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Development of an isotope labeling ultra-high performance liquid chromatography mass spectrometric method for quantification of acylglycines in human urine

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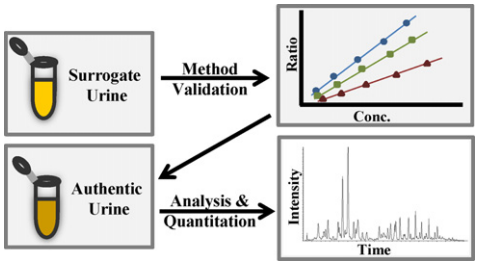
GRAPHICAL ABSTRACT



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ABSTRACT

Acylglycines play a crucial regulatory and detoxification role in the accumulation of the corresponding acyl CoA esters and are an important class of metabolites in the diagnoses of inborn errors of metabolism. Sensitive quantification of a large number of acylglycines not only improves diagnosis but also enables the discovery of potential new biomarkers of diseases. We report an ultra-high performance liquid chromatography tandem mass spectrometry (UPLC–MS) method for quantifying acylglycines in human urine with high sensitivity. This method is based on the use of a newly developed isotope labeling reagent, p-dimethylaminophenacyl (DmPA) bromide, to label acylglycines to improve detection sensitivity. Eighteen acylglycines, namely acetylglycine, propionylglycine, isobutyrylglycine, butyrylglycine, 4-hydroxyphenylacetylglycine, 2-furoylglycine, tiglylglycine, 2-methybutyrylglycine, 3-methylcrotonylglycine, isovalerylglycine, valerylglycine, hexanoylglycine, phenylacetylglycine, phenylpropionylglycine, glutarylglycine, heptanoylglycine, octanoylglycine and suberylglycine, were measured. This method uses calibration standards prepared in surrogate matrix (un-derivatized urine) and stable-isotope labeled analytes as the internal standards. The analysis was carried out in the positive ion detection mode using multiple reaction monitoring (MRM) survey scans. The calibration curves were validated over the range of 1.0–500 nM. The method achieved a lower limit of quantitation (LLOQ) of 1–5 nM for all analytes, as measured by the standard derivations associated with calibration curves and confirmed in surrogate matrix; the signal-to-noise ratio at LLOQ ranged from 12.50 to 156.70. Both accuracy (% RE or relative error) and precision (% CV) were <15%. Matrix effects were minimized using the surrogate matrix. All eighteen analytes were stable in urine for at least 5 h at room temperature, autosampler (4 °C) for 24 h, 7 weeks at −20 °C and after three freeze/thaw cycles. This surrogate matrix approach was validated using a standard addition experiment. As an example of

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applications, the endogenous concentrations of all eighteen analytes in urine samples of 20 healthy individuals collected in three consecutive days (i.e., 60 samples) were determined; there was no significant correlation found between the acylglycine profile and gender or body mass indices.

1. Introduction

The analysis of acylglycines in biofluids is of major interest in diagnosing inborn errors of metabolism (IEMs) because of their prominent role in metabolic pathways [1–3]. IEMs change the way the body processes nutrients and uses them for energy production. These disorders are characterized by a defect in a single enzyme or multiple enzymes, resulting in reduced or absence of catalytic activity, or by the impaired activity of transporters or cofactors along the metabolic pathway. This can result in an accumulation of metabolites that can become substrates for enzymes in alternative metabolic pathways. In disorders of amino acid and fatty acid metabolisms, if the intermediary metabolism of amino acids or fatty acids is blocked, the intermediary product formed will be in excess and must be conjugated to carnitine or glycine to facilitate excretion or a balance of coenzyme A. Glycine conjugation serves an important detoxification role and occurs mainly in the liver. Therefore acylglycines in urine is an indicator of the accumulation of the corresponding acyl CoA esters and can be important in the diagnoses of some IEMs, such as fatty acid metabolism disorders and amino acid metabolism disorders. In order to diagnose as many IEMs as possible, a wide range of acylglycines should be quantified in urine.

Traditionally acylglycines in urine have been analyzed by gas chromatography–mass spectrometry (GC–MS) [4,5] and are usually derivatized before analysis. GC–MS provides the combination of a high resolution separation in gas chromatography and mass spectrometric detection, which reduces the number of false positives. Metabolic profiling can also be done by GC–MS analysis. Although GC–MS methods offer these advantages, they can be tedious and time consuming. Direct infusion electrospray ionization (ESI) tandem mass spectrometry (MS/MS) [6] has been used to analyze acylglycines, and it is a fast and powerful diagnostic tool, but it lacks the capability to distinguish between isomeric and isobaric species and is limited in diagnosing certain IEMs. A few LC–MS methods have been reported for quantification of acylglycines [7–9] with varying degree of sensitivity and detectability; the lower limit of quantification, as defined by the signal-to-noise ratio at 10 where the noise was measured in aqueous solution (not the same matrix solution as urine), was in low to high nanomolar range. In a recent study [10], we have analyzed acylglycines using ultra-high performance liquid chromatography (UPLC) MS/MS and have qualitatively detected many acylglycines that were previously undetected in normal human urine. Many of them were found to be present at low concentrations as the ion signals were relatively low in UPLC MS/MS.

The objective of this present work is to develop a sensitive UPLC–MS method for quantification of acylglycines including the low abundance ones in human urine. To improve the detection sensitivity as well as the accuracy and precision of UPLC–MS, an isotope labeling approach based on a newly developed isotope reagent, p-dimethylaminophenacyl (DmPA) bromide [11], is combined with a "surrogate matrix" strategy for quantifying acylglycines with high performance.

2. Experimental

2.1. Chemicals and reagents

Optima grade acetonitrile, methanol and water were purchased from Fisher Scientific (Ottawa, ON). Triethylamine (TEA) and formic acid were obtained from Sigma–Aldrich (Oakville, ON). Acetylglycine, propionylglycine, isobutyrylglycine, butyrylglycine, 2-methylbutyrylglycine, isovalerylglycine, valerylglycine, hexanoylglycine, heptanoylglycine, tiglylglycine, glutarylglycine and suberylglycine were purchased from Dr. Herman J. ten Brink (Amsterdam, Netherlands). 3-Methylcrotonylglycine, 2-furoylglycine, phenylacetylglycine, phenylpropionylglycine, and 4-hydroxyphenylacetylglycine standards were obtained from the Human Metabolome Database (HMBD) compound library (University of Alberta, Edmonton, AB). Octanoylglycine was purchased from Sigma–Aldrich. Synthesis of $^{12}C_2$- and $^{13}C_2$-p-dimethylaminophenacyl bromide was done according to the published procedure [11].

2.2. Urine samples

Urine samples were collected from 10 males and 10 females, who were known to be not on any special diet or medications or have any inborn errors of metabolism. This study was conducted in accordance with the Arts, Science & Law Research Ethics Board policy at the University of Alberta. The volunteers were all adults, aged 25–30, with body mass indices (BMIs) ranging from 18 to 34.3. The urine samples were all collected as second morning void for 3 consecutive days (a total of 60 samples). The urine was centrifuged, filtered, aliquoted and stored at −80 °C without any added preservatives, until analysis.

Creatinine in urine was determined using an assay based on a kinetic Jaffe reaction using the QuantiChrom™ Creatinine Assay kit (Gentaur, Montreal, QC) with a linear detection range of 8.0 μM to 4.4 mM. Acylglycine levels reported herein were normalized to a creatinine concentration of 200 nmol.

2.3. Sample preparation

A standard stock solution was prepared by dissolving each accurately weighed acylglycine into acetonitrile to give a concentration of 1.0 mM. A mixture of all acylglycines at a concentration of 10 μM in 20% water in acetonitrile was mixed with 100 μL of either $^{12}C_2$-DmPA or $^{13}C_2$-DmPA (30 mg mL^{-1}) and 100 μL TEA (30 mg mL^{-1}) in acetonitrile. The mixture was vortexed and heated in a heating block at 90 °C for 1 h. The reaction was then terminated with 100 μL acetic acid dissolved in acetonitrile (10%, v/v) reacted at 90 °C for 15 min. The reaction scheme is shown in Supplemental Scheme S1. The resulting mixture was evaporated to dryness and reconstituted in 100 μL 0.1% formic acid in 50% acetonitrile (50/50, v/v) before analysis by UPLC–MS.

