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Determination of metformin in human plasma using hydrophilic interaction liquid chromatography–tandem mass spectrometry

Ang Liu*, Stuart P. Coleman

Matrix BioAnalytical Laboratories, 25 Science Park at Yale, New Haven, CT 06511, United States

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ABSTRACT

A sensitive, simple and rapid assay based on hydrophilic interaction liquid chromatography (HILIC) with tandem mass spectrometry was developed and validated for the quantitative analysis of metformin in human plasma using protein precipitation. Plasma samples were prepared using a protein precipitation solution containing acetonitrile, 0.5% formic acid and the internal standard, metformin-D_6. The analytes were separated on a GL Sciences Inertsil HILIC column using an isocratic mobile phase consisting of water/acetonitrile (30:70, v/v) and 0.1% formic acid. Metformin and internal standard were recorded using multiple reaction monitoring in positive ion electrospray mode with transitions of m/z 130–71 and m/z 136–77, respectively. No endogenous components in plasma were found to interfere with metformin measurements. The lower limit of quantification (LLOQ) was 0.5 ng/mL (0.1 pg on-column). The linear range was 0.5–500 ng/mL with an average correlation coefficient of 0.999 using weighted ($1/x^2$) linear least-squares regression. Dilutional linearity was evaluated up to 5000-fold dilution and the results indicate no influence on the accuracy of analysis. The absolute extraction recovery was 81% for metformin. Intra-day and inter-day precision (CV, %) ranged from 0.73% to 7.18%, and accuracy within ±10.98% from nominal. The analyte was found to be stable for at least 38 days at −20 and −80 °C, 24 h at room temperature, and stable for four freeze–thaw cycles. The processed extracts were stable for 88 h at 4 °C.

1. Introduction

Type-2 diabetes, formerly called non-insulin-dependent diabetes mellitus (NIDDM), is characterized by insulin resistance and relative insulin deficiency, leading to high blood glucose level and a series of complications such as renal dysfunction, retinopathy, neuropathy and cardiovascular disease [1–3]. The American Diabetes Association reported in 2009 that 23.6 million people in the United States, or 7.8% of the population, have diabetes, 90% of whom are type-2 [4]. Treatment of type-2 diabetes includes oral administration of hypoglycemic agents that help in reducing blood glucose levels.

Metformin (Fig. 1), an insulin-sensitizing agent, has become one of the most commonly prescribed medications for type-2 diabetes [5]. Oral administration of metformin can improve peripheral insulin sensitivity, inhibit hepatic gluconeogenesis and reduce hepatic glucose production in type-2 diabetic patients [6,7]. Metformin has been widely used either as a monotherapy agent or as a part of combination therapy. Determination of plasma concentration of metformin is important for pharmacokinetics studies, for therapeutic drug monitoring and for optimization of dosing and dosing regimen in antidiabetic therapy.

Metformin is difficult to extract and measure in biological fluids due to its high polarity ($\log P$ octanol/water = −2.64). Various analytical strategies have been used for metformin measurement including normal phase chromatography (silica and cyano) [8–10], cation exchange chromatography [11,12], ion pair chromatography [13,14] and reversed phase chromatography [15–18] with UV or mass spectrometric detection. However, most of these methods exhibited low sensitivity with the lower limit of quantification (LLOQ) ranging from 7.8 to 30 ng/mL [8–12,14,16–18], required large sample volumes [8,11–14,16–18], used complex sample preparation procedures [9,11,13], and required long chromatographic run time [8,9,12,13,17]. Hydrophilic interaction liquid chromatography (HILIC), first introduced in 1990 [19], has demonstrated to be a powerful technique for the analysis of polar compounds [20–22]. A recently published study described a HILIC–UV method for the determination of metformin and its two prodrugs in human and rat blood [23]. However, the reported sensitivity was low, with the LLOQ of 1 μg/mL.

In the present study, a sensitive, simple and rapid HILIC method with tandem mass spectrometric detection (HILIC–MS/MS) was developed and validated for the quantitative analysis of metformin in human plasma using protein precipitation. This novel

* Corresponding author. Tel.: +1 203 298 4452; fax: +1 203 789 6123.
E-mail addresses: ang.liu@matrixbioanalytical.com, akingno@gmail.com (A. Liu).

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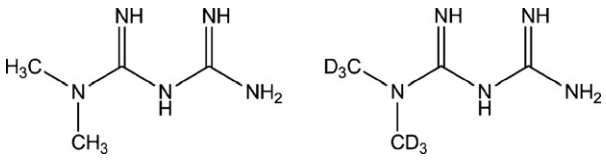

Fig. 1. Structures of metformin and the internal standard, metformin-D_6.

HILIC–MS/MS method exhibited excellent performance in terms of sensitivity, robustness, simplicity of sample preparation and relatively short analysis time. This method has a higher sensitivity (LLOQ, 0.5 ng/mL) than previously reported methods for metformin measurement.

