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Quantitative determination of potential urine biomarkers of respiratory illnesses using new targeted metabolomic approach

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Abstract

The diagnosis of asthma and chronic obstructive pulmonary disease (COPD) can be challenging due to the overlap in their clinical presentations in some patients. There is a need for a more objective clinical test that can be routinely used in primary care settings. Through an untargeted ^{1}H-NMR urine metabolomic approach, we identified a set of endogenous metabolites as potential biomarkers for the differentiation of asthma and COPD. A subset of these potential biomarkers contains 7 highly polar metabolites of diverse physicochemical properties. To the best of our knowledge, there is no liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that evaluated more than two of the target metabolites in a single analytical run. The target metabolites belong to the families of monosaccharides, organic acids, amino acids, quaternary ammonium compounds and nucleic acids, rendering hydrophilic interaction liquid chromatography (HILIC) an ideal technology for their quantification. Since a clinical decision is to be made from patient data, a fully validated analytical method is required for biomarker validation. Method validation for endogenous metabolites is a daunting task since current guidelines were designed for exogenous compounds. As such, innovative approaches were adopted to meet the validation requirements. Herein, we describe a sensitive HILIC-MS/MS method for the quantification of the 7 endogenous urinary metabolites. Detection was achieved in the multiple reaction monitoring (MRM) mode with polarity switching, using quadrupole-linear ion trap instrument (QTRAP 6500) as well as single ion monitoring in the negative-ion mode. The method was fully validated according to the regulatory guidelines. Linearity was established between 6 to 21000 ng/mL and quality control samples demonstrated acceptable intra- and inter-day accuracy (85.7%-112%), intra- and inter-day precision (CV% <11.5%) as well as stability under various storage and sample processing conditions. To illustrate the method's applicability, the validated method was applied to the analysis of a small set of urine samples collected from asthma and COPD patients. Preliminary modelling of separation was generated using partial least square discriminant analysis (R^2 0.752 and Q^2 0.57). The adequate separation between patient samples confirms the diagnostic potential of these target metabolites as a proof-of-concept for the differentiation between asthma and COPD. However, more patient urine samples are needed in order to increase the statistical power of the analytical model.

Keywords: targeted metabolomics; asthma; COPD; HILIC-MS/MS; validation; urine

1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory conditions of the respiratory airways with similar symptoms. Each disease can be differentiated based on their pathologic inflammation profiles and airway function tests [1]. However, for the typical doctor's office, accurate diagnostic tests are not readily available [1-3]. Moreover, the misuse and underuse of spirometry at the primary and secondary levels of care have been shown [2-4]. Diagnosis becomes more challenging with the increased prevalence of asthmatic smokers [5], non-asthmatic COPD patients [6], patients with late-onset asthma [2, 7] and patients with asthma-COPD overlap syndrome [5]. The insufficient sensitivity of the currently available clinical tests and reliance on therapy trials have drained health resources with a negative impact on the overall quality of life of patients [2, 3, 8-10].

In search for better diagnostic tests, recent investigations have focused on the impact of asthma and COPD on the human metabolome [11]. Metabolomics has demonstrated potentials in linking altered biochemical pathways to therapeutic interventions through biomarkers discovery experiments [12, 13]. Urine exhibits several advantages over other bodily matrices. It is non-invasively collected and is rich in metabolites while low in cell and protein contents in comparison to plasma [14-16].

Urine metabolomics has been reported to differentiate stable and unstable asthmatic patients, to determine the severity stage of COPD in patients or to compare either asthma or COPD to healthy participants [17-22]. Limited research, however, has been directed towards the differential diagnosis of other conditions that can mimic asthma [23] or COPD [24]. To the best of our knowledge, only our previous paper has reported potential biomarker metabolites in urine between asthma and COPD [25]. Using ^{1}H-NMR, 50 metabolites were suggested [25].

Clinical validation of potential biomarkers requires robust quantitative analytical platforms [26, 27], and we have been developing liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods to meet this purpose. The metabolites were divided into 4 subgroups, based on chemical nature or concentration. Groups 1 and 2 were quantified using differential isotope labelling strategies [28, 29] . Group 3 is the focus of the current study and it contains 1-methylnicotinamide (1MN), choline (COL), creatine (CRE), D-glucose (GLC), guanidine acetic acid (GAA), pyruvic acid (PYA) and uracil (URC). The use of differential isotope labelling methods [28] was not possible for these metabolites, as they lacked a common functional group. In addition, the higher polarity of the metabolites rendered the use of C_{18} column inappropriate. For this reason, we investigated the development and validation of a hydrophilic interaction liquid chromatography (HILIC)-MS/MS method for their quantification in urine.

HILIC has several advantages over conventional normal- and reversed- stationary phases rendering it ideally suited for polar metabolites [30]. To the best of our knowledge, a validated HILIC-MS/MS method that combines more than two of our metabolites of interest does not exist. The novelty of the work described herein lies in the combination of HILIC-MS/MS method development and validation for that specific set of 7 endogenous metabolites that had been previously shown to demonstrate a biomarker importance for asthma and COPD diagnosis. The method was validated according to the guidelines issued by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) [31, 32]. Patient urine samples were analyzed for potential diagnostic accuracy by the validated methodology.

2. Experimental

2.1. Reagents and reference standards

1-Methylnicotinamide chloride, choline chloride, GLC, GAA, PYA and URC were purchased from Sigma Aldrich (ON, CA). CRE was purchased from Spectrum Laboratories Inc. (CA, USA). 1-methylnicotinamide-d_3 chloride, choline-d_9 chloride, creatine-d_3 monohydrate, glucose-d_2 (GLC-IS), guanidine acetic acid-d_2 (GAA-IS) and uracil-d_2 (URC-IS) were purchased from C/D/N Isotope Inc. (QC, CA). Pyruvic acid-$^{13}C_3$ sodium salt was obtained from Omicron Biochemical Inc. (IN, USA). Optima® LC-MS grade acetonitrile (ACN), and water as well as formic acid (FA) and ammonium formate (NH_4FA) were purchased from Fischer Scientific (ON, CA). Figure 1 displays the structures of the selected internal standards (ISs). Creatinine measurement was achieved using Quantichrom™ Creatinine Assay Kit (QC, CA) [33].

2.2.Standards and ISs preparation

All preparations were conducted in water. 1MN, 1-methylnicotinamide-d_3 (1MN-IS), COL, choline-d_9 (COL-IS), CRE, Creatine-d_3 (CRE-IS), GLC, GLC-IS, PYA and pyruvic acid-$^{13}C_3$ (PYA-IS) were prepared at 3 mg/mL. GAA, GAA-IS, URC and URC-IS were prepared at 1 mg/mL due to their lower solubility in water.. The metabolites working stock solution contained 24, 8, 80, 280, 100, 60 and 68 µg/mL of 1MN, COL, CRE, GLC, GAA, PYA and URC, respectively. The ISs working stock solution, prepared from individual ISs stocks, contained 1MN-IS, COL-IS, CRE-IS, GLC-IS, GAA-IS, PYA-IS and URC-IS at 15, 5, 30, 600, 120, 60 and 80 µg/mL, respectively.

