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Quantification of urinary zwitterionic organic acids using weak-anion exchange chromatography with tandem MS detection

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Abstract

A rapid and accurate quantitative method was developed and validated for the analysis of four urinary organic acids with nitrogen containing functional groups, formiminoglutamic acid (FIGLU), pyroglutamic acid (PYRGLU), 5-hydroxyindoleacetic acid (5-HIAA), and 2-methylhippuric acid (2-METHIP) by liquid chromatography tandem mass spectrometry (LC/MS/MS). The chromatography was developed using a weak anion-exchange amino column that provided mixed-mode retention of the analytes. The elution gradient relied on changes in mobile phase pH over a concave gradient, without the use of counter-ions or concentrated salt buffers. A simple sample preparation was used, only requiring the dilution of urine prior to instrumental analysis. The method was validated based on linearity ($r^2 \geq 0.995$), accuracy (85–115%), precision (C.V. < 12%), sample preparation stability (≤5%, 72 h), and established patient ranges. The method was found to be both efficient and accurate for the analysis of urinary zwitterionic organic acids.

Keywords: Weak Anion Exchange; Formiminoglutamic acid; Pyroglutamic acid; 5-hydroxyindoleacetic acid; 2-methylhippuric acid; LC/MS/MS; Organic acids

1. Introduction

Metabolic disorders, and related disease, are quickly emerging as the most prevalent cause of preventable death in the United States [1]. These findings are not unique to the U.S., as many industrialized nations experience an ever-growing population afflicted with varying forms of metabolic disorder. This is a result of increased exposure to toxins coupled with poor diet and exercise, facilitated by current customs in developed countries [2,3]. A direct consequence of these factors is the prevalence of obesity, which has been related to many metabolic disorders [4]. Growing knowledge of these disorders, along with an increased use of testing in conjunction with technological advancement has lead to early detection and treatment of many metabolic diseases [5]. However, delayed detection and treatment has significant draw backs that can alter the long-term health of patients with disease [6]. There is a need for rapid and accurate testing to facilitate early detection of metabolic disease. In an attempt to lower cost and improve availability, many tests for metabolic diseases have been developed that provide non-invasive sample collection that requires little or no supervision by a healthcare professional [5,7].

The measurement of urinary formiminoglutamic acid (FIGLU), L-pyroglutamate (PYRGLU), 5-Hydroxyindole-3-acetic acid (5-HIAA), and 2-methylhippurate (2-METHIP) has been reported in the assessment of specific metabolic disorders and toxicity. The quantification of FIGLU and PYRGLU has been related to the status of specific metabolic pathways. The functional state of folate metabolism and the evaluation of formiminotransferase deficiencies have been related to FIGLU excretion [8,9]. The measurement of PYRGLU has been used in the assessment of glycine insufficiencies and the diagnosis of 5-oxoprolinuria [10,11]. The major form of metabolized serotonin, 5-HIAA has been reported as a marker for the content and turnover of gastrointestinal serotonin [12,13]. It has also been related to carcoid syndrome as well as a number of neurological disorders [14–17]. The measurement of urinary 2-METHIP has been associated with a specific form of toxic

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exposure, arising from contact with substances containing xylene and toluene [18–20].

The most common analytical technique used for the evaluation of low molecular weight biological organic acids has been gas chromatography/mass spectrometry (GC/MS) [21]. However, in recent years the introduction of liquid chromatography/tandem mass spectrometry (LC/MS/MS) has allowed for more rapid analytical techniques to be developed [22]. The decrease in time needed for analysis is attributed to the high degree of selectivity offered by LC/MS/MS instrumentation. However, many published reports for the rapid analysis of small compounds are flawed by improper use of the mass spectrometer or by their lack of adequate chromatography [23]. In some cases, the selectivity of small molecules is not sufficient enough to offer total accuracy in the absence of chromatographic separation [24]. The chromatographic separation developed must provide enough retention to separate compounds from interferences and avoid matrix effects seen by co-elution within the column void volume [23–25].