For the urine samples, volumes of urine equivalent to 200 nmol creatinine were aliquoted into vials and evaporated to dryness. The dried urine was then reconstituted in 100 μL of 20% water in acetonitrile and vortexed. To the mixture was added 100 μL triethylamine (30 mg mL^{-1}) and 100 μL DmPA (30 mg mL^{-1}) and the mixture heated in a heating block at 90 °C for 1 h. The reaction was terminated with 100 μL acetic acid dissolved in acetonitrile (10%, v/v) reacted at 90 °C for 15 min. The resulting mixture was evaporated to dryness and reconstituted in 100 μL 0.1% formic acid in 50% acetonitrile (50/50, v/v) before it was subjected to UPLC–MS analysis.

2.4. UPLC–MS

Liquid chromatography was performed on an Agilent 1290 Series LC (Mississauga, ON). Chromatographic separation was done on two Phenomenex Kinetex 1.7 μm minibore 50 mm × 2.1 mm C_{18} columns. Elution conditions were as follows: linear gradient of 18–28% mobile phase B over 17 min, 28–43% B over 18 min, and 43–90% B over 10 min, where mobile phase A consisted of 2% acetonitrile, 0.1% formic acid in water and mobile phase B consisted of 2% water, 0.1% formic acid in acetonitrile. The columns were maintained at 25 °C and autosampler set at 4 °C. Flow rate was set at 0.250 mL min^{-1} and 5.0 μL of each sample was injected. The regeneration pump performed column wash and equilibration steps in parallel on the second C_{18} column using 100% mobile phase B and 18% mobile phase B, respectively.

Mass analysis was carried out on an AB Sciex 4000 QTRAP® hybrid triple quadrupole linear ion trap mass spectrometer (Concord, ON), equipped with an electrospray ionization (ESI) interface. The ESI source was set to perform in the positive ion mode. The optimized parameters were as follows: spray voltage, 4800; curtain gas, 10; CAD, high; temperature, 250 °C; GS1, 40; GS2, 30; declustering potential, 45; collision energy, 25 eV. The acquisition method consisted of an information dependent acquisition (IDA) scan using multiple reaction monitoring (MRM) as a survey scan and two dependent enhanced product ion (EPI) scans. The EPI scans were in the range 50–800 Da, scanned at 4000 Da s^{-1}. Data was acquired and processed using Analyst®, version 1.5.1 software (Concord, ON).

2.5. Method validation

The method was validated by examining the followings: selectivity, linearity of ISTD and analytes, accuracy and precision. Carry-over and matrix effects were also evaluated. All required statistical analyses were performed using R script 2.11.1 and Igor Pro 6.01.

2.5.1. Selectivity and carry-over

Selectivity of the method was determined by analyzing the following: an instrument blank (injecting only mobile phase A), a solvent blank [consisting of 0.1% formic acid in 50% acetonitrile (50/50, v/v)], a reaction blank (reaction mixture without analytes), blank surrogate matrix and authentic matrix. These were all analyzed using UPLC–MS and were evaluated for the presence of any interfering signals. Carry-over was assessed by analyzing instrument blanks after analysis of 5 replicates of upper limit of quantitation (ULOQ) standards, 5 replicates of high quality control (QC) standards and 3 replicates of high concentration samples.

2.5.2. Linearity

To increase the efficiency of the surrogate matrix method (see below), a stable isotope labeled internal standard (ISTD) should be used whenever possible. In order to determine the suitability of the $^{13}C_2$-DmPA-labeled analytes as the ISTD, two sets of separate calibration curves were constructed: one for each analyte and one for each corresponding ISTD. Two sets of standards, one containing eighteen ^{12}C-DmPA-labeled acylglycines at concentrations of 1, 5, 10, 50, 100, 500, 1000 nM and another containing the $^{13}C_2$-labeled acylglycines at the same concentrations were made. The calibration standards were prepared in the solvent mix and surrogate matrix separately and analyzed in triplicates. Similarities in detector response for the analytes and their ISTD were evaluated by comparing the slopes (sensitivity) of the regression curves obtained in surrogate and authentic matrix using a modified Student *t*-test, at the 95% confidence level [12]. The test statistic is:

$$t = \frac{b_1 - b_2}{s_{b_1-b_2}}, \text{ where } s_{b_1-b_2}\sqrt{\frac{(s^2_{Y.X})_p}{(\Sigma x^2)_1} + \frac{(s^2_{Y.X})_p}{(\Sigma x^2)_1}} \text{ and } (s^2_{Y.X})_p = \frac{(\text{residual SS})_1 + (\text{residual SS})_2}{(\text{residual DF})_1 + (\text{residual DF})_2}$$

The parameters used: 'b' is the slope of the regression line, '$s_{b_1-b_2}$' is the standard error of the difference between regression coefficients, $(s^2_{Y.X})_p$ is the pooled residual mean square and the subscripts 1 and 2 refer to the two regression lines being compared. Critical value has $(n_1 + n_2 - 4)$ degrees of freedom.

The linearity of the ESI response for the DmPA-labeled acylglycines was examined by analyzing 5 replicates at 7 different concentration levels. Serial dilutions of a mixture of eighteen $^{12}C_2$-DmPA-labeled acylglycines (10 μM) were performed to obtain concentrations of 1, 5, 10, 50, 100, 500, 1000 nM in surrogate urine. Here, the surrogate matrix used is pooled underivatized urine prepared by mixing equal volumes of the urine samples. A fixed volume of $^{13}C_2$-DmPA-labeled acylglycines (final concentration 100 nM) was added to the urine as internal standard. Peak area ratios were calculated by dividing the peak area of each analyte by the peak areas of the corresponding ISTD. Calibration curves were constructed by plotting peak areas ratios against concentration of each acylglycine. Different weighting factors were evaluated and the curves were fitted to linear regression analysis with a weighting of $1/y$. The limit of detection (LOD) and the lower limit of quantitation (LLOQ) were determined for each acylglycine using the equations $3 \times (\sigma_B)/m$ and $10 \times (\sigma_B)/m$, respectively, where σ_B is the standard deviation of the response and m is the slope derived from the calibration curve. The standard deviation of the blank response was estimated from the regression parameter, standard deviation of the y-intercept [13,14]. The LLOQ of each analyte was confirmed experimentally by analyzing five replicates of surrogate matrix spiked with the analytes at a concentration equal to the LLOQ.

2.5.3. Authentication of the surrogate matrix

Calibration standards were prepared as described above by spiking the ^{12}C-DmPA-labeled analytes in derivatized pooled urine (known in this study as authentic matrix) at 7 different concentration levels and analyzed by the same UPLC–MS method. The suitability of the surrogate matrix as the calibrator matrix was evaluated by comparing the slopes (sensitivity) of the regression curves obtained in surrogate and authentic matrix using the modified Student *t*-test described above, at the 95% confidence level.

A standard addition experiment was performed to evaluate the suitability of underivatized urine as a surrogate matrix. The endogenous concentrations of acylglycines in a derivatized pooled urine sample were determined by analyzing three replicates of the samples by UPLC–MS and using the surrogate matrix calibration curve as described above. The pooled urine sample was then used to prepare a standard addition curve by adding a series of derivatized standards in increasing concentration. The concentration of each acylglycine in the pooled urine was determined by extrapolating to determine the magnitude of the x-intercept. This concentration was compared to the concentration of acylglycines in pooled urine derived from the surrogate matrix calibration curve. Percent relative error (% RE) was calculated using the following equation: (surrogate matrix concentration − standard addition concentration)/standard addition concentration × 100.

2.5.4. Dilution linearity

Three volumes of pooled derivatized urine were fortified with 100 nM derivatized standards. Dilutional linearity was determined by serially diluting pooled derivatized urine 2-, 5-, 10-, and 20-fold with surrogate urine. The measured concentrations of the diluted samples were plotted against expected concentrations for each analyte and a linear regression was performed.