2.3.Calibration standards and quality control (QC) samples preparation

A set of 8 calibration standards covering a linearity range extending over 2 orders of magnitude was prepared. Aliquots from the metabolites working stock solution were serially diluted with water and for each calibration standard, 15 µL from the appropriate diluted solution were mixed with 80:5 µL of ACN: ISs working solution. The final mixtures were vortexed and transferred into HPLC vials equipped with 100 µL inserts for analysis.

Quality control (QC) samples, used in validation experiments, were prepared at four concentration levels. For the preparation high (HQC), medium (MQC) and low quality control (LQC) samples, the endogenous levels of the investigated metabolites were measured in pooled urine from 7 healthy participants. A volume of 20 µL from this standardized pooled urine was fortified with 20 µL or 10 µL of the metabolites working stock solution for the preparation of HQC or MQC, respectively. The volume in each QC solution was then completed to 60 µL with water. The LQC sample was prepared via the 50 fold dilution of HQC with water. For the 3 levels of QC samples, an aliquot of 15 µL from the appropriate concentration was mixed with 80:5 µL of ACN: ISs working solution. The mixtures were centrifuged at 14000 rpm for 10 min for protein precipitation. The supernatant was transferred into HPLC vials equipped with 100 µL inserts for analysis. The lowest calibration standard, i.e. lower limit of quantification (LLOQ), also served as the fourth level of QC samples as per the regulatory guidelines [31, 32].

2.4.Urine sample preparation

Following collection, urine specimen cups (Starplex Scientific Inc, ON, CA) were promptly placed in -80 ºC freezer. Samples were subjected to one freeze-thaw cycle for aliquots preparation. At the time of analysis, sample aliquots were thawed to room temperature, diluted 3 folds with water and 15 µL from the appropriate diluted solution were mixed with 80:5 ACN: ISs working solution. The mixtures were centrifuged at 14000 rpm for 10 min for protein precipitation. The supernatant was transferred into HPLC vials equipped with 100 µL inserts for analysis.

2.5.Patient characteristics

Patient samples were obtained from participants who gave informed consent, as approved by the Human Ethics Board of the University of Saskatchewan BIO#13-89. Urine was analyzed in

subjects with asthma (n=8; 63% female; mean age 57±8.7 years; BMI 29.3±4.0) and COPD (n=8; 75% female; mean age 60.5±7.1; BMI 24.1±9.2). Our previous study had demonstrated the insignificant influence of diet and time of collection on the variation of the investigated metabolites between patient groups [25], accordingly, random urine samples with no dietary restrictions were collected. Control urine samples were collected as random mid-stream urine from 7 healthy participants (25-40 years of age) currently not taking any medications.

2.6.Instrumentation

Chromatographic and MS parameters are compiled in Supplemental Materials section.

2.7.Method validation

In general, the validation of the developed method was conducted according to the guidelines issued by the FDA and the EMA [31, 32]. Due to the endogenous nature of these metabolites, slight modifications in the methodology of these guidelines or their acceptance criteria were expected to accommodate unforeseen challenges. Nonetheless, the validation results of the developed method were not compromised (discussed below).

2.7.1. Selectivity

Due to the endogenous nature of the investigated metabolites, the assessment of method selectivity as described by the FDA and the EMA guidelines was not feasible [31, 32]. Alternatively, a double blank solution (80% ACN, 6 replicates) was injected and the interference observed at the metabolites channel at their expected retention times was compared to the mean absolute peak area of the LLOQ. The method was deemed selective if the observed interference is less than 20% of the LLOQ [31].

The assessment of method selectivity for the ISs was conducted as described by the FDA and EMA guidelines [31, 32]. Briefly, 6 urine samples from 6 different participants (3 asthma and 3 COPD) were processed as previously described with the replacement of the ISs working stock solution with water. The interference observed at the ISs channel at their expected retention times was compared to the mean absolute peak area of the LLOQ. The method was deemed selective if the observed interference is less than 5% of the LLOQ [31].

The presence of isotopic impurities in the ISs can compromise method selectivity. Accordingly, the isotopic purity of the ISs was assessed by injecting five blank solutions containing only ISs in 80% ACN. The interference observed at the metabolites channel was measured and compared to the mean absolute peak area of the LLOQ. An interference less than 20% of the LLOQ was acceptable [28, 31, 34].

Finally, since GAA, GLC and URC differ from their corresponding ISs by 2 mass unit difference; the second natural C_{13} isotopic peak of these metabolites is expected to interfere at the ISs channel. In order to investigate this effect, the upper limit of quantification (ULOQ) was prepared in 5 replicates without the inclusion of the ISs. The interference observed at the ISs channel was measured and the isotopic contribution was compared to the mean areas of the ISs peaks; measured in blank solutions [28, 34].

2.7.2. Matrix effects

The matrix effects were evaluated according to the EMA guidelines [31], since detailed methodology for matrix effects assessment is lacking in the FDA guidelines [32]. Six random patient urine samples were spiked with aliquots of the metabolites working stock solution equivalent to 2×LLOQ and 66.67% of ULOQ to produce low and high concentrated samples, respectively. The samples were processed as previously described in 2.4. The absolute peak areas were corrected for endogenous metabolites levels through subtraction and then compared to analogues low and high concentrated samples prepared similarly in a neat solvent (80% ACN).

For each metabolite and its IS in each urine matrix, the matrix factor (MF) was calculated according to equation 1. The IS-normalized MF was calculated according to equation 2 and the coefficient of variation values (CV%) were generated. According to the EMA, the CV% of the IS-normalized MF should not be greater than 15% [31].

Equation 1: Matrix factor (MF)= B/A

Equation 2: IS-normalized MF= MF metabolite/MF IS

Where B is the corrected absolute peak area of the metabolite in the urine matrix and A is the absolute peak area of the metabolite in the neat solvent (80% ACN).

2.7.3. Carry-over effects

Carry-over effects were investigated according to the EMA guidelines [31], in which blank samples were injected after the ULOQ, HQC and high concentrated patient urine samples. The responses in the metabolites channels were compared to the LLOQ. Carry-over effects can be deemed insignificant if they are less than 20% of the LLOQ [31].

2.7.4. Linearity

The $^{1}/_{x}$ weighed least square regression equation for each metabolite was generated using the (metabolite/IS) peak area ratios *vs.* the corresponding concentrations of a set of 8-point calibration standards. For accepting a calibration curve; the back-calculated concentration of the calibration standards must fall within 15% of their respective nominal concentration with the exception of the LLOQ, in which 20% difference is acceptable. At least 75% (6 out of 8) of the calibration points must fulfil this criterion [31, 32, 35].

2.7.5. Accuracy and precision

The intra- and inter-day accuracy and precision were assessed on 3 non-consecutive days using four levels of QC samples. According to the FDA and EMA guidelines [31, 32], the LQC should be within 3 times the LLOQ, the MQC should be around 50% of the calibration range and the HQC should be at least 75% of the ULOQ. As such, the preparation of the QC samples was optimized such that the LQC was, at most, 1.7 times the LLOQ and the MQC was at 51% ±9.4 of the calibration range. Due to the varying levels of metabolites in the pooled urine from 7 healthy participants, the HQC was marginally below 75% of the ULOQ for URC and PYA, i.e. 71.1% and 73.8%, respectively. Increasing the concentration of these metabolites would ultimately result in higher LLOQ, which was not preferred due to the low concentration of URC and PYA in patient samples. In order to accept the accuracy from a single run, the mean calculated concentration should be within 15% of the nominal values of each QC level (excluding the LLOQ), while a CV% of less than 15% is adequate to achieve the required precision of each QC level (excluding the LLOQ) [31, 32]. A 20% acceptance value was adopted with the LLOQ for evaluating accuracy and precision [31, 32].