LC separations are commonly performed using reversed phase chromatography, even though retention of small polar biological compounds is difficult without derivatization [26]. A major disadvantage of analyte derivatization is the increased sample preparation, which may be less cost effective for clinical settings. Many methods which do not require derivatization, rely on changes in mobile phase pH or ion pairing reagents to promote retention. In either case, the mobile phase additives have been shown to reduce sensitivity on LC/MS/MS systems [24]. Ion exchange chromatography offers an alternative approach, which can provide excellent retention of small polar biological compounds. However, classical ion-exchange methods require the use of mobile phases that contain high concentrations of non-volatile salts or other counter-ions, which can lead to ion suppression [27].

The method presented utilizes weak-anion exchange chromatography for compound separation. Four small biological compounds are separated on an amino stationary phase using a pH gradient with an organic modifier. The underivatized compounds require little sample preparation and are adequately retained on column to provide separation from interferences and to minimize loss of sensitivity due to matrix effects. This method was validated for clinical use and was found to be rapid, robust, and reproducible.

2. Experimental

2.1. Instrumentation and reagents

The chromatographic separations were performed on a Waters (Milford MA, USA) 2695 high-performance liquid chromatograph. Samples were analyzed on a Waters Quattro-micro tandem mass spectrometer equipped with an electrospray ionization source. All collected data was processed using MassLynx V4.0.

HPLC grade acetonitrile and formic acid were purchased from VWR (VWR International, North America), and ammonium formate was obtained from Sigma (St. Louis, MO, USA). The standards, 5-hydroxyindole-3-acetic acid and L-pyroglutamate were purchased from Sigma. 2-methylhippurate was purchased from Aldrich (St. Louis, MO, USA). Formiminoglutamic acid was obtained from PharmAgra Laboratories (Brevard, NC, USA). Internal standards, *N*-benzoylalanine (NBA) and 5-fluoroindole-3-acetic acid (5-FIAA) were obtained from Sigma. Glutamic acid, 2, 4, 4,-*d*3 (D3GLU), was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Chromatographic conditions

The separation was performed on a Phenomenex (Torrance, CA, USA) Luna Amino column, 50 mm × 2.0 mm, 5 μm maintained at 40 °C throughout the experiment. The three mobile phases consisted of 0.2% formic acid in de-ionized water (MPA), 20.0 mM ammonium formate in de-ionized water adjusted to pH 5.0 with formic acid (MPB), and acetonitrile with 0.15% formic acid (MPC). Twenty five micro litres of the sample was injected under these initial mobile phase conditions: 40% MPA, 35% MPB, and 25% MPC at a flow rate of 0.3 mL/min. A concave gradient was employed over the first 2 min (0–2 min) to 25% MPA, MPB 0%, and 75% MPC. These conditions were held for 1 min (2–3 min) following the gradient. Finally, the mobile phase composition was returned to the initial conditions after 3 min (3.01–8), and the flow rate increased to 0.5 mL/min to minimize total run time (0–8 min). The gradient employed in this method utilized a present instrument gradient, Waters #8, to perform a concave gradient from initial conditions to the final elution conditions (0–3 min). The gradient changes were concave up for MPA and MPC. However, the MPB concentration changes from 35% to 0% were concave down. The overall elution profile results in a change in mobile phase composition from a buffered environment to one that is un-buffered, acidic, and containing a large proportion of organic phase.

2.3. Mass spectrometer conditions

All compounds were detected in electrospray positive ionization mode, with the desolvation gas set to 800 L/h. To increase sensitivity the cone gas was not used. Capillary voltage was maintained at 3.5 kV, with source and desolvation temperatures at 150 and 350 °C, respectively. Each MRM was collected at unit mass resolution with a dwell time of 0.1 s. The cone and collision settings were established individually for each compound for multiple reaction monitoring (MRM) detection. The conditions for detection of all analytes were obtained by direct infusion of a standard solution in line with the HPLC at initial mobile phase conditions. The MRM transitions and appropriate detection settings are presented in Table 1.

