization method. Osmolality is a measure of the number of particles per kilogram of solvent and is commonly used to characterize the ionic strength of urine. In situations where total urine volume is not available (as in animal studies when urine collection is impractical or complicated by such factors as urinary loss due to cage design and bedding and/or food absorption) osmolality can be used as a normalization factor to more accurately reflect changes in urine component concentration [45]. In a controlled clinical setting, however, total urinary output is often available and can be used as a normalization factor when reporting concentrations of urinary components. The human urine samples analyzed for our studies were collected during a controlled clinical study and total urinary volume was recorded. We therefore reported levels of 1,5-AG after normalizing to individual 24 h urinary output.

3.5. Analysis of human diabetic patient samples

One of the primary objectives of this study was to monitor the urinary output pattern of 1,5-AG in humans to observe if levels of 1,5-AG in urine, like glucose, are subject to diurnal variation. Urine was collected from 47 diabetic patients receiving either placebo or 25 mg of a proprietary compound known to alter levels of 1,5-AG. Samples were collected on days 1 and 14. The amount of 1,5-AG in the urine was normalized to total urinary volume output. The clinical data are summarized in Fig. 6. And show that urinary levels of 1,5-AG within treatment groups remained fairly constant over 24 h. The clinical implication of this observation is that 1,5-AG urine levels may have the potential of being used as a spot-check of glycemic status. The elimination of tedious 24-h urine collection (for glucose determination) would be a huge relief to patients, clinicians, and hospital staff.

The proprietary compound dosed in these studies was known to alter levels of 1,5-AG in urine, and the data in Fig. 6 also illustrate that 1,5-AG responded to treatment as expected.

4. Conclusions

A selective and sensitive LC/MS^3 method combining HILIC and ion trap mass spectrometry was successfully developed for the determination of 1,5-AG, a glycemic marker, in human urine. The method employed a fast dilution based sample preparation step and the method was sufficiently rugged to analyze large numbers of urine samples. The method was successfully used to monitor the behavior of 1,5-AG in human diabetic urine. The procedure described in this paper offers a relatively fast, simple, and sensitive method to measure 1,5-AG from patient urine. Though not fully validated, the method could easily be adapted to clinical sample analysis, especially through use of a stable-labeled internal standard.

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