2.7.6. Extraction recovery

Urine sample preparation includes protein precipitation with ACN/centrifugation for 10 min at 14,000 rpm. To evaluate the extraction efficiency, the amount of metabolites recovered after protein precipitation was compared between pre-spiked and post-spiked urine samples. A *pre-spiked* sample was prepared so that urine was spiked with metabolites standard solution and then extracted, while a *post-spiked* sample referred to a urine sample that was first extracted and then spiked with metabolites working stock solution. For the preparation of *pre-spiked* samples; a pooled urine sample was 3 fold diluted with 3 concentration levels of the metabolites working stock standards; low, medium and high; equivalent to final concentrations of 2.6, 26 and 66.67 times the LLOQ. A 15 µL aliquot from each *pre-spiked* urine sample was extracted with 85 µL ACN containing 5 µL of ISs working stock solution. After centrifugation, the supernatant was

transferred into HPLC vials equipped with 100 µL inserts for analysis and the metabolites peak areas were calculated.

An equivalent set of *post-spiked* urine samples was prepared by the proper dilution of pooled urine aliquots with water and extraction with ISs-containing ACN. Aliquots from the extracted urine were spiked with proper volumes of the metabolites working stock solution equivalent to final concentrations of 2.6, 26 and 66.67 times the LLOQ. *Post-spiked* samples were transferred into HPLC vials equipped with 100 µL inserts for analysis and the metabolites peak areas were calculated. Volumes used in *post-spiked* samples preparation were adjusted to accommodate the change in sample preparation methodology. However, the consistency of ISs, ACN and endogenous metabolites contents across all samples was maintained. The consistency of spiked metabolites concentration across each comparative pair of samples (pre/post spiked) was also kept constant. Extraction recovery was evaluated by comparing the absolute peak area of the metabolites *post-spiked* to that of the *pre-spiked*, after correcting for the endogenous level of metabolites through subtraction in both samples.

2.7.7. Dilution integrity

For the evaluation of dilution integrity, 50 µL of pooled urine were mixed with 100 µL of the metabolites working stock solution. The mixture was then 10, 20, 100 fold diluted with water, in 5 replicates, and 15 µL from each diluted mixture were processed as described under section 2.4. Dilution integrity were accepted if the accuracy and precision were meeting the criteria set by the EMA, i.e. within ±15% [31].

2.7.8. Stability

Stability was assessed at conditions that are encountered during sample preparation and analysis as well as at different storage conditions using HQC and LQC samples and freshly prepared calibration curves [31, 32]. Five sets of QC samples (HQC, LQC; n=5 at each level) were prepared as described in section 2.4 and subjected to one of the following conditions; (A) room temperature for 4 hours (benchtop stability), (B) 4 ºC for 36 hours (autosampler stability), (C) after 1 month of storage at -80 ºC (short-term stability), (D) after 3 month of storage at -80 ºC (long-term stability), and (E) after 3 freeze-thaw cycles. For the last stability study, QC samples were frozen at -80 ºC for at least 24 hours then thawed at room temperature and were refrozen to -80 ºC for at least 12 hours before the next freeze-thaw cycle [31, 32]. For the evaluation of metabolite working stock solution stability, a 3-month stock was used for the preparation of two calibration standards; 4×LLOQ and 50% of ULOQ which were then analyzed against calibration curves, prepared from fresh stock solutions. Since the stability of the stock is the main purpose of this experiment, pooled urine was not spiked in this specific set of validation samples. Samples were deemed stable if they were within ±15% of their respective nominal concentration [31].

2.8. Analysis of patient urine samples

Patients' data was acquired following method validation. Urine samples were included in an analytical batch along with double blank, blank, calibration set (eight standards) and 3 levels of QC samples (LQC, MQC and HQC) that were prepared in duplicates and injected at intervals based on the total number of samples. The acceptance criteria for the calibration curve and QC samples as described in sections 2.7.4 and 2.7.5 were adopted [31, 32]. Moreover, in order to accept the analytical run, at least 67% of the QC samples and at least 50% of their replicates should be within ±15% of the nominal concentration [31, 32].

To account for differences in hydration, values of each metabolite were referenced to the participant's creatinine level. The data was log-transformed and then exported to SIMCA® software (SIMCA-P 11, Umetrics, Sweden) for partial least square discriminant analysis (PLS-

DA). Metabolites consistently differing between patient groups were used for the creation of the statistical model and they are displayed by the software as a coefficient of variation (CoV) plot and a variables of importance plot (VIP). GraphPad Prism software (version 6, CA, USA) was used for prediction score plot generation.

3. Results and Discussion

3.1.Method development

HILIC column combines the advantages of reversed and normal phase chromatography [30]. While using water containing organic mobile phases, it allows the separation of polar and ionic metabolites that are typically non-retainable on reversed phase columns [30]. In addition, it solves the challenge of limited solubility in the organic phases typically employed while using normal phase chromatography [30]. ZIC-HILIC bears a zwitterion stationary phase with a terminal negatively charged sulfonic acid group thus allowing hydrophilic and ionic interaction with the selected metabolites [30].

3.1.1. Optimization of the matrix

According to the FDA and the EMA, it is recommended to validate the analytical method in the biological matrix of the samples [31, 32]. An exception is given to matrices of limited availability, such as cerebral spinal fluids, which can be replaced by suitable artificial alternatives [31, 32, 36]. Another exception is when the analytes are endogenous in nature and an analyte-free biological matrix cannot be prepared. In this case, alternative matrices such as buffers may be used [32].

In our method, considerable endogenous levels of the investigated metabolites are present in urine, regardless of the pathological condition of the participant. Accordingly, the method was initially developed in artificial urine, prepared from major reported human urine components (supplemental tables, Table 1) [37, 38]. However, 3 main concerns rendered such matrix unsuitable. First, during selectivity assessment, an unacceptable interference was observed in CRE and PYA MRM channels (184% and 120% of the LLOQ, respectively). This interference was due to impurities within the reagents used during artificial urine preparation and can be theoretically addressed by increasing the LLOQ for these metabolites. However, unlike CRE, PYA was endogenously present at relatively low concentrations and a quantification method of high sensitivity was needed.

The second concern was the inability of artificial urine to simulate the composition of human urine, especially during GLC and PYA determination. As shown in Figure 2, the concentration of GLC is lower than its closely eluting isomers. Accordingly, the validation of accuracy and precision of GLC in urine was crucial to exclude any potential interference from other naturally existing hexoses. Such validation experiments would not be reflective of patient urine samples if the matrix used for calibration and QC samples is artificial urine. As for PYA, the chromatogram baseline in the proximity of PYA in urine was relatively higher than in artificial urine. The influence of the elevated baseline on accuracy, precision and LLOQ of PYA determination in urine is needed to be carefully assessed. As for the final concern, it was observed that all signals of ISs (except PYA-IS) were suppressed only in human urine, reaching up to 60.1% ±3.6 ion suppression in URC-IS, for example. Therefore, it was evident that the preparation of calibration and QC samples in artificial urine would not allow for obtaining valid results from patient samples.