2.4. Standard and working solutions

Standard stock solutions were prepared as follows: Working stock solution A (StkA) was made by the addition of 0.032 g of FIGLU and 0.0015 g of 2-METHIP to a 500 mL volumetric flask and brought to volume with de-ionized water. Working stock solution B (StkB) was prepared by the addition of 0.08 g

Table 1
MRM transitions and detection settings for all analytes and internal standards

Analytes and I.S.	Parent ion [M + H]	Product ion	Cone potential (V)	Collision energy (eV)
FIGLU	175	82.9	20	20
PYRGLU	130	83.9	25	15
5-HIAA	192	146	20	15
2-METHIP	194	119	15	10
D3GLU	151	86.8	15	15
NBA	194.1	104.9	20	15
5-FIAA	194	148	20	15

of PYRGLU and 0.005 g of 5-HIAA to a 100 mL volumetric flask containing 33 mL of StkA and brought to volume with de-ionized water. The internal standard solution was prepared by the addition of 0.001 g of NBA, 0.001 g of D3GLU, and 0.002 g of FIAA to a 500 mL volumetric flask and brought to volume with de-ionized water.

2.5. Calibration standards

Calibration standards were prepared by serial dilution of StkB for the desired calibration range established from collected patient data. Six calibration standards, including a blank, were prepared for each analyte as follows: FIGLU, 0.00, 0.480, 0.960, 1.92, 2.88, 3.84, 15.4 mg/L; PYRGLU, 0.00, 25.0, 50.0, 100, 150, 200, 800 mg/L; 2-METHIP, 0.00, 0.0310, 0.0630, 0.125, 0.188, 0.250, 1.00 mg/L; 5-HIAA, 0.00, 1.56, 3.13, 6.25, 9.38, 12.5, 50.0 mg/L.

2.6. Urinary creatinine measurement

Urinary creatinine concentration was measured on a Cobas Mira Plus using a creatinine assay kit purchased from Roche (Quebec, Canada) following Jaffe's picric acid method [28].

2.7. Patient ranges

Adult patient ranges were established using intra-laboratory samples, following method validation. All concentration measurements were normalized to creatinine. Data was taken from approximately two hundred patient samples to calculate a working within laboratory range for both normal and elevated results. Normal patient ranges were established within the 95% confidence level. Any result outside the established normal range was considered to be elevated. The ranges established for each analyte are relevant to patients 13 years of age and older.

2.8. Preparation of quality control samples

Normal controls were prepared from pooled urine. The pooled sample was also used in the preparation of the elevated controls. Elevated controls were spiked with a known amount of standard salt which was dissolved in normal control urine. The amount of standard added elevated the normal value of each analyte to a level within the calibration range and above the observed normal patient range.

2.9. Method validation

The method was validated based upon linearity, accuracy, precision, and sample preparation stability. Linearity was evaluated using a six-point calibration curve. Accuracy was established by measuring spike recoveries for all analytes in a pooled urine sample when spiked with mid-level and high-level calibrators. The urine samples were spiked with no more than 10% of initial urine volume and calculated based on the average of three successive measurements for each level. Precision, within and between run, was calculated using normal and elevated ($n = 30$) controls collected over a five-day period. Sample preparation stability was evaluated from quantitative results of three samples taken over three days.

2.9.1. Linearity

The linearity of the calibration curve was evaluated by linear regression, including the intercept ($y = mx + b$), weighted by $1/x$. Linear curves were comprised of six calibration levels, run in duplicate and quantified from a standard curve to evaluate precision and accuracy. All calculations were performed using EP Evaluator 6 software (RHOADS, Kennett Square, PA, USA).

2.9.2. Limits of detection (LOD) and quantification (LOQ)

The LOD (S/N = 3) and LOQ (S/N = 10) were determined using the regression approach based upon the linear regression of calibration from the established linear range [29]. The sensitivity of the present method was determined from these measurements.

2.9.3. Evaluation of matrix effects

The effects of sample matrix on calibration were measured using a simple matrix matching experiment. A pooled urine sample was prepared using intra-laboratory samples. To prepare calibration standards in matrix, a volume of pooled urine (500 μL) was added to 16 mm × 100 mm glass tubes and blown to dryness under a steady stream of nitrogen in a water bath maintained at 50 °C. The dried urine was reconstituted in 500 μL of calibration solutions, absent of matrix, corresponding to the calibration range used to establish linearity [30]. The matrix matched calibration solutions were measured using the same method described in this report and compared with measured calibration solutions at the corresponding calibration levels [31]. The data from matrix matched and unmatched calibrators were taken to generate linear regression plots using Microsoft Excel (2003). Two plots were examined, the first comparing the area

under the curve (AUC) versus calibration concentration and the second comparing the response factor of analyte area corrected by internal standard area versus calibration concentration. Within each plot, matrix effects were statistically evaluated by comparison of the slopes for each regression line using Student's *t* test.