2.5.5. Matrix effects

Matrix effects were assessed by comparing the slopes of the calibration curves prepared in solvent mix to those prepared in surrogate (underivatized) and authentic (derivatized) urine. The modified *t*-test, at the 95% confidence level, was used to compare the slopes to evaluate if the slope differences are statistically significant and indicate matrix effects. In addition ion suppression/enhancement was also calculated according to the following equation:

$$\%\,\text{Supperssion/enhancement} = \frac{\text{slope of curve in matrix}}{\text{slope of curve in solvent}} \times 100 - 100$$

2.5.6. Inter-day and Intra-day precision

The between-day and within-day precision were determined by analyzing a series of 10 injections of a derivatized pooled urine sample over 3 consecutive days under the same operating conditions. Intra-day precision was evaluated for the 10 injections (total of 10 replicates per day) and inter-day precision was evaluated for a period of three days (total of 30 replicates). Precision was evaluated by calculating coefficients of variations (% CV) from the measured concentrations.

2.5.7. Method reproducibility

To measure the reproducibility of the derivatization method, five replicates of pooled urine were derivatized by the method described above and analyzed by UPLC–MS. Method precision was evaluated by calculating coefficients of variations (% CV) from the measured concentrations.

2.5.8. Accuracy

To demonstrate the applicability of the method to accurately determine the concentrations of acylglycines in urine, three sets of pooled derivatized urine was fortified with derivatized analytes at three concentrations (three replicates per level): 10 nM (low QC), 150 nM (medium QC) and 400 nM (high QC). The accuracy was calculated by subtracting the amount of endogenous acylglycine in an un-spiked sample from the amount found in fortified sample and comparing to the added amounts. Percent relative error (% RE) or deviation from the added concentration was calculated as a measure of accuracy.

2.5.9. Stability

Three pooled derivatized urine samples were diluted 1:10 and fortified with derivatized analytes at a concentration of 100 nM to study the stability of the analytes. The urine stability samples were analyzed immediately after spiking (labeled "fresh" samples) and at selected times over the storage period. Stability was assessed after each freeze–thaw cycle for three cycles, after 24 h in the autosampler at 4 °C, and after 5 h on a benchtop at room temperature. Freezer stability was assessed by analyzing the sample after storage at −20 °C for two and eight weeks. The percent recovery of each analyte was determined by calculating the concentration at each condition and expressed as a percentage of the fresh sample.

Table 1
Masses, abbreviations and retention times of protonated DmPA-labeled acylglycines.

DmPA-labeled acylglycine	Abbreviation	Protonated mass (Da)	RT (min)
Acetylglycine	AG	279	3.46
Propionylglycine	PG	293	5.12
Isobutyrylglycine	IBG	307	7.86
Butyrylglycine	BG	307	8.12
4-Hydroxyphenylacetylglycine	HPAG	371	8.57
2-Furoylglycine	FG	331	9.44
Tiglylglycine	TG	319	11.36
2-Methylbutyrylglycine	2MBG	321	11.86
3-Methylcrotonylglycine	3MCG	319	12.19
Isovalerylglycine	IVG	321	12.35
Valerylglycine	VG	321	13.34
Hexanoylglycine	HG	335	19.87
Phenylacetylglycine	PAG	355	20.65
Phenylpropionylglycine	PPG	369	20.82
Glutarylglycine	GG	512	22.88
Heptanoylglycine	HpG	349	23.69
Octanoylglycine	OG	363	26.60
Suberylglycine	SG	554	26.68

2.6. Measurement of endogenous levels of acylglycines in human urine

For absolute quantification of acylglycines, three volumes of urine from each individual equivalent to 200 nmol creatinine were dried and derivatized with $^{12}C_2$-DmPA as explained above and analyzed by UPLC–MS. Peak area ratios for each acylglycine were calculated and quantification was performed using the equations derived from the calibration curves in surrogate matrix.

Relative quantification was done on putatively identified acylglycines, for which there were no standards available. Putative identification was done using fragmentation patterns based on a previous study [10]. Urine samples were prepared as described above for absolute quantification and analyzed using identical conditions. Peak area ratios were calculated using the peak area of each acylglycine and peak area of the closest eluting ISTD. Quantification was done using the equations derived from the calibration curves in surrogate matrix.

3. Results and discussion

3.1. Effect of derivatization on chromatography

Chromatographic separation of polar analytes before mass spectrometric detection enhances their detection sensitivity, partly because it reduces ion suppression. Acylglycines are polar molecules and they elute in a higher percentage of aqueous solvent when using a reversed phase (RP) column, especially the smaller and more polar acylglycines, such as acetylglycine, glutarylglycine and propionylglycine. The addition of the labeling group or tag, which contains an aromatic ring increases the hydrophobicity of the acylglycines and therefore increases their retention on a RP column. This results in a higher efficiency in separation and isomers are better separated when compared to the unlabeled species. Under the experimental conditions of the present method, the analysis run time was 37 min and the retention times of the eighteen derivatized acylglycines and their abbreviations are shown in Table 1. The incorporation of a stable-isotope labeling tag does not display any isotope effect in the RP separation. This is illustrated in Figure 1A where the ^{12}C- and $^{13}C_2$-labeled analytes, ^{12}C-/$^{13}C_2$-DmPA-hexanoylglycine (HG), perfectly co-elute. Co-elution of the light and heavy labeled analyte and ISTD respectively are essential for accurate quantitation by UPLC–MS. Differences in retention time of analyte and ISTD can cause significant variability in the

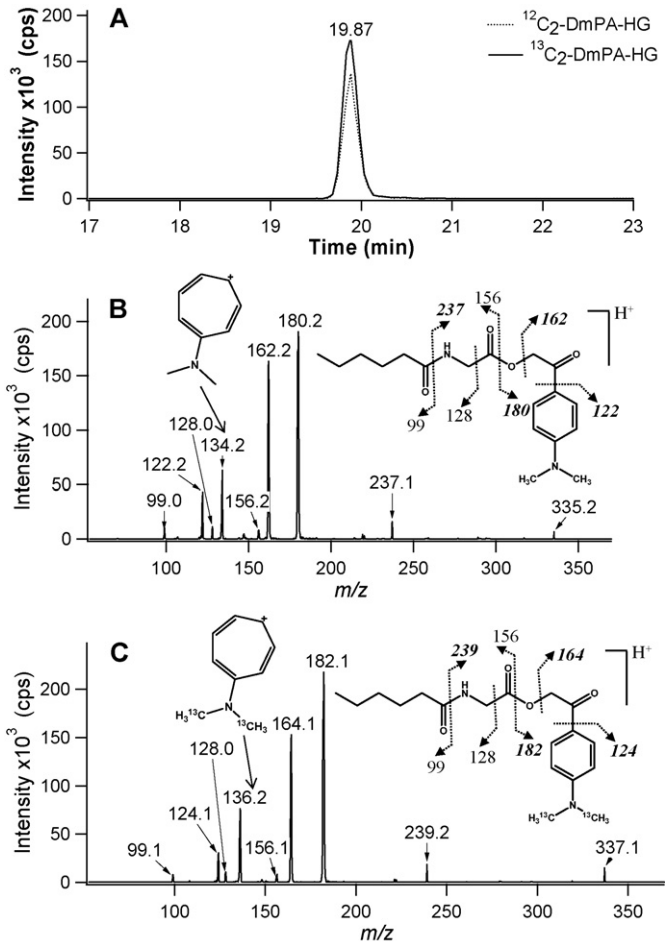

Fig. 1. (A) Extracted ion chromatograms of analyte $^{12}C_2$-DmPA-labeled hexanoylglycine (HG) and internal standard $^{13}C_2$-DmPA-labeled hexanoylglycine (HG). (B) Product ion spectrum of $^{12}C_2$-DmPA-HG showing the structure and fragmentation. (C) Product ion spectrum of $^{13}C_2$-DmPA-HG showing the structure and fragmentation. Fragments in bold italics are the fragments of the DmPA tag. Fragments *m*/*z* 134 and *m*/*z* 136 are shown as insets in B and C, respectively.