As such, we investigated the use of diluted pooled human urine as an alternative approach. Figure 3 is a double blank prepared in 1000 fold diluted pooled urine. It still contains significant interference that reaches up to 136% of the LLOQ in URC, rendering urine dilution unsuitable. A final option was the use of the standard addition method [39], however, this technique required the

preparation of a calibration set for every sample being analyzed, which would be inapplicable when high throughput is expected [39, 40].

Based on the above findings, the calibration standards were prepared in 80% ACN. The QC samples (HQC, MQC and LQC) were prepared by spiking known concentrations of the working stock solution into pooled urine that was pre-quantified (standardized) against the calibration curves. As such, the HQC and the MQC contained the same urine content as patient samples (i.e. 20 fold diluted urine). The LQC was prepared by employing 50 fold dilution of the HQC in water, and due to the endogenous metabolites levels, the preparation of LLOQ in 80% ACN was inevitable.

3.1.2. Optimization of sample preparation

Due to the significantly higher concentrations of GLC and CRE in human urine, relative to other metabolites, an initial 3 fold dilution step with water was needed. Protein precipitation was achieved through 6.6 fold dilution with ACN spiked with ISs. We were interested in investigating the effect of filtration on method's sensitivity. The extracts of 6 urine samples were filtered using 0.2 µm PVDF 13 mm syringe filter (GE *Healthcare* Life Sciences, NJ, USA). No change in the peak area ratios following filtration was observed and consequently, higher sensitivity was not obtained. Moreover, there was a significant loss of URC and PYA following filtration as determined by paired student *t*-test (*t* values; 2.78 and 2.81 for URC and PYA, respectively), and accordingly, filtration was avoided (Supplemental materials, Figure 1).

3.1.3. Optimization of the chromatographic separation

Initial experiments conducted using different ratios of a binary isocratic mobile phase of NH_4FA (10-50 mM) and ACN achieved limited success in the separation of the investigated metabolites in the standard mixture and in urine. Consequently, gradient systems were explored while applying different column temperatures. The elution of GLC as a split peak as well as the use of single ion monitoring for PYA required vigilant optimization for their separation from closely eluting isomers (Figure 2). Ten mM NH_4FA was less optimal than higher strengths (concentrations $\geq$20 mM) for 1MN/COL and CRE/GAA separation. However, it imposed the least ion suppression effects on URC and was consequently employed as mobile phase A. Overall, peak shape was improved through the addition of 0.1% FA and 5% 10 mM NH_4FA in ACN (mobile phase B), with metabolites separation in 6.5 min (Figure 4).

The optimized chromatographic conditions also resulted in the separation of the α- and β- anomers of GLC which were both used for quantification (Figure 4). Interestingly, Fu et al [41]. reported that GLC can elute as single or split peak depending on the column being used. In our work, increasing the ionic strength of the mobile phase ($\geq$50 mM NH_4FA) resulted in the co-elution of both anomers as a single peak; however, this cannot be used as it resulted in significant suppression of URC. Finally, a time of 3 min was adequate to equilibrate the column prior to the following injection.

3.1.4. Optimization of mass spectrometric conditions

The MS/MS fragmentation pattern of the investigated metabolites was generated and rationalized (supplemental materials, schemes 1-7). Table 1 summarizes the MRM transitions used for quantification (quantifier ion) and confirmation of metabolites identity (qualifier ion).

The MS/MS of PYA did not result in the formation of product ions with adequate abundance to be used for quantification (supplemental materials, scheme 7). Consequently, its quantification was obtained in the single ion monitoring mode at *m/z* 87, while employing a CE of -10 V (Instrument default minimum CE setting) (Table 1). Other studies have reported its quantification using MRM transitions of *m/z* 175→87 [42] and *m/z* 87→43 [43], which were not generated in high intensities

in our work. Such difference can be attributed to the significantly higher LLOQ in the aforementioned studies [42, 43] or due to the low *m/z* value of the detected ion.

Due to the intrinsically lower specificity of the single ion monitoring mode in comparison to MRM mode, an additional experiment was conducted during PYA quantification to ensure the absence of interferences at unit resolution. The ratios of the qualifier (*m/z* 87→43)-to-quantifier (*m/z* 87→87) ions of PYA were calculated in patient samples and compared to that of the calibration standards [44-46]. Specificity in patient urine samples was demonstrated with ratios within ± 20% of that of the calibration standards.

GLC predominantly formed a stable formic acid adduct ion (supplemental materials, Figure 2A) [47] (Table 1), while other reported adducts, such as $[M+Na]^+$ [48, 49] were not formed. Interestingly, the MRM transition of *m/z* 225→179 was at least 15 times of higher intensity than other MRM ion transitions detected at the optimized MS/MS conditions (supplemental materials, Figure 2D). A similar fragmentation behaviour was observed with GLC-IS, and consequently, an MRM transition of *m/z* 227→181 was initially selected for it. However, when GLC-IS was spiked in urine, it was masked by a significant interference detected at the same MRM channel (supplemental materials, Figure 2B). For this reason, a transition of *m/z* 227→121 was employed (supplemental materials, Figure 2C). However, due to its intrinsically lower intensity, GLC-IS had to be spiked at a concentration above the ULOQ of GLC (Table 1), which is against the conventional use of ISs usually spiked at concentrations within the linearity range of their analytes [34].

Since the chromatographic peak of GLC closely eluted with other hexoses in urine and it was split due to anomer separation, the ratios of the qualifier-to-quantifier ions of GLC were calculated in patient samples and calibration standards and compared [44-46]. All ratios in patient urine samples were within ±20% of those of calibration standards, thus excluding the possibility of interference in chromatographic peaks of GLC.

3.2.Method validation

3.2.1. Selectivity

Table 2 demonstrates the acceptable selectivity of the method for the investigated metabolites in 80% ACN as per the regulatory requirements [31], in which the interferences observed at the metabolites channels were lower than 20% of the LLOQ, with a maximum value observed for URC (10.2% ±2.68 of the LLOQ).

The analysis of ISs-free urine revealed interferences in the ISs channel less than 1.6% ±2.5 of the LLOQ, indicating acceptable selectivity as defined by the EMA [31] for all metabolites except for PYA-IS (Table 2). PYA-IS experienced unacceptable interference in urine (19.40%±5.57 of the LLOQ) (Table 2), which was persistent even with the use of 6 other different sources of urine. PYA-IS was detected at *m/z* 90.01 in the single ion monitoring mode, rather than the MRM mode. With the absence of selective product ion of PYA-IS and the use of unit resolution MS, there are higher possibilities of interference from the urine matrix. Therefore, we opted to increase the concentration of the spiked PYA-IS (3000 ng/mL) to the extent where the contribution of this interference was below 0.6%. As for the isotopic purity of the IS, all ISs were sufficiently isotopically pure, in which a maximum interference of 6.25% of the LLOQ from PYA impurity in PYA-IS was observed (Table 2).