2.9.4. Accuracy

Accuracy was evaluated by spike recovery from pooled urine samples. This baseline urine level was spiked with two levels of calibrators (mid-level and high-level). Both spiked samples were prepared by the addition of 10% (v/v) of the specific calibrate to the baseline urine. Mid-level solutions were spiked using calibrator level 5 (FIGLU 3.84 mg/L, PYRGLU 200 mg/L, 2-METHIP 0.25 mg/L, 5-HIAA 12.5 mg/L). High-level spikes were spiked using working stock solution B (FIGLU 15.36 mg/L, PYRGLU 800 mg/L, 2-METHIP 1.0 mg/L, 5-HIAA 50 mg/L). The baseline samples were prepared in a similar way i.e. made with 10% de-ionized water in the place of calibrate. The baseline, mid-level, and high-level samples were run in duplicate and quantified using a standard curve.

2.9.5. Precision

Precision was measured by the variation of normal and elevated control values for each analyte over a five-day period. Within run data was evaluated statistically for each control group. Between run data was evaluated based upon values for all control group data.

2.9.6. Stability of sample preparation

Stability of the sample preparation was measured over a period of three days. A prepared sample of elevated control urine was measured once per day, starting with an initial measurement and evaluated at 24 h intervals for three consecutive days. During the course of the experiment, the sample was stored on instrument at 5 °C. The preparation stability for each analyte was evaluated based upon the percent deviation of the analytes from the initial measurement.

2.9.7. Sample preparation

Urine samples were collected in tubes containing 20 uL of thymol (0.05 mg/mL) as a preservative, and stored at −20 °C. Samples were prepared by diluting 100 μL of urine with 100 μL of internal standard solution and 300 μL of buffer solution matching the initial mobile phase conditions (40% MPA, 35% MPB, 25% MPC).

3. Results and discussion

3.1. Mass spectrometry

FIGLU, PYRGLU, 5-HIAA, and 2-METHIP are not conventional organic acids because each compound exhibits a zwitterion. This structural characteristic allows for both positive and negative electrospray ionization. The sensitivity for either mode of detection can be enhanced by mobile phase pH. Each analyte was evaluated in both modes at varying pH, from 2.0 to 11.0. The conditions that provided the greatest sensitivity for

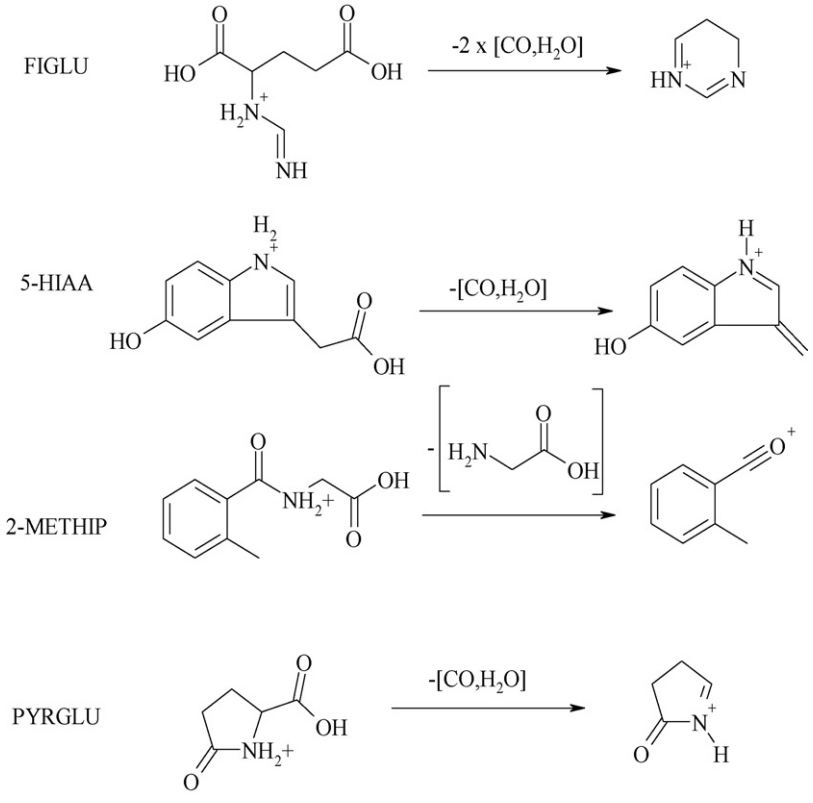

Fig. 1. Prediction of fragmentation ions for FIGLU, 5-HIAA, 2-METHIP, and PYRGLU.