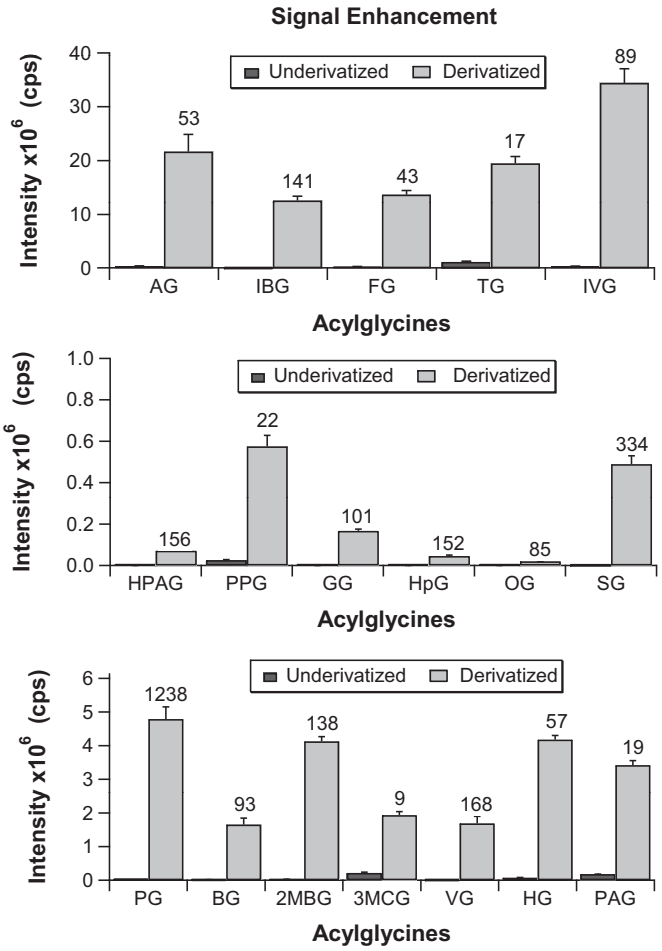

Fig. 2. Signal enhancement using the DmPA tag. Acylglycines are grouped by peak area ranges. Numbers in bold above the bars are the fold increase of derivatized to underivatized.

ion suppression effect in the detection of both and can result in quantitation errors.

3.2. MS and MS/MS analysis

The structures of a representative derivatized acylglycine, DmPA-hexanoylglycine (^{12}C-form), and its internal standard ($^{13}C_2$-form) are shown in panels B and C of Fig. 1, respectively. For these two compounds the protonated precursor molecules were observed at *m*/*z* 335 and *m*/*z* 337 for hexanoylglycine and ISTD, respectively. During the IDA experiment, the precursors are subjected to collision-induced dissociation to yield product ion spectra. The product ion spectra of hexanoylglycine and ISTD are also shown in Fig. 1B and C, along with the proposed fragmentation schemes. Under optimized conditions to yield the highest sensitivity of the analytes, the most abundant fragment ions produced is *m*/*z* 180 and *m*/*z* 182 for hexanoylglycine and ISTD, respectively. These two fragments are generated from the loss of a neutral ketene (on the side of the acylglycine) and resulting in a protonated DmPA tag. These fragments are generated in the product ion spectra of all the analytes and their corresponding internal standards and thus were used in the MRM survey scans, with the Q1 ions being the precursor ions of either the analyte or ISTD and Q3 ions being either *m*/*z* 180 or *m*/*z* 182, respectively. A list of Q1 and Q3 ions used for the MRM survey scan and instrument parameters are shown in Supplemental Table S1. Fragment ions at *m*/*z* 162, *m*/*z* 134 and *m*/*z* 122 are produced from the further fragmentation of the DmPA tag and are present in all product ion spectra of the analytes. Fragment ions at *m*/*z* 99, *m*/*z* 128 and *m*/*z* 156 are produced from dissociation of the acylglycine portion of the molecule and are more diagnostic in nature. These result from neutral losses of 236, 207, and 179, respectively, and these losses were used as diagnostic losses to detect other acylglycines. Explanation of the fragmentation pattern can be found in a previous study [10]. The fragment ion at *m*/*z* 237 is also diagnostic because it corresponds to the protonated mass of (DmPA + glycine).

An IDA experiment was employed to generate simultaneous product ion spectra, which were used to confirm the identity of the acylglycine detected. With 63 precursor ions scanned in the MRM survey scan and a dwell time of 15 ms, peak shape integrity was maintained and a minimum of 11 points across a peak could still be obtained. No cross-talk was detected in the mass spectrometric analysis. This proposed method enabled the determination of low-level concentrations of derivatized acylglycines in human urine.

3.3. ESI signal enhancement

Three volumes of a urine sample were analyzed before and after derivatization. The ESI signals of DmPA-labeled acylglycines were compared to their unlabeled counterparts and can be represented as a bar chart in Fig. 2 (same amount injected). The signals for the labeled acylglycines were approximately 1–3 orders of magnitude

higher than the signal for the corresponding unlabeled acylglycines. Also, despite the differences in the chain lengths and aromaticity of the fatty acid portion of the glycine conjugates, compared to the unlabeled acylglycines, less variation of responses was observed for all the acylglycines in the ESI (note the similarity of their calibration slopes, see below). For some of the acylglycines, equimolar concentrations of standards produce similar responses in ESI. The signal enhancement can be due to several factors. First, the increased pK_a of the labeled acylglycines, due to the presence of the dimethylamine moiety, leads to preferential protonation in solution, which is advantageous for the generation of gas-phase ions in ESI. And second, the increase in hydrophobicity of the labeled acylglycines allows them to be less solvated and increases their chances of staying on the surface layer of the droplets formed in electrospray. In comparison with the native acylglycines, they are more likely to have a higher surface activity and are therefore more likely to be significantly enriched in the offspring droplets, which are eventually desolvated and form gas-phase ions. Ionization efficiency is also enhanced because the labeled analyte now elutes in a higher percentage organic solvent rather than in a solvent with higher water content as with the corresponding native analyte. As the retention time increases in reversed-phase chromatography, the eluting analytes are less hydrated and therefore more hydrophobic.

3.4. Surrogate matrix approach

One of the major challenges faced in quantitation experiments is the complication of quantifying naturally occurring (endogenous) analytes in biological matrices, due to background interferences from the analytes being present in the blank matrix. Method validation is primarily done in the authentic blank matrix, which is often analyte-free and preparation of calibrators and reference samples in the matrix are quite straightforward. In the presence of endogenous analytes, however, calibrators and reference samples have to be prepared in a different way, using alternate analytes and matrices, and validation methods have to be adjusted to fit the approach used. Another major challenge is the lack of regulatory guidelines on accurate method validation. Many researchers apply the same validation procedures as those used for exogenous compounds and these may not be accurate or applicable in many cases. An important part of method development and validation is to construct calibration curves in the matrix of interest for quantitation and assessment of matrix effects. This is usually problematic because the matrix contains unknown quantities of the analytes of interest. The use of standard addition method is a suitable and accurate approach for endogenous analyte quantitation, which eliminates any matrix effect issues. This method consists of the addition of a series of standard solutions in increasing concentration to each individual sample and sample concentration is determined by the *x*-intercept of the calibration curve. The problem with this approach, however, is that it can be impractical when there are many samples or limited sample volumes and the method is only accurate when the sample concentration is greater than matrix baseline concentration. In this study, we choose to address the issue of endogenous interference by using a strategy called the "surrogate matrix" approach, which uses a calibration curve constructed in an alternate matrix. Pure aqueous or organic solutions [15,16], synthetic matrices [17,18], or matrices from alternate species [19,20] have been previously used, but these may not account for recovery in sample preparation and matrix effects in LC–MS analyses.