Finally, the isotopic contribution from the 2^{nd} natural isotopic peak of GAA, GLC and URC on their analogues ISs was monitored due to the presence of only 2-mass unit difference in each analyte/IS pair. This contribution becomes more profound towards the upper end of the calibration curve leading to a false increase in the peak area of the ISs and a false negative estimation of the

analyte concentration [34, 50]. We addressed a similar challenge in our recent work [28] in which the concentration of the IS was increased so that the effect of the isotopic contribution from its corresponding metabolite became negligible [28, 34]. Since the maximum contribution is expected to arise from the ULOQ, 5 replicates of the ULOQ (without ISs) were injected and the interference observed at the ISs channel of GAA, GLC and URC was measured. The interference was then compared to the areas of the optimized concentrations of the ISs prepared in blank solutions. The isotopic contribution from the ULOQ of GAA, GLC and URC minimally impacted their corresponding ISs, in which a maximum contribution of 0.9% was observed from the 2nd natural isotopic peak of GLC (Table 2).

3.2.2. Matrix effects

With the exception of PYA, all metabolites and their ISs suffered from varying levels of ion suppression (Table 3). Ion suppression is mostly attributed to the competition and interference from co-eluting moieties for charge during ionization [51]. However, ion suppression was also observed for the quaternary ammonium compounds, COL and 1MN which bear a permeant charge. This observation can indicate that non-volatile urine components might be affecting the efficiency of droplet formation and evaporation as well [52]. The only ion enhancement effect was observed for PYA with a matrix factor of 2.54 (Table 3). Despite ion suppression/enhancement effects, the use of isotopically labelled ISs provided the best correction of matrix effects. As can be seen in Table 3, the IS-normalized MF values for all metabolites were close to unity (0.91-1.05), indicating the ability of the ISs to account for other interferences from the urine matrix. In addition, the CV% values of the IS-normalized MF for all metabolites were less than 12.5%, thus meeting the specifications set by the EMA (Table 3) [31].

3.2.3. Carry-over effects

In order to avoid cross-contamination between samples, a needle-washing step with 80% ACN was employed between injections. In addition, blank samples were injected after the ULOQ and the HQC samples as well as after every 3 patient samples. No specific recommendations are compiled within the FDA guidelines [32] for evaluating carry-over effect, accordingly, the EMA guidelines [31] were followed. Table 3 demonstrates the negligible carry-over effects observed during the analysis of a batch of patient urine samples.

3.2.4. Lower limit of quantification and linearity

The LLOQ was determined so that a minimum signal-to-noise ratio of 5:1 was obtained [31, 32]. Achieving the lowest possible limit of quantification was specifically crucial for URC and PYA, due to their low levels within patients' urine samples. In addition, for each metabolite, the linear range was optimized based on its average endogenous levels observed during preliminary urine sample analysis. The linear ranges, therefore, varied vastly among the investigated metabolites (Table 4). Following sample analysis, the generated $^{1}/_{x}$ weighed least square regression equations met the specifications set by the FDA and the EMA guidelines [31, 32], in which at least 6 points out of 8 (including ULOQ and LLOQ) are within 15% of their nominal values (20% in LLOQ). Table 4 summarizes the statistical parameters of linearity which can be deemed adequate from the close-to-unity correlation coefficients ($r>0.998$).

3.2.5. Accuracy and Precision

The accuracy and precision of the developed method were assessed using 4 QC levels. A pooled urine sample whose endogenous metabolites levels were predetermined using the calibration curves, was spiked with low, medium and high concentrations of the working stock solution to generate LQC, MQC and HQC, respectively. Acceptable intra- and inter- day accuracy and precision were demonstrated for all metabolites at all levels according to the FDA and EMA

guidelines [31, 32]. As can be seen in Table 2, supplemental tables, recovery % values for intra-day accuracy were between 85.7% and 112%, while the CV% values were less than 10.5% for all metabolites at all levels. The inter-day recoveries were within 100 ±8% and the inter-day precision was with a CV% less than 11.5% (Table 3, supplemental tables).

3.2.6. Extraction recovery

Matuszewski et al. [53] described the extraction recovery as the ratio of the peak areas of the standards spiked before extraction in the matrix to the peak areas of the standards spiked after extraction. This definition, as such, is not applicable when the metabolites are endogenous in nature and a metabolite free matrix cannot be obtained. For this reason, a modified extraction recovery procedure was adopted, in which standards were spiked into pooled urine before and after extraction of endogenous metabolites using ACN spiked with ISs. Theoretically, the level of endogenous metabolites is not expected to change between *pre-spiked* and *post-spiked* pooled urine. Accordingly, the peak areas of endogenous metabolites in pooled urine, prepared similarly without spiking, were subtracted from the *pre-spiked* and *post-spiked* samples prior to peak areas ratio generation.

The FDA [32] recommends the evaluation of extraction recovery at the 3 concentration levels. It sets no criteria for the acceptable extent of recovery as long as the extraction process is consistent and precise. As shown in Table 4, supplemental tables, protein precipitation with ACN did not compromise the recovered concentration of the metabolites. The extraction recovery% ranged from 95.9% to 102.5% across low, medium and high levels. The extent of extraction was found consistent and precise across all levels with a CV% of less than 5% (Table 4, supplemental tables).

3.2.7. Dilution integrity

The EMA recommends the use of blank matrix for the dilution of the matrix that had been spiked above the ULOQ with the analyte standards. However, it still accepts the use of other justifiable matrices for sample dilution [31]. Due to the absence of a metabolite-free matrix, we selected water for this task, since it is the solvent used for standards preparation and it matches the aqueous nature of the urine. The analysis of urine samples revealed that GLC and CRE can occur at concentrations above the ULOQ (15% of samples). COL was concentrated in 5% of the processed samples. Patient samples also revealed the occasional occurrence of GAA and 1MN outside their linear ranges. For this reason, dilution integrity was investigated over 10, 20 and 100 folds of dilution for all metabolites. As demonstrated in Table 4, supplemental tables, acceptable accuracy and precision were maintained across all dilutions, in which the accuracy ranged from 90.3% to 103.5%, while the CV% values were lower than 8.4%.

3.2.8. Stability

Conditions that are encountered during sample handling and storage were employed for stability assessment. An average of 1 hours is the time needed for the preparation of a set of 10 patient urine samples along with calibration standards and QC samples. Accordingly, benchtop stability was evaluated by storing LQC and HQC samples at room temperature for 4 hours. Autosampler sample stability for 36 hours at 4 ºC can also allow processing a large batch of patient urine samples in an analytical run overnight. Table 5, supplemental tables, shows the stability results of the investigated metabolites under different conditions. Metabolites were found stable when kept for 4 hours at room temperature, for 36 hours at 4 ºC, for 1 and 3 months at -80 ºC or after 3 freeze-thaw cycles. As can be seen from Table 5, supplemental tables, recovery % values were between 88% and 107% with a CV% values less than 11%. As for PYA, the HQC samples demonstrated a subtle unacceptable accuracy (>84%) for freeze-thaw stability and benchtop stability. However,

such findings were not concerning since we have intentionally assessed the benchtop and freeze-thaw stability at levels higher than we would typically encounter during patient sample analysis.