all analytes were electrospray positive at low pH (<4.0). Internal standards were chosen with similar chemical structures and ionization potential. The most dominant molecular ion formed at low pH, $[M+H]^+$, resulted from the ionization of the nitrogen containing functional group within the compounds. Solvent adducts were not observed. Stable product ions were formed for all analytes and internal standards. The proposed mechanism of fragmentation for each ion is displayed in Fig. 1. The optimized mass spectrometer conditions provided good sensitivity for each analyte.

The selectivity of each analyte was evaluated by monitoring all MRM channels during individual injections of single analytes made from high concentration calibration solutions. No cross-channel interference was observed greater than 5% of the lower limit of detection. However, the selectivity of each analyte MRM in matrix was not adequate enough to negate the need for chromatography. Interferences were observed in the MRMs for PYRGLU, 5-HIAA, 2-METHIP, 5-FIAA, and NBA. Baseline separation of all analyte peaks from interferences was achieved with the exception of PYRGLU and 2-METHIP. Resolution from the interferences of PYRGLU was achieved at 30% peak height and 2-METHIP at 10% peak height. Alternative MRMs were explored for all analytes that contained matrix interferences, however no MRMs were found to provide selectivity or sensitivity greater than those presented.

3.2. Chromatography

The separation of all target analytes and internal standards from interferences and the column void volume is important for the overall accuracy of the method. In developing this method, reversed phase chromatography was evaluated for retention, resolution, and efficiency. The reversed phase columns used varied in both hydrophobic character (C8, C18) and manufacturer. Experiments were performed using simple gradients with aqueous and organic mobile phases modified with formic acid to lower the pH (∼2.5). The results of these experiments were similar for hydrophilic compounds, FIGLU and PYRGLU, yet varying slightly for compounds with more hydrophobic character, 5-HIAA and 2-METHIP. The retention of FIGLU and PYRGLU was not found to be adequate for any reversed phase column used. However, 5-HIAA and 2-METHIP were well retained and easily manipulated with gradients of organic mobile phase. In each case, the lack of retention for two of the compounds was unsuitable for accurate measurement, while the retention of the other analytes would require extended run times and column equilibration.

Ion exchange chromatography is well suited for the analysis of small biological compounds. However, the most common ion exchange methods require mobile phase additives that can drastically reduce sensitivity when using LC/MS/MS systems. A variation of traditional ion exchange relies on a change in pH and the addition of organic mobile phase to promote elution. The retention of compounds, using weak anion exchange, is a result of electrostatic interactions between the carboxylic acid and the stationary phase. These interactions can be mediated by controlling the pH of the mobile phase. As the pH is lowered below the pKa of the carboxylic acid, the ionic character of the compound is decreased and retention shifts to a reversed phase mechanism. Compound retention is then mediated by hydrophobic interactions which can be manipulated by increasing the concentration of organic mobile phase. As a result, weak ion exchange chromatography can be accomplished without the use of salts or counter-ions. The resulting chromatography is illustrated in Fig. 2.

3.3. Method validation

3.3.1. Linearity

Linearity was evaluated based on the average of six calibrators ($n=2$) and a blank calculated from a standard curve. The curves were fit to a linear equation of slope and intercept ($y=mx+b$) weighted by $1/x$. All slopes had r^2 values greater than 0.995. Deviations from the standard values, based upon recovery, were less than 15% for all analytes. Residuals about the line of regression were less than 15% of the target value for all analytes.

3.3.2. Limits of detection (LOD) and quantification (LOQ)

The LOD (S/N = 3) and LOQ (S/N = 10) were measured for each analyte based upon the linear regression of calibration from the established linear range. The calculated values are shown in Table 2. Both LOD and LOQ indicate adequate sensitivity for this method given that clinically significant results are much higher than the LOQ.