3.5. Selection of surrogate matrix

We have established that one of the major challenges in performing method validation of endogenous compound analysis is obtaining a true blank matrix without the presence of analytes in order to construct a calibration curve. Initially, a solvent mix, comprising of water/acetonitrile (50/50, v/v) in 0.1% formic acid was used to prepare the calibration curve. Water alone could not be used because of solubility issues of the derivatized acylglycines. However, when comparing the acylglycines concentrations using standard addition and using a calibration equation in solvent mix, the relative differences were higher than the recommended accuracy guideline of 15% [13]. Also, as will be discussed below, matrix effects were observed in the urine. Using diluted derivatized urine was not an option because several of the analyte levels were too high and the dilution would have to be greater than 10-fold. For the measurement of derivatized acylglycines in urine, pooled underivatized urine (equal volumes from all samples) was used as the surrogate matrix because the latter does not contain the derivatized acylglycines. Not only does it not contain the target analytes, it is quite similar to the authentic matrix; more so than the urine obtained from other mammalian species. The use of urine of other mammalian species is also complicated by the fact that most mammalian species would have endogenous levels of acylglycines.

3.6. Method validation

3.6.1. Selectivity and carry-over

In this study of endogenous analytes, selectivity was more difficult to evaluate because an analyte-free control matrix was difficult to obtain. Selectivity assessment included the analysis of an instrument blank, a solvent blank, a derivatized blank and blank surrogate matrix to determine interferences with the analyte and internal standard signals and analysis of the authentic matrix to determine interferences with the internal standard signal. Carryover was also assessed by analyzing instrument blanks after analysis of several replicates of high concentration standards and samples. Representative chromatograms of each type of blanks, surrogate and authentic matrices are shown in Fig. 3A–D. The chromatograms of the solvent blank, derivatized blank and surrogate blank in panels A, B and D, respectively, reveal that there are no interfering peaks at the retention times where each analyte and ISTD are expected to elute, indicating sufficient selectivity of the method. No significant carryover ($\leq$5% of LLOQ) originating from the method was observed, as illustrated in blank (Fig. 3C) after running the ULOQ calibration standard. In Fig. 3E, the MRM chromatogram with the transitions corresponding to the internal standards in authentic matrix is shown. Only two of the peaks at retention time 3.44 min and 11.41 min are observed, corresponding to the isotopic contribution from acetylglycine and tiglylglycine (two of the most abundant acylglycines in urine). The isotopic contribution of the carbon 12 of the analytes can be accounted for and subtracted from the internal standard signal. Panels F and G in Fig. 3 display a standard mix (1 nM) and an internal standard mix (100 nM) in surrogate matrix, respectively. No interferences were observed.

3.6.2. Linearity of ISTD and analytes

The concept behind the use of an internal standard methodology is that it is chosen to mimic the analyte of interest so that any changes in extraction or instrumental techniques would be reflected in a change in the ISTD as well. In most methods, a calibration curve is set up by analyzing a set of standards containing various concentrations of the analyte and one concentration of ISTD. The assumption here is that the ISTD has the same detector response as the analyte and that a calibration curve of the ISTD will have the same linearity as that of the analyte. Choosing the concentration of the ISTD then becomes important because there could be a chance that the linearity of the analyte is different from the ISTD and the chosen concentration of the ISTD might lie in a nonlinear region of the curve. As suggested by others [21], the more

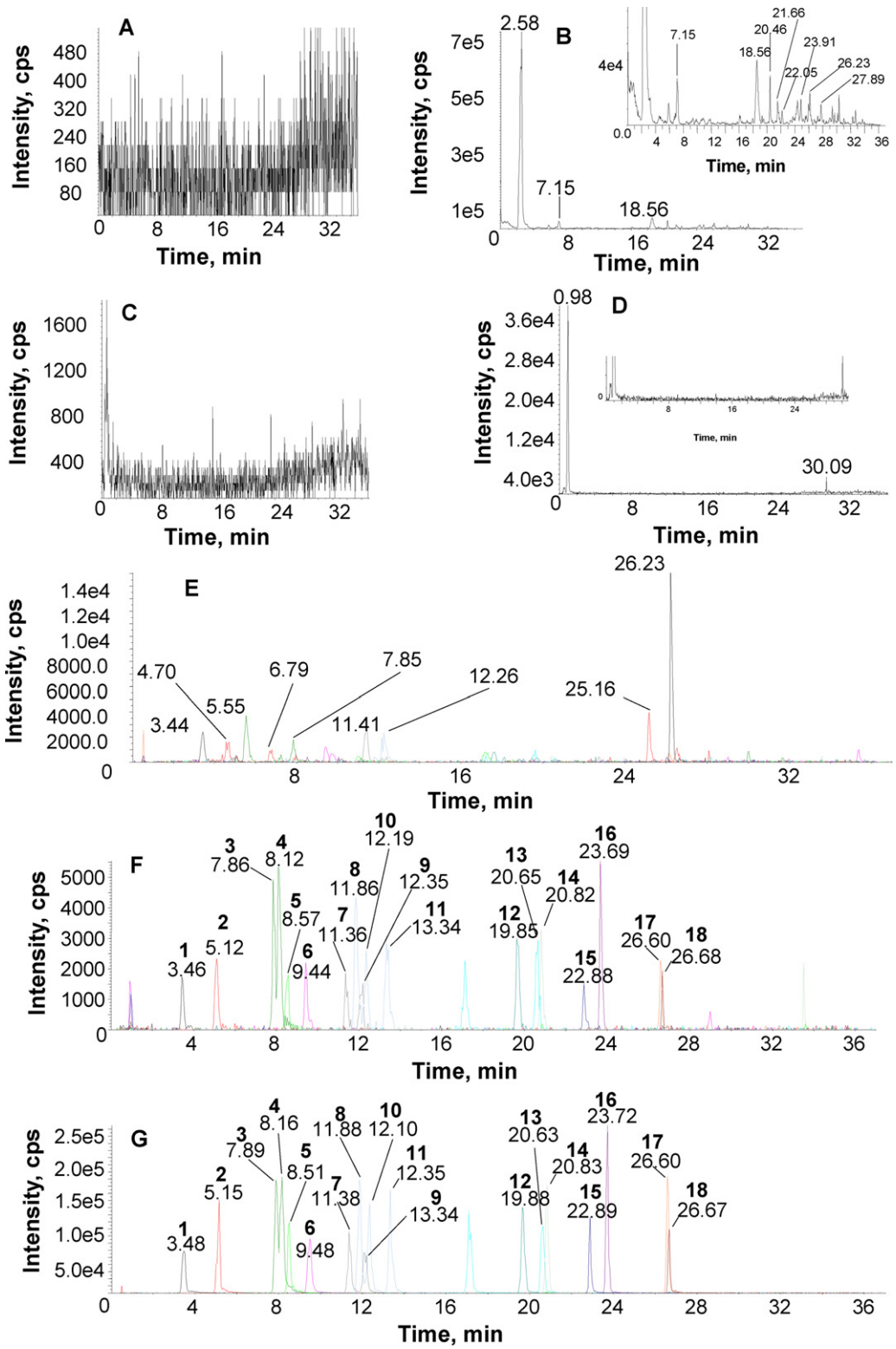

Fig. 3. Representative UPLC–MS chromatograms of (A) solvent blank, (B) derivatization blank; inset shows expanded region, (C) blank after running ULOQ calibration standard ($n = 5$), (D) surrogate blank; inset shows expanded region from 0 to 30 min, (E) internal standards (100 nM) in authentic matrix, (F) analytes (1 nM) in surrogate matrix and (G) internal standards in surrogate matrix. Compounds: (1) acetylglycine; (2) propionylglycine; (3) isobutyrylglycine; (4) butyrylglycine; (5) 4-hydroxyphenylacetylglycine; (6) 2-furoylglycine; (7) tiglylglycine; (8) 2-methylbutyrylglycine; (9) 3-methylcrotonylglycine; (10) isovalerylglycine; (11) valerylglycine; (12) hexanoylglycine; (13) phenylacetylglycine; (14) phenylpropionylglycine; (15) glutarylglycine; (16) heptanoylglycine; (17) octanoylglycine; (18) suberylglycine.

accurate way to calculate the concentration of the analyte when using a single ISTD calibration point is to ensure linearity around the chosen ISTD concentration. The linearity studies comparing ^{12}C-DmPA and $^{13}C_2$-DmPA-labeled acylglycines can be seen in Supplemental Table S2. For each of the eighteen analytes, a calibration curve was constructed and the slopes recorded as a comparison of detector response. The slopes were then compared statistically and calculated t-values compared to the critical values of t at the

Table 2
Comparison of slopes in surrogate and authentic matrices and calibration parameters of calibration curve in surrogate urine.