3.3.Analysis of patient samples

To illustrate the usefulness of the method, a small cohort of patient samples was analyzed. Metabolite concentrations varied vastly among the processed patient urine samples. URC and CRE were below their LLOQ in 2 different patients. Four different samples required 100 fold dilution for the determination of CRE or GLC. After correction for dilution, data was log transformed and imported to SIMCA® software for PLS-DA. The constructed model demonstrated regression coefficient (R^2) of 0.752 and predictive coefficient (Q^2) of 0.57 with satisfactory separation between patient groups (Figure 5A). The VIP plot (Figure 5B) shows that URC, PYA and GAA were the most important metabolites in the model. Removing metabolites of low significance can enhance the R^2 and the Q^2 of the model [54, 55]. However, since no clinical decision is to be drawn from the current study, we opted against the exclusion of any metabolites at this stage. In addition, the expected final diagnostic model will contain additional significant metabolites using additional methods in a large sample cohort [28]. This report shows proof-of-concept to the appropriateness of the validated HILIC-MS/MS method in quantifying the selected metabolites in patient samples The altered levels of the investigated metabolites in response to either asthma [18, 56] or COPD [57, 58] have been previously reported in various biological fluids such as exhaled breath condensate (EBC) [57], serum [56, 58] and urine [18]. The investigated metabolites are involved in more than one biochemical process, thus complicating the characterization of the most significant underlying metabolomic pathways that contribute to their differential expression. For instance; COL, PYA, CRE and GAA are common intermediates in the pathway of glycine, serine and threonine metabolism, while PYA, CRE and GAA are common intermediates in arginine and proline metabolism [59]. PYA is also involved in other biochemical pathways including the pantothenate and CoA biosynthesis pathway along with URC [59]. PYA shares the pentose phosphate pathway with GLC and the nicotinamide metabolism pathway with 1MN [59], which is known to exhibit anti-inflammatory properties through oxygen radicals scavenging [60].

4. Conclusion

A novel HILIC-MS/MS method was developed for the quantification of 7 endogenous urinary metabolites. Due to the absence of metabolite free matrix, solvents were used for the preparation of calibration curves while quality control samples were prepared using pooled urine. The method was fully validated as per the FDA and EMA guidelines. The method was successfully applied for the quantification of the target metabolites in urine samples collected from asthma and COPD patients. A larger sample subset will be analyzed in the future, using the developed method along with other methods covering the remaining target metabolites.

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Table 1. MRM parameters of the investigated metabolites and their respective internal standards. *values in parenthesis represent CE of the qualifier product ion

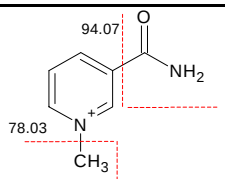
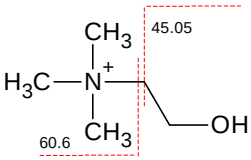
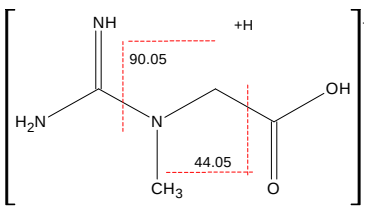
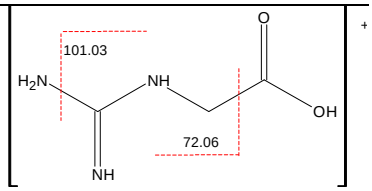
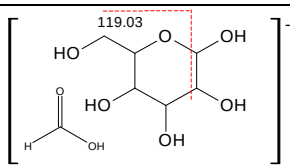
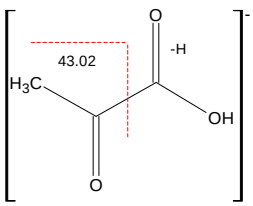
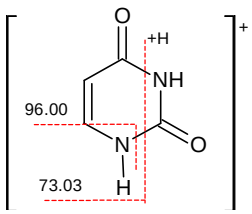
Metabolite	Ionization mode	Precursor ion (Da)	Product quantifier ion (Da)	Product qualifier ion (Da)	Collision energy (CE)*	Dwell time	Fragmentation pathway
1MN	Positive	137.07	94.07	78.03	15(20)	22	
1MN-IS		140.09	97.08		15	22	
COL	Positive	104.11	60.08	45.03	12(15)	22	
COL-IS		113.16	69.14		12	22	
CRE	Positive	132.08	90.05	44.05	27(18)	22	
CRE-IS		135.10	93.07		27	22	
GAA	Positive	118.06	101.03	72.06	14(14)	22	
GAA-IS		120.07	103.04		22	22	
GLC	Negative	225.06	179.06	119.03	-11(-11)	22	
GLC-IS		227.07	121.05		-11	22	
PYA	Negative	87.01	87.01	43.02	-10(-10)	150	
PYA-IS		90.01	90.01		(-10)	100	
URC	Positive	113.03	96.00	70.03	22 (22)	100	
URC-IS		115.05	98.00		22	100	

Table 2. Selectivity assessment of the developed HILC-MS/MS method for the quantification of seven endogenous metabolites in urine.

Metabolite	Mean % response in double blank at metabolite channel ±SD* n=6	Mean % response in urine at internal standard channel ±SD* n=6	Mean % response in blank at metabolite channel ±SD* (isotopic purity of internal standard) n=5	Mean % isotopic contribution from upper limit of quantification on internal standard channel ±SD* n=5
1MN	0.15±0.1	0.64±0.4	0.28±0.2	0.05±0.0
COL	3.84±1.1	0.33±0.2	1.41±0.9	0.01±0.0
CRE	0.47±0.3	1.55±2.4	4.80±8.4	0.23±0.0
GLC	0.68±0.1	0.58±0.6	0.70±0.3	0.90±0.0
GAA	1.88±1.2	3.65±0.9	3.56±1.7	0.65±0.0
PYA	7.49±6.4	**19.40±5.6**	6.25±3.2	0.05±0.0
URC	10.20±2.7	0.85±0.6	5.49±2.7	0.81±0.1

***** Standard deviation

Table 3. Evaluation of the matrix and carry-over effects in the HILIC-MS/MS method for the quantification of seven endogenous metabolites in urine

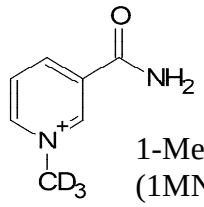
Metabolite	MF±SD* (n=12)	MF(IS)±SD* (n=12)	IS normalized MF±CV% (n=12)	Carry-over effects** (Blank peak area/LLOQ peak area ×100)±SD*, n=6
1MN	0.59±0.2	0.66±0.2	0.91±12.1	0.35±0.2
COL	0.68±0.2	0.71±0.2	0.96±7.2	3.53±1.7
CRE	0.82±0.2	0.79±0.2	1.03±10.4	0.33±0.1
GAA	0.87±0.2	0.83±0.1	1.05±9.7	13.29±1.7
GLC	0.55±0.1	0.57±0.1	0.97±7.0	5.53±3.1
PYA	2.54±0.7	2.61±0.7	0.97±6.2	6.77±3.3
URC	0.64±0.1	0.64±0.1	1.00±4.7	3.41±1.8

* Standard deviation

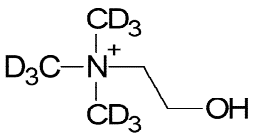
****** calculated from blanks injects after ULOQ, HQC and patient urine samples

Table 4: Regression parameters for the developed HILIC-MS/MS method for the quantification of seven endogenous metabolites in urine