3.3.3. Evaluation of matrix effects

Two linear regression plots were generated for all analytes comparing AUC and the response factor of the analyte versus calibration concentration. The variations observed in the slopes of matrix matched and unmatched calibrators within each plot were evaluated to determine the existence of any significant matrix effects. The assessment of plotted AUC data was to determine if any inherit matrix effects were present that would significantly alter analyte response. The data taken from graphs of response factor were used to determine if the internal standards appropriately correct for analyte specific matrix effects. The variations between the mean ($n=2$) slopes of matrix matched and unmatched calibration plots were evaluated using Student's t test at the 95% confidence level (Table 3). Fig. 3 shows the average linear regression of both AUC and response plots for 2-METHIP to illustrate the effect of matrix and internal standard

Table 2
The calculated LOD and LOQ for all analytes

Analyte	LOD[a] (mg/L)	LOQ[b] (mg/L)
PYRGLU	0.437	1.46
2-METHIP	0.0172	0.0574
5-HIAA	0.280	0.933
FIGLU	0.286	0.953

[a] LOD was calculated at S/N = 3.
[b] LOQ was calculated at S/N = 10.

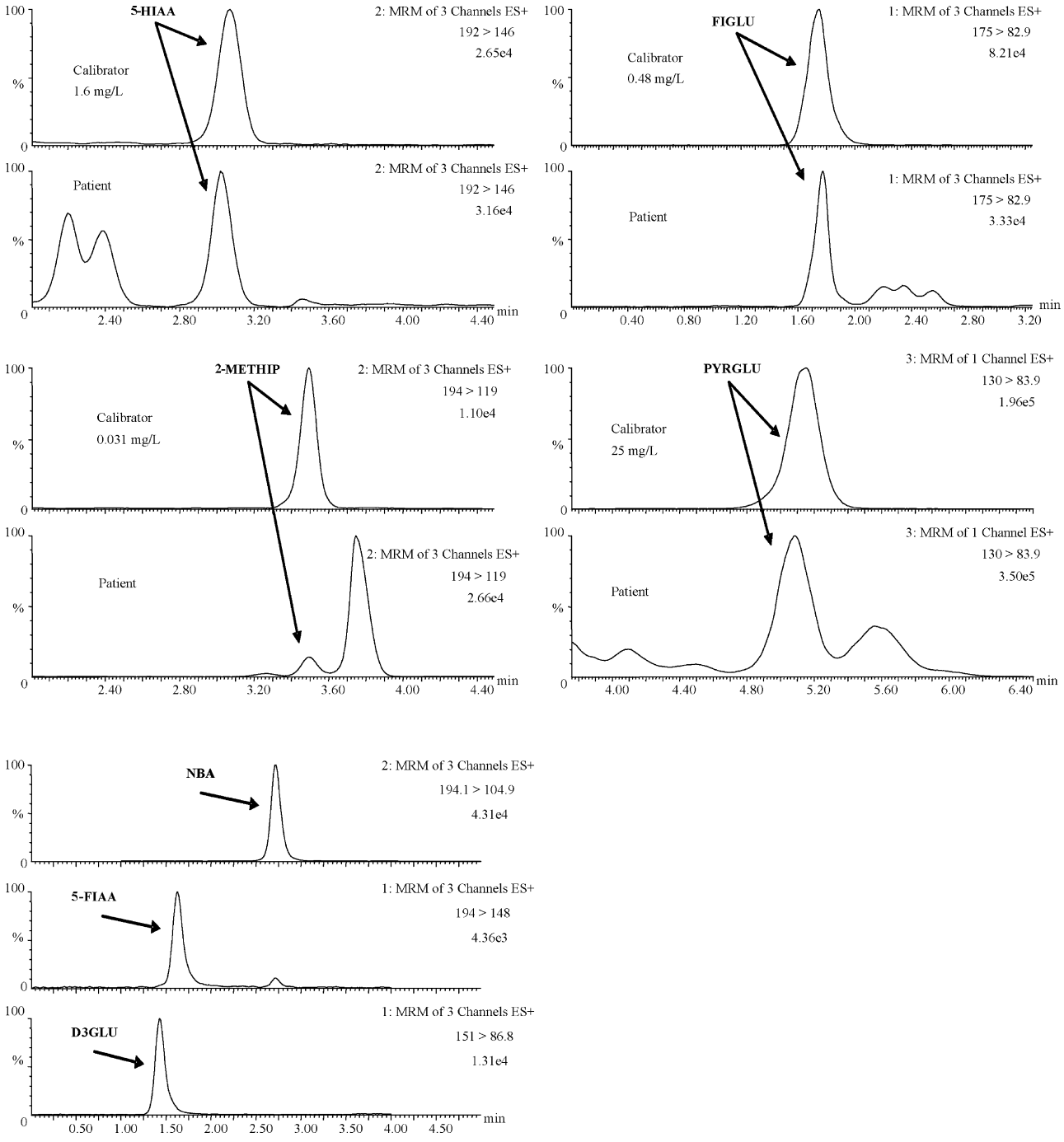

Fig. 2. Representative chromatograms of 5-HIAA, FIGLU, 2-METHIP, PYRGLU, and internal standards. For the analyte chromatograms, the upper channels show a calibration standard and the lower channels show a normal patient. The internal standards shown are taken from the calibration blank.

correction on the slope of each plot. FIGLU and 2-METHIP have significant statistical variations in slope ($t_{calculated} > t_{table}$) for AUC plots, indicating that matrix effects exist for these analytes. These variations were not observed for the AUC plots of PYRGLU and 5-HIAA, indicating that no significant matrix effects were present for these analytes. For all