Acylglycines	Urine dilution	Slopes (surrogate)	Slopes (authentic)	*t*-Value	Linearity (R^2)	LOD (nM)	LLOQ (nM)	Linear dynamic range	Overall %CV
AG	10×	8.61	9.06	0.48	0.9993	1	3	3–500	11.99
PG	1×	9.15	8.77	1.25	0.9996	1	3	3–1000	8.18
IBG	10×	10.42	10.90	1.93	0.9996	1	2	2–500	14.91
BG	1×	10.00	10.31	1.97	0.9992	1	4	4–500	12.83
HPAG	1×	8.69	8.48	1.43	0.9985	1	5	5–500	10.76
FG	10×	8.90	8.90	0.01	0.9997	1	2	2–500	9.96
TG	10×	8.55	8.81	0.52	0.9986	1	5	5–500	11.7
2MBG	1×	9.24	9.14	0.75	0.9992	1	3	3–500	8.46
3MCG	10×	9.38	9.69	1.95	0.9987	1	4	4–500	9.13
IVG	10×	10.09	9.39	1.94	0.9994	1	3	3–500	11.93
VG	1×	9.21	9.14	0.50	0.9983	2	5	5–500	7.11
HG	10×	9.25	8.86	1.91	0.9997	1	2	2–500	10.83
PAG	10×	9.33	9.62	1.93	0.9995	1	3	3–500	10.81
PPG	1×	9.95	9.75	1.37	0.9996	1	2	2–500	10.07
GG	1×	8.80	8.69	1.61	0.9995	1	2	2–500	12.45
HpG	1×	9.19	9.02	0.95	0.9987	1	4	4–500	10.52
OG	1×	9.48	9.42	0.20	0.9994	1	3	3–500	8.08
SG	1×	8.33	8.61	2.14	0.9987	1	4	4–500	14.88

95% confidence level. As is observed in Table S2, the *t*-values are all less than the critical values indicating that there are no significant differences between the slopes. A concentration of 100 nM was chosen for the ISTD and linearity was ensured for higher as well as lower concentration around this point. Because of ion suppression and enhancement that can be observed in electrospray ionization, the concentration of the ISTD can be lower or higher than expected and this linearity test ensures the accuracy of the method.

3.6.3. Calibration, authentication of surrogate, LOD, LOQ

The linearity of the method was investigated in solvent, surrogate matrix and authentic matrix, covering the range of 1.0–1000 nM. Slopes, intercepts, coefficients of determination (R^2) and standard errors were determined by the least-squares linear regression model. Due to the heteroscedasticity of the data, the best linear fit and residuals of the model was achieved using a weighting factor of $1/y$. The calibration curve was linear over the range 1.0–1000 nM for the solvent and 1.0–500 nM for the surrogate and authentic matrices, except for propionylglycine (linear range 1.0–1000 nM). The endogenous levels of eight acylglycines, acetylglycine, isobutyrylglycine, tiglylglycine, 3-methylcrotonylglycine, isovalerylglycine, 2-furoylglycine, hexanoylglycine, and phenylacetylglycine, were on the upper end of the linear dynamic range and standard addition calibration curves of these eight analytes were non-linear. Surrogate and authentic matrices were therefore diluted 1:9 with solvent and fortified with increasing concentrations of these eight acylglycines and calibration curves were then constructed.

In order to determine if the response of the analytes were similar in surrogate and authentic matrices, a comparison of slopes were performed using the modified *t*-test. Similarities in the detector responses in both matrices would authenticate the use of the surrogate matrix in the validation process. Table 2 summarizes the calibration parameters for both surrogate and authentic matrices. Comparison of the sensitivity for acylglycines in surrogate and authentic matrices using the modified *t*-test indicates that the two slopes were not significantly different at the 95% confidence level (calculated *t*-values were all less than the critical values of *t* at that confidence level). This indicates that the surrogate matrix can be used as a substitute for the authentic matrix and application of the method will yield accurate quantitation results. The R^2 values of the weighted calibration curves were all higher than 0.998 for each acylglycine. Overall % CV values, calculates as an average of the % CV at each level, are all less than 15%.

LOD and LLOQ values were calculated using the standard deviation of the *y*-intercept and the slope, derived from the linear regression model. The LOD ranged from 1.0 to 2.0 nM and LLOQ ranged from 2.0 to 5.0 nM. This information can also be found in Table 2. Confirmation of the LLOQ was performed by analyzing five replicates of the LLOQ concentration in surrogate matrix. The % RE and % CV derived from the replicate analysis at the LLOQ concentration of each acylglycine ranges from 17.47 to 7.34 and 3.67 to 15.21, respectively, indicating good accuracy and precision. This information is summed up in Supplemental Table S3. It is important to note that the signal-to-noise ratios of the LLOQ at the respective concentrations were significantly higher than 10 for all analytes, except for glutarylglycine. This indicates that these values of LLOQ would be considerably lower if LLOQ was measured using the signal-to-noise method (see the last column in Supplemental Table S3) and thus our method is significantly more sensitive than the current methods [7,9].

3.6.4. Standard addition results

Due to the presence of endogenous acylglycines in derivatized urine, the use of underivatized urine as a blank matrix was validated using a standard addition experiment. Comparison of the acylglycine concentrations determined using a standard addition method and calibration in surrogate matrix can be observed in Supplemental Table S4. Percent relative differences calculated were all less than 15%, except for HpG, OG and HPAG, whose endogenous levels are below the LLOQ. These values of % RE support the use of underivatized urine as the surrogate urine.

3.6.5. Dilution linearity

The premise of the parallelism and dilution linearity is that if the diluting matrix is suitable and there are no matrix interferences from either matrix then the concentration of the diluted sample and the back calculated concentrations should be the same. The dilution linearity was assessed for all analytes by plotting observed results vs. expected results and performing linear regression. Results are shown in Supplemental Table S5. The slope of the resulting line and the correlation coefficient (R^2) is given for each analyte. Parallelism is proven when the slopes are nearly 1. Acylglycine concentrations at different dilutions showed good agreement indicating that the method had good dilution linearity. These results also indicate that there are no matrix interferences when surrogate urine is the diluent and that the surrogate is a suitable matrix. Surrogate matrix would be used to dilute samples with concentration higher than the ULOQ.

3.6.6. Evaluation of matrix effect

The most common methods of evaluating matrix effects are post-column infusion and post-extraction addition. These methods are only appropriate when there are blank matrices available. Another option available when there are endogenous levels of analytes is a comparison of slopes in matrix or matrix-match solutions with the slopes obtained in matrix-free standard solution [22,23]. One indicator that the sample matrix affects the analysis is a deviation in the slopes. The slopes of the calibration curves in solvent were compared to the slopes in both surrogate and authentic urine using the modified *t*-test defined above and the results are reported in Supplemental Table S6, as well as calculations of matrix effects according to the equation defined in the experimental section. Negative values signify suppression, whereas positive values indicate enhancement. All analytes, with the exception of isobutyrylglycine, experienced suppression in the urine samples, though the suppression was rather modest (less than 20%). The modified *t*-test demonstrated that for most of the analytes, the slopes in solvent were significantly different from those in both of the urine samples. On the other hand, Table 2 shows that the slopes in surrogate and authentic matrices are not significantly different at the 95% confidence level. To minimize matrix effects and for a more accurate validation and quantification, all calibrations and validations were done using either surrogate or authentic urine samples.

3.6.7. Method reproducibility

Method reproducibility was defined through the analyses of five experimental replicates under the same reaction conditions. The obtained results are presented in Supplemental Table S7. Results are expressed as % CV and the values are all under 10% for all acylglycines, except for heptanoylglycine and octanoylglycine, whose concentrations were at or below the LLOQ. The reported % CV values are 12.54 and 14.76% respectively, which are still under 15% signifying that the current derivatization method demonstrated adequate precision.