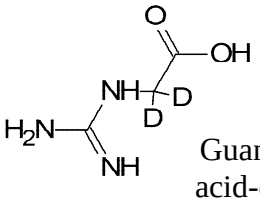
Metabolite	Linear range (ng/mL)	Slope	Intercept	Coefficient of determination (R^2)	Concentration of IS (ng/mL)
1MN	18-1800	5.12E-03	-1.16E-04	0.9999	750
COL	6-600	5.92E-03	4.55E-03	0.9998	250
CRE	60-6000	5.80E-04	4.00E-03	0.9998	1500
GAA	75-7500	5.74E-04	1.82E-02	0.9998	6000
GLC	210-21000	4.71E-04	1.80E-02	0.9998	30000
PYA	45-4500	2.43E-04	2.65E-03	0.9998	3000
URC	51-5100	2.39E-04	1.69E-03	0.9995	4000



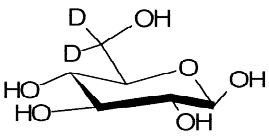
1-Methylnicotinamide-d_3 (1MN-IS)



Choline-d_9 (COL-IS)

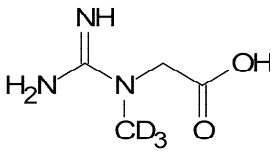


Guanidine acetic acid-d_2 (GAA-IS)



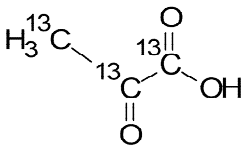
Glucose-d_2 (GLC-IS)

l structu

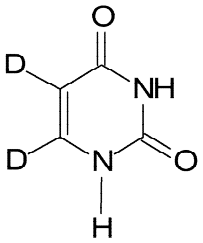


Creatine-d_3 (CRE-IS)

standa



Pyruvic acid-$^{13}C_3$ (PYA-IS)



Uracil-d_2 (URC-IS)

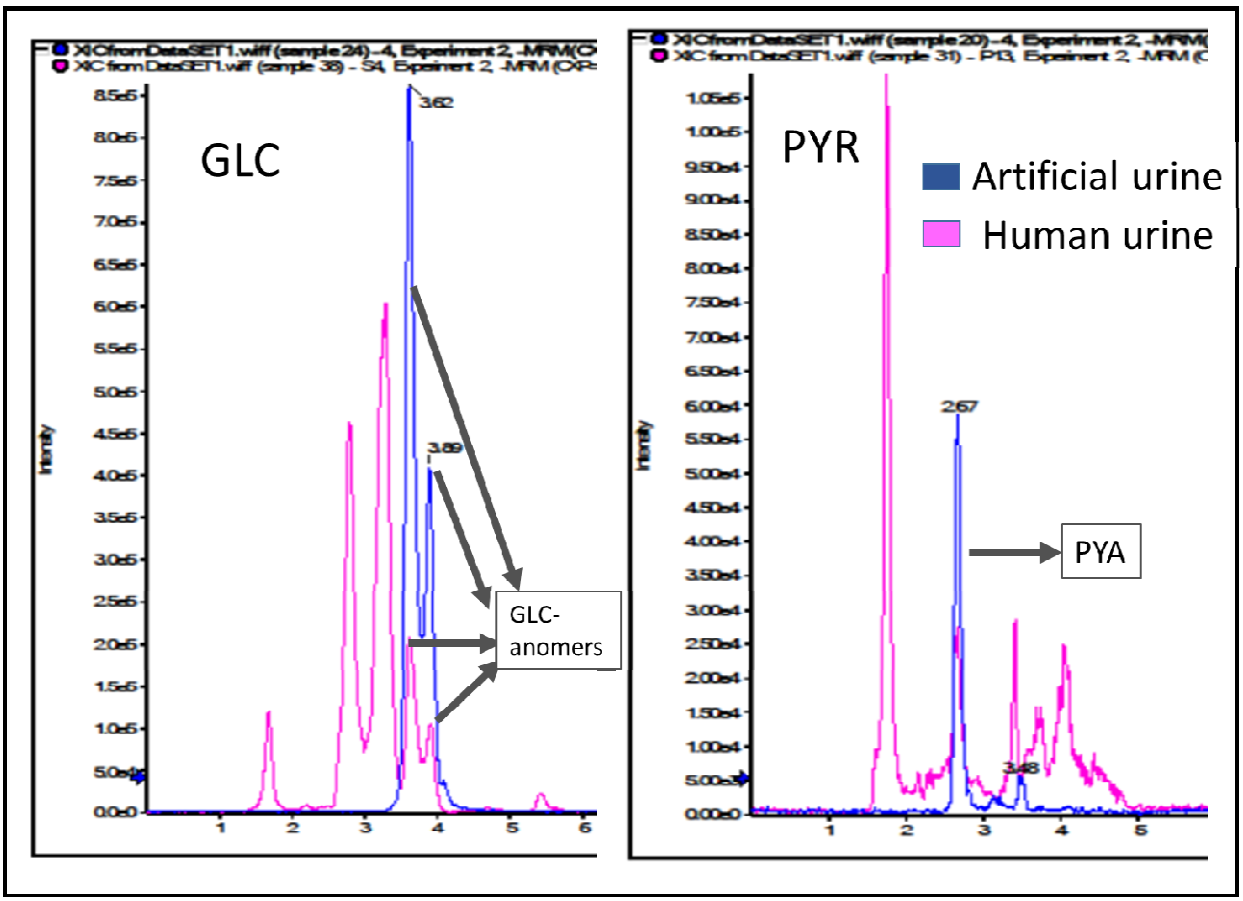


Figure 2. Extracted ion chromatogram (XIC) of GLC and PYA in artificial and human urine in negative ionization. Closely eluting hexoses were observed during the quantification of GLC in urine, however that was not reflected in the artificial urine. Similarly, elevated baseline levels in PYA quantification was only observed during urine analysis.

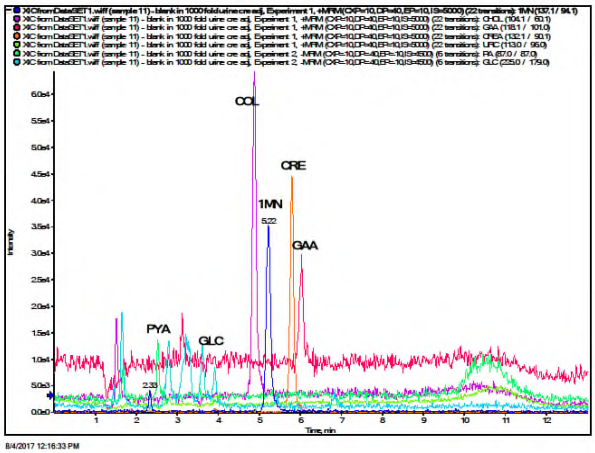


Figure 3. Extracted ion chromatogram (XIC) of the investigated metabolites in a double blank prepared in 1000 fold diluted pooled urine. The presence of significant endogenous levels of the metabolites rendered the 1000 fold diluted urine an unsuitable matrix for method development. Positive ionization is employed for all metabolites except for PYA and GLC.