analytes, no significant statistical variations in the slopes for response plots were observed. These findings indicate the effectiveness of internal standards to correct for any matrix effects. Therefore, matrix effects were observed for some analytes, however, the use of response factors as a ratio of analyte area to internal standard area adequately compensated for any error that may have resulted from these effects.

3.3.4. Accuracy

The accuracy of each analyte was evaluated based on the percent recovery for two levels of spiked samples compared with a baseline of pooled urine. The percent deviation from the theoretical value for the recovered spike was less than 15%. The average recoveries for each analyte are found in Table 4.

Table 3
Statistical comparison between the slopes of matched and unmatched calibrators

Analyte	Cal. solution	Slope (AUC vs. conc.)	*t*-value[a]	Slope (resp. factor vs. conc.)	*t*-value[a]
FIGLU	H_2O	1076 ± 32	7.16	0.2457 ± 0.0023	2.16
	Matrix	1256 ± 15		0.2772 ± 0.0205	
PYRGLU	H_2O	661 ± 10	3.07	0.0219 ± 0.0006	2.45
	Matrix	537 ± 56		0.0230 ± 0.0002	
5-HIAA	H_2O	1891 ± 44	1.84	0.8918 ± 0.0051	1.80
	Matrix	1530 ± 274		0.9571 ± 0.0510	
2-METHIP	H_2O	8570 ± 487	7.78	0.2831 ± 0.0032	2.16
	Matrix	5840 ± 96		0.2452 ± 0.0245	

[a] Calculated *t*-value at 95% confidence level ($n = 2$); table *t*-value at 95% confidence level ($n = 2$) is 4.303. The difference is significant if $t_{calculated} > t_{table}$.

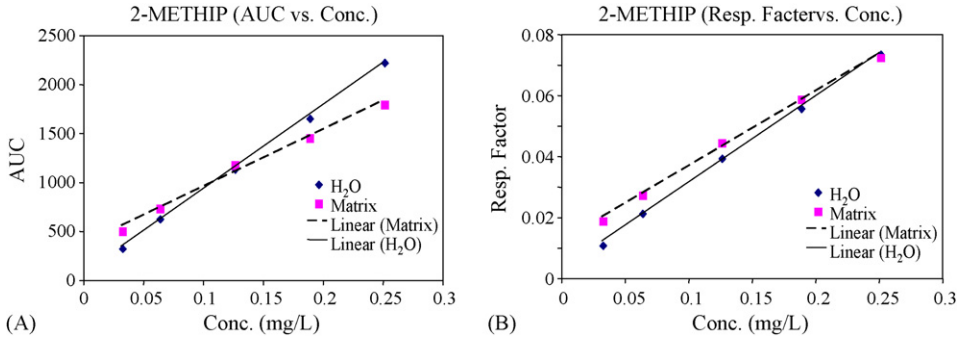

Fig. 3. The plots of calibration solutions in H_2O and in urine matrix. (A) is the linear regression plot of AUC vs. conc. used to determine the effects of matrix. (B) is the linear regression plot of resp. factor vs. conc. used to determine the correction of internal standard on the slope. The variations in slope between each curve in 3A and 3B were determined using the Student's *t* test at the 95% confidence level.