3.6.8. Precision and accuracy

Intra- and inter-day precision of the proposed method were also determined and presented in Supplemental Table S7. Results are expressed as % CV and intra-day values varied between 2.50% and 11.40% and inter-day values varied between 3.11% and 11.70%. Accuracy was investigated for three concentrations, 10.0, 150.0, 400.0 nM and the results are summarized in Supplemental Table S8. The results are expressed as % RE and % CV and both sets of values are lower than 15% for all analytes at all three concentrations. These results indicate that the proposed method demonstrated satisfactory accuracy and precision.

3.6.9. Stability

Samples were considered to be stable if the concentration of analytes were within ±15% (i.e., 85–115%) of initial concentration in the fresh samples. Results of the stability study conducted under various conditions are expressed as percent recovery and are summarized in Supplemental Table S9. All eighteen acylglycines were within the acceptable limits and were shown to be stable in human urine samples after 3 freeze/thaw cycles and up to 8 weeks storage at −20 °C. It was also demonstrated that they were stable for up to 5 h at room temperature and 24 h at 4 °C in the autosampler.

3.7. Measurement of endogenous levels

The validated UPLC–MS method was then used in the analysis of urine samples from 20 healthy individuals, collected over 3 consecutive days (total of 60 samples). Each sample was derivatized and analyzed in triplicates. Concentrations were back-calculated using the peak area ratios of analyte/ISTD from a calibration curve constructed in surrogate urine.

Table 3
Concentration ranges of acylglycines in human urine.

Acylglycines	Concentration (μmol mol^{-1} creatinine)		
	Day 1	Day 2	Day 3
AG	596.5–9788.9	493.9–1848.4	418.0–3466.0
PG	86.4–378.3	59.1–402.4	60.9–630.0
IBG	215.7–1621.7	174.2–1249.0	164.3–838.6
BG	8.1–134.6	6.5–170.4	12.1–237.0
HPAG	<2.5–7.9	<2.5–9.7	<2.5–4.8
FG	154.4–2183.1	81.0–3278.5	167.9–5191.6
TG	568.8–5034.2	559.8–3519.3	470.3–3190.1
2MBG	54.5–261.9	34.8–364.3	64.0–225.2
3MCG	55.9–645.5	40.0–623.6	65.1–444.2
IVG	592.5–7151.3	542.4–5656.1	477.1–4772.2
VG	8.6–215.2	8.0–146.9	11.7–90.1
HG	21.3–199.0	26.8–375.3	33.8–198.3
PAG	36.6–136.1	39.4–169.5	37.0–131.4
PPG	<1.0–65.6	<1.0–65.7	2.0–57.3
GG	3.5–56.5	2.5–51.7	1.2–58.6
HpG	<2.0–15.5	<2.0–8.5	<2.0–3.7
OG	<1.5–34.7	<1.5–41.4	<1.5–48.9
SG	7.8–68.1	3.5–51.5	<2.0–39.0

3.7.1. Absolute quantitation

The ranges of concentrations of each acylglycine over three days are shown in Table 3. The concentrations are represented in μmol mol^{-1} creatinine. The values obtained in this study are comparable with published data for those that have been measured (e.g., PG, BG and PPG) [7,9]. A more expanded table consisting of the daily concentrations of each of the 20 individual can be found in Supplemental Table S10. The concentration of each day is shown in the columns and is represented as the mean ± standard deviation of the three experimental replicates. The concentrations are represented in μmol mol^{-1} creatinine. For most of the individuals, day-to-day variations in acylglycine concentrations were relatively small. The identities of the eighteen analytes were confirmed by two factors: the co-elution of the internal standard and the interpretation of the product ion spectra.

3.7.2. Acylglycine pattern

The acylglycine profile of the individual was investigated to determine if there was any dependence of the acylglycine excretion pattern on parameters such as gender and BMI. The influence of gender on the acylglycine pattern is seen in Fig. 4. The box plots were generated using the full range of values. Fifteen acylglycines were plotted (only acylglycines with values greater than LLOQ were plotted) and each value plotted is an average of three days. There are differences between males and females but there does not seem to be a consistent pattern. There is no discernible pattern between short- and medium-chain acylglycines. Thus, it appears that gender does not play a role in the excretion pattern of acylglycines in urine.

Similar results were observed for the dependence of the profile on BMI. BMI was calculated by dividing the individual body mass in kilograms by the square of his/her height in meters and the units are expressed in kg m^{-2}. The BMIs were divided into four categories, according to the Canadian Guidelines for body weight classification: (a) underweight-BMI less than 18.5; (b) normal weight-BMI between 18.5 and 24.9; (c) overweight-BMI between 25 and 29.9 and (d) obese-BMI of 30 and more. The number of individuals that fit into each category is $n = 1$ (underweight), $n = 15$ (normal weight), $n = 2$ (overweight) and $n = 2$ (obese). The mean values of BMI between males and females were very similar, 23.5 in males and 22.2 in females. The influence of BMI can be seen in the

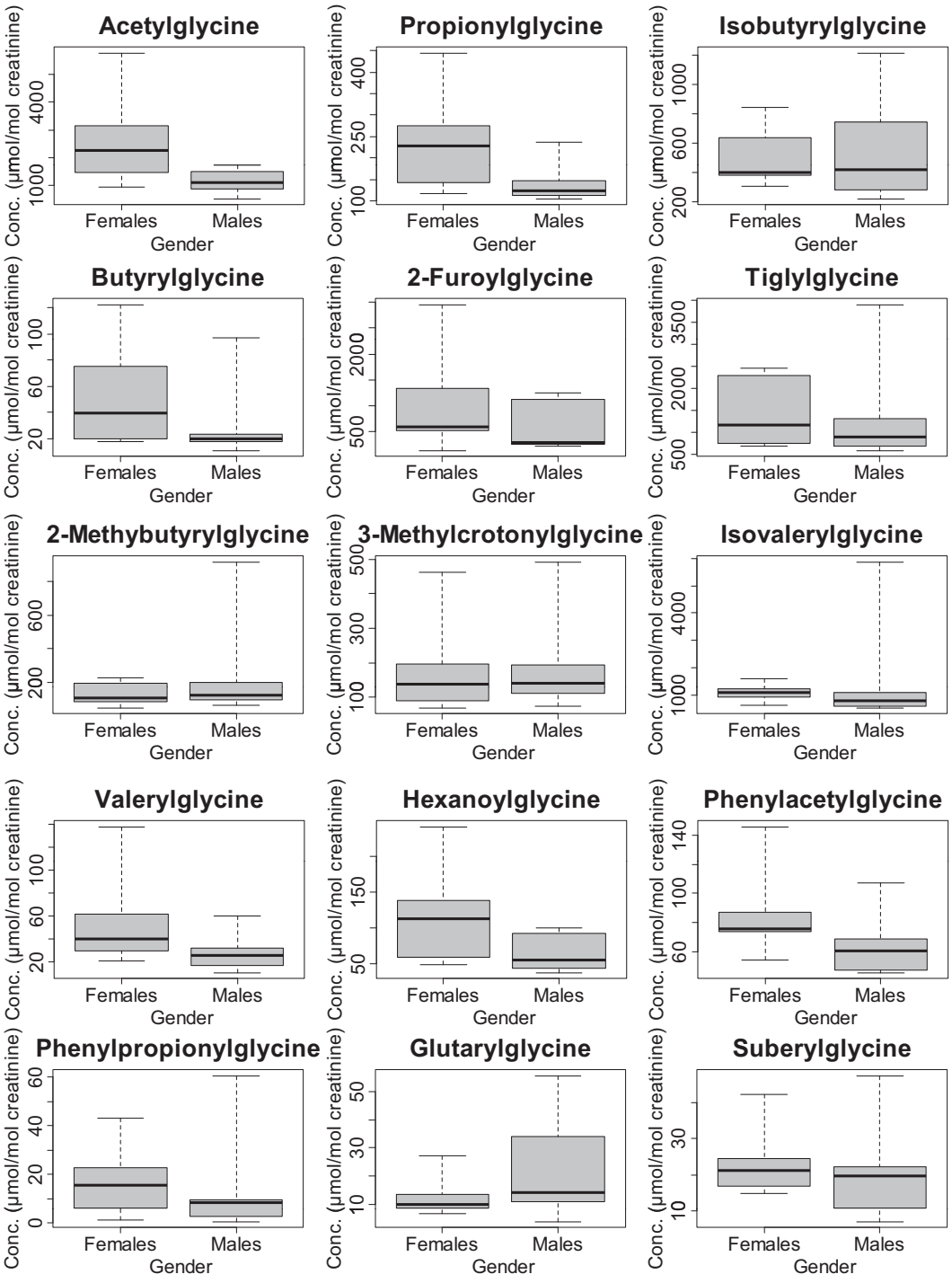

Fig. 4. Influence of gender on the acylglycine excretion profile ($n = 10$ for both males and females).

bar charts in Fig. 5. Values plotted are an average of acylglycine values over the three days. Acylglycines are arranged based on similar concentration ranges. As can be observed, the available data does not indicate any influence of BMI on the excretion pattern. However, it should be noted that, due to the small number of samples investigated, the biological interpretation of the data remains limited. Nevertheless, the results shown in Figs. 4 and 5 illustrate that the proposed UPLC–MS method can be used to investigate the acylglycine profile changes. The application of this method for comparison of acylglycine levels from different cohorts of healthy and diseased individuals will be reported in future.