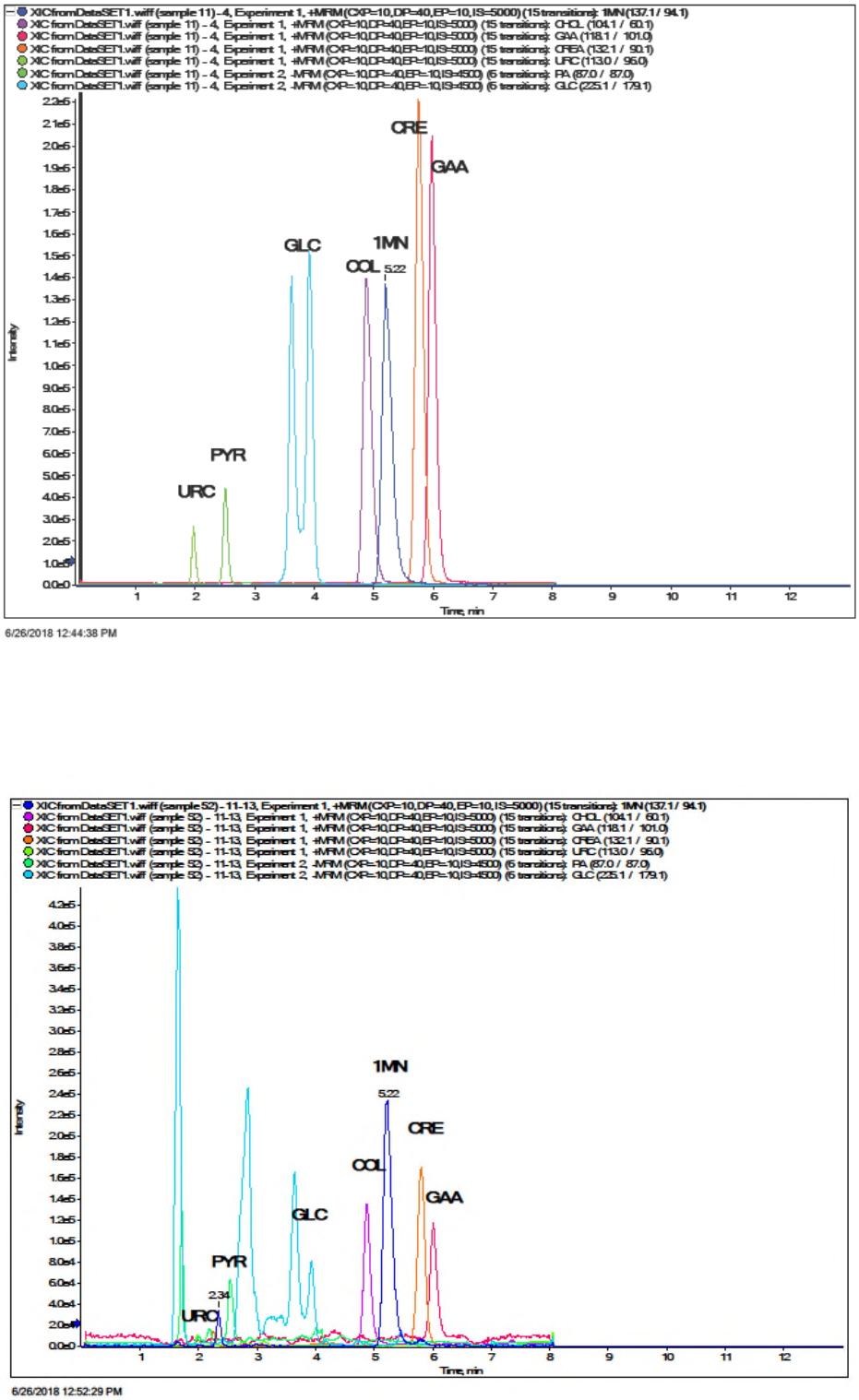


Figure 4. Extracted ion chromatogram (XIC) of the seven investigated metabolites in standard mixture (top) and in patient urine sample (bottom) using the validated HILIC-MS/MS method. Positive ionization is employed for all metabolites except for GLC and PYA where negative ionization was employe

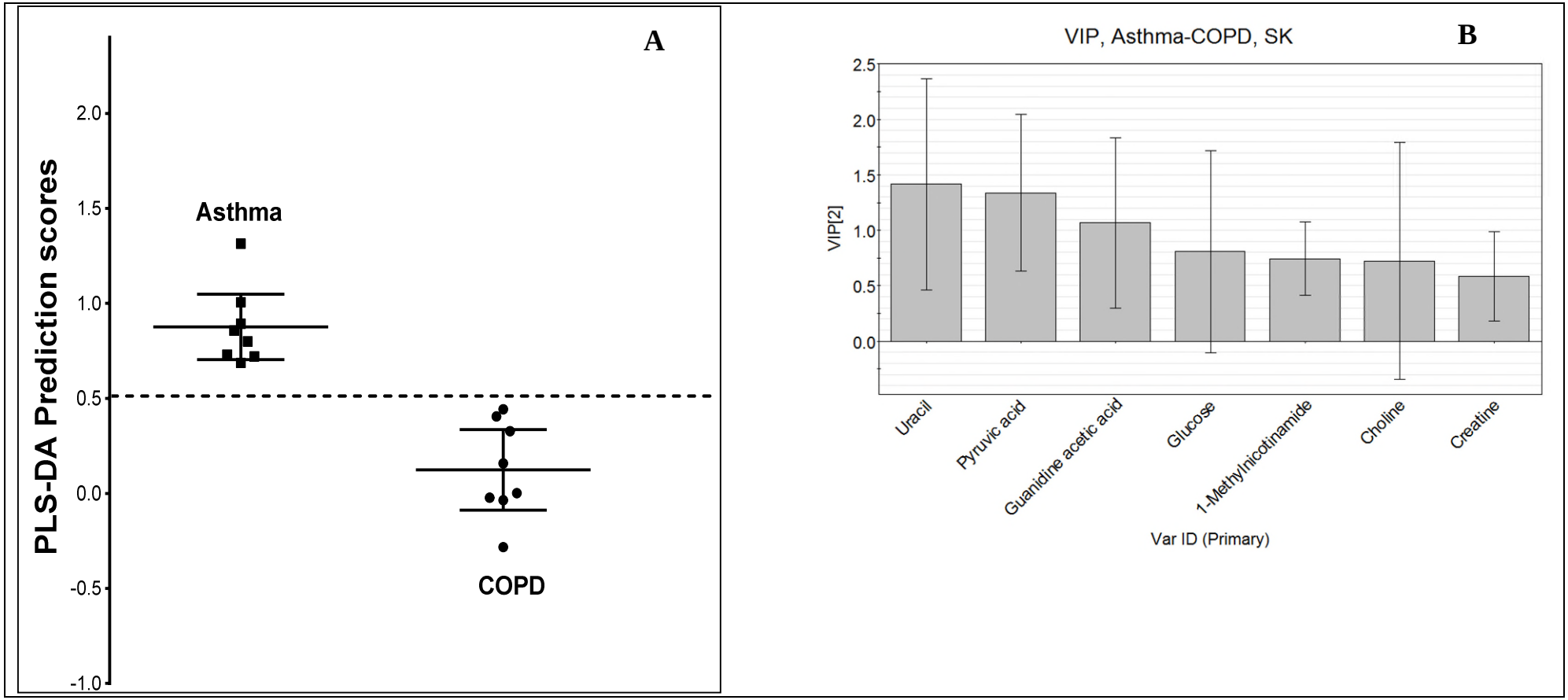


Figure 5. (A) PLS-DA prediction score for each subject, with error bars representing medians and interquartile ranges. (B) VIP ranking the metabolites according to their significance in the mode