3.3.5. Precision

Precision was calculated from two control values, normal and elevated, over five days. To establish precision, ten samples of each control were evaluated in one batch. Five samples of each control were run once per day for the remaining 4 days of the study. The with-in and between run precision for all days are displayed in Table 5. The within-run precision was less than 8% for all normal controls and no greater than 8.1% for ele-

Table 4
% Recovery of FIGLU, PYRGLU, 2-METHIP, 5-HIAA from pooled urine

Spike solution	% Spike recovery (mean ± S.D., $n = 3$)			
	FIGLU	PYRGLU	2-METHIP	5-HIAA
Mid-level	96.92 ± 0.01	105.1 ± 0.3	96.0 ± 0.1	116.3 ± 0.2
High-level	101.3 ± 0.1	107.9 ± 4.0	97.7 ± 0.1	112.0 ± 0.3

Table 5
Precision of normal and elevated controls in urine

Analyte	Control	Within-run[a]		Between-run[b]	
		Conc. (mg/L)	%CV	Conc. (mg/L)	%CV
FIGLU	NC	0.293 ± 0.022	7.57	0.291 ± 0.024	8.33
	EC	2.19 ± 0.17	7.96	2.22 ± 0.22	9.78
PYRGLU	NC	40.0 ± 1.9	4.75	39.6 ± 2.5	6.35
	EC	292 ± 14	4.86	295 ± 19	6.44
5-HIAA	NC	2.96 ± 0.13	4.26	2.91 ± 0.33	11.2
	EC	29.0 ± 1.6	5.66	28.7 ± 2.2	7.49
2-METHIP	NC	0.0329 ± 0.0025	7.66	0.0331 ± 0.0033	10
	EC	0.321 ± 0.012	3.86	0.320 ± 0.015	4.79

[a] Mean concentration ($n = 5$) of control values with S.D.
[b] Mean concentration ($n = 30$) of control values with S.D.

Table 6
Sample preparation stability, % deviation over 3 days

Analytes	24 h	48 h	72 h
FIGLU	−10.2	−13.3	−11.7
PYRGLU	−8.62	1.03	−4.48
5-HIAA	−6.16	3.86	0.389
2-METHIP	−3.14	−0.314	−3.77

Table 7
Normal and elevated ranges (μg/mg Crea.) ($n \sim 200$)

	Normal	Elevated
FIGLU	0.00–2.90	>2.90
PYRGLU	0.00–96.0	>96.0
5-HIAA	0.75–8.70	>8.70
2-METHIP	0.00–0.10	>0.10

vated controls. The between-run precision was less than 10% for FIGLU and PYRGLU. 5-HIAA and 2-METHIP had the greatest between run variation but were no more than 11.2%.

3.3.6. Sample stability

The data corresponding to sample preparation stability is presented in Table 6. All analytes deviated less than 14% from the initial values over a 72 h period. The deviation observed for all analytes was not great enough to affect the clinical relevance of the measurement. Given the precision of the collected data along with no negative observable trend in stability suggests that each analyte was stable on instrument for 72 h at 5 °C.

3.4. Patient ranges

Adult patient ranges were taken from approximately two hundred intra-laboratory samples. The values for normal and elevated results were taken from these ranges. The established ranges, corrected for creatinine, are presented in Table 7. Although no current patient ranges for FIGLU, PYRGLU, and 2-METHIP have been reported, urinary ranges for 5-HIAA are well established. The normal patient ranges determined by this method for 5-HIAA compare well with previously reported ranges [32,33].

4. Conclusions

An analytical method for the evaluation of four urinary metabolites using weak ion-exchange chromatography and tandem MS detection was developed and validated. The analytes were resolved using a pH gradient without high concentrations of counter-ion or buffer. Sample preparation was minimal, requiring only a simple dilution before analysis. Prepared samples were found to be stable on instrument for up to 72 h, insuring that large patient batches can be assayed accurately. The method was found to be accurate and precise. The method is rapid allowing for high-through-put analysis and screening for metabolic disorders and toxicity.

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