3.7.3. Relative quantitation

Finally, we note that the proposed method can also be used for relative quantification of acylglycines if the standards are not available. In the relative quantitation of the derivatized acylglycines, for which there were no standards, putative identification was done by interpretation of the product ion spectra generated. In the spectra of the unknown acylglycine the following factors were investigated: (a) fragments corresponding to the tag, (b) fragments corresponding to the dissociation of the acylglycine, i.e. neutral losses of 236, 207, and 179, (c) fragment ion m/z 237 and (d) logical retention time. The fragmentation of the acylglycine portion of the molecule resulted in low abundant fragments that are

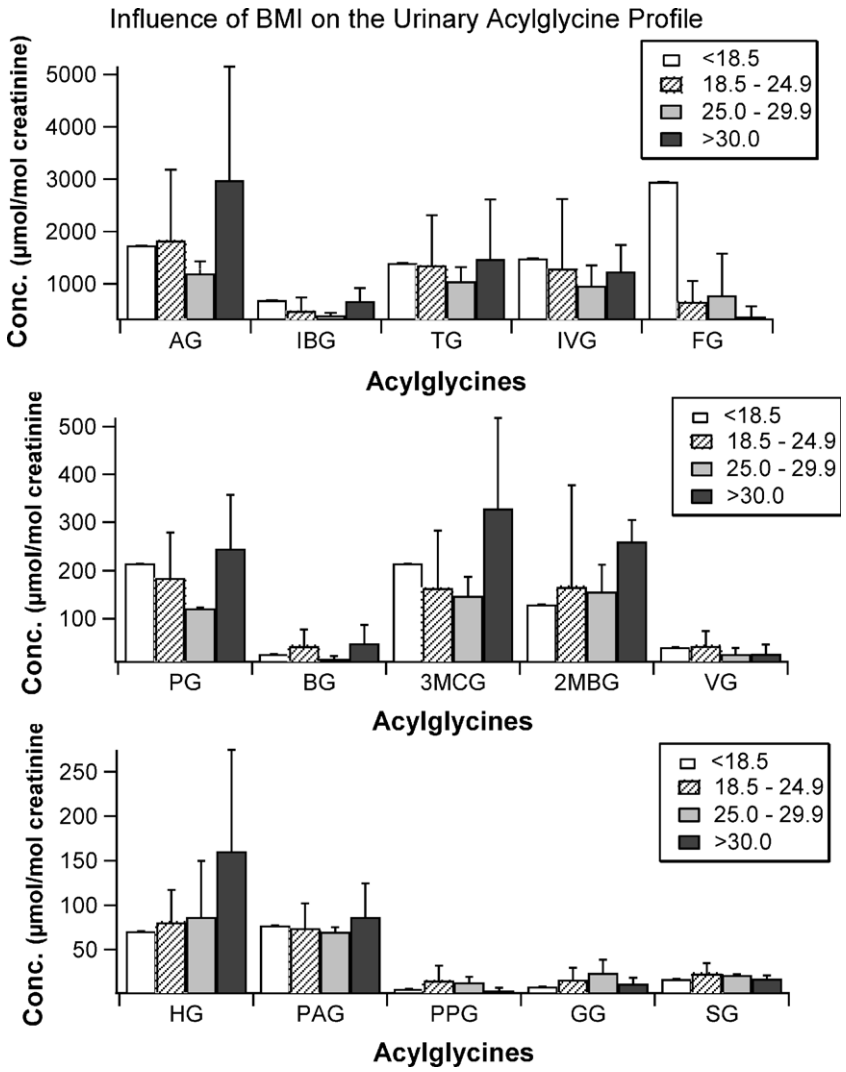

Fig. 5. Influence of BMI on the acylglycine excretion profile.

Table 4
Concentration ranges for putatively identified acylglycines in urine.

Acylglycines	RT (min)	Concentration (μmol mol^{-1} creatinine)		
		Day 1	Day 2	Day 3
C_5:1-G	12.47	12.9–646.9	9.4–652.3	23.2–524.9
C_6:1-G	16.50	4.3–93.7	4.1–48.0	5.8–50.9
C_6-G	19.07	17.9–317.4	13.3–133.8	21.9–118.4
C_8:1-G	23.89	104.4–792.2	77.7–650.3	113.4–531.9
C_8:1-G (2)	24.22	3.8–121.0	3.9–82.7	7.2–66.9
C_9:1	25.94	<1.5–34.8	<1.5–71.0	<1.5–61.6

often too low to observe. We believe that there are many more acylglycines that exist in the samples, but with low abundance fragment ions it was difficult and often impossible to putatively identify these compounds. Due to that fact, only six compounds could be putatively identified. These are C_5:1-G (pentenoylglycine or isomer), retention time, 12.47 min; C_6:1-G (hexanoylglycine or isomer), retention time, 16.50 min; C_6-G (methyl valerylglycine or isomer), retention time,19.07 min; C_8:1-G (cis-3,4-methylene heptanoylglycine or isomer), retention time, 23.89 min; C_8:1-G (octenoylglycine or isomer), retention time, 24.22 min; and C_9:1-G (cis-3,4-methylene octanoylglycine or isomer), retention time, 25.94 min. The ranges of concentrations for these acylglycines over three days are shown in Table 4. The concentrations are represented in μmol mol^{-1} creatinine. A more expanded table consisting of the daily concentrations of each of the 20 individual can be found in Supplemental Table S11. The concentration of each day is shown in the columns and is represented as the mean ± standard deviation of the three experimental replicates. The concentrations are represented in μmol mol^{-1} creatinine. EPI product ion spectra used in the putative identification of each acylglycines are shown in Supplemental Figure S1.

4. Conclusions

This paper describes a sensitive UPLC–MS method for the quantification of acylglycines in human urine. The method was validated using underivatized urine as the surrogate matrix and proved to be reliable in terms of selectivity, linearity, accuracy and precision. The

complications associated with performing validation for endogenous compounds have been satisfactorily dealt with and method performance was evaluated based on a fit-for-purpose approach. The surrogate matrix calibration method was successfully applied and demonstrated an advantage over the use of solvent (water) as a suitable surrogate matrix. Parallel experiments were used during validation to evaluate any bias originating from the differences between authentic and surrogate matrices. The typical acylglycine excretion profile in healthy individuals shows no dependence on BMI or gender. Except for a few individuals, day-to-day variations are relatively minor. It is important to note, however, that no biological significance can be drawn from such a limited number of samples.

We believe that the high performance isotope labeling UPLC–MS method offers many merits over previously reported methods, namely a more accurate method using a surrogate matrix for validation, potentially lower limits of detection, overall signal enhancement and the ability to create internal standards for each standard obtained (i.e., no need to synthesis isotope labeled analogs). This method can be adopted for the accurate quantification of many endogenous biomarkers that can be readily derivatized, with little or no modifications. Although this paper mainly focuses on the methodology of quantifying acylglycines, this method can easily be applied to clinical samples where the concentrations of the analytes are expected to increase for IEMs and similar disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.05.006.

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