2. Experimental

2.1. Chemicals and reagents

Metformin (97% purity) was purchased from Sigma–Aldrich (St. Louis, MO). Metformin-D_6 (internal standard, 98% purity) was purchased from Medical Isotopes, Inc. (Pelham, NH). K_2EDTA human plasma was purchased from Bioreclamation, Inc. (Liverpool, NY). HPLC grade acetonitrile and water were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). A.C.S. grade formic acid was purchased from Sigma–Aldrich (St. Louis, MO) and A.C.S. grade 2-propanol was purchased from VWR International (West Chester, PA).

2.2. LC–MS–MS conditions

LC–MS/MS was carried out using a Shimadzu (Columbia, MD) Prominence HPLC system interfaced to an Applied Biosystems (Concord, Ontario, Canada) Sciex API 4000 triple quadrupole mass spectrometer equipped with a TurboIonspray™ source.

The chromatographic separation was achieved using isocratic conditions on a GL Sciences (Torrance, CA) Inertsil HILIC column, (2.1 mm × 150 mm, 5 μm) with a pre-column filter. The mobile phase consisted of water/acetonitrile (30:70, v/v) with 0.1% formic acid at a flow rate of 650 μL/min. Each chromatographic run was completed within 4 min. Solution of water/2-propanol/acetonitrile (15:35:50, v/v/v) with 2% formic acid was used as needle and valve wash solution to eliminate the potential carryover in autosampler. The column oven was set to 42 °C and the mobile phase was maintained at the same temperature using a Selerity Technologies (Salt Lake City, UT) Caloratherm™ mobile phase temperature conditioner.

Positive ion electrospray mass spectrometry was used for the detection of metformin and its internal standard, metformin-D_6. The source temperature was set to 600 °C. The ion spray voltage and entrance potential (EP) were maintained at 5000 and 10 V, respectively. UHP nitrogen (99.999%) at 30, 50 and 6 psi were used as the nebulizer gas, curtain gas and collision gas, respectively. Analytes were detected using multiple reaction monitoring (MRM) and the optimized collision energy was 27 V for metformin and 29 V for metformin-D_6. The transitions of m/z 130–71 and m/z 136–77 were monitored for metformin and metformin-D_6, respectively, at a dwell time of 150 ms per transition.

2.3. Sample preparation

Two stock solutions of metformin were prepared by dissolving the accurately weighed reference standards in water/acetonitrile (20:80, v/v) to reach a final concentration of 3 mg/mL. The equivalence of separate weighings and stock solutions were examined by comparing area ratio per ng after repeat injections of neat solutions and the separate weighings were within ±2% of each other (data not shown). A stock solution of metformin-D_6 was prepared at 0.098 mg/mL using pure acetonitrile. All stock solutions were stored at 4 °C.

Standards and quality control (QC) samples were prepared using an initial spike of stock solution into K_2EDTA human plasma blank, which was then diluted to obtain different concentration levels. Nine standards at the concentrations of 0.5–500 ng/mL were used for the calibration curve. Double blanks (blank processed without internal standard) and single blanks (blank processed with internal standard) were prepared using the same matrix. Quality control samples of 1.5 ng/mL (Low QC), 40 ng/mL (Mid QC) and 400 ng/mL (High QC) were prepared for the evaluation of accuracy, precision and storage stability. Standards and quality control samples were then pipetted into polypropylene tubes and stored at different temperatures of −80, −20, 4 °C, and ambient temperature until analysis. A set of double blanks, single blanks, LLOQ QC (0.5 ng/mL) and the upper limit of quantification (ULOQ) QC (500 ng/mL) were prepared using six individual lots of human plasma (three from male subjects and three from female subjects) for the evaluation of selectivity and matrix effects. An ultrahigh dilution QC (10,000 ng/mL) was prepared and diluted to three concentrations of 2, 50 and 450 ng/mL to verify that samples which contain more analyte than the highest standard may be diluted and reprocessed without affecting the accuracy of the analysis.

Samples were thawed at room temperature followed by mixing to ensure homogeneity. A simple protein precipitation method was used to reduce sample preparation time. The protein precipitation solution was prepared by spiking acetonitrile containing 0.5% formic acid with the initial internal standard stock solution to a concentration of 5 ng/mL of metformin-D_6. An aliquot of 50 μL plasma was transferred into a well of 96-deep-well plate and then mixed with 200 μL of protein precipitation solution. For double blanks, acetonitrile with 0.5% formic acid was used to precipitate the proteins in plasma. The mixture was vortexed vigorously for 10 min, centrifuged for 10 min at 1522 × g, 4 °C, and an aliquot of 1 μL was injected during LC–MS/MS analysis.

2.4. Method validation

The validation experiments were carried out in accordance with Matrix BioAnalytical Laboratories Standard Operating Procedures (SOPs) and included ones to determine the characteristics of calibration curve, the lower limit of quantification, accuracy and precision, assay recovery, matrix effects, and stability. The design and results of method validation experiments are presented in Section 3.

2.5. Data processing and quantification

Data acquisition was performed using Analyst™ version 1.4.1 software. For each analytical batch, the peak area ratios of metformin to its internal standard (metformin-D_6) were plotted versus the nominal concentrations of calibration standards using a linear least-squares regression with a weighting factor of $1/x^2$. The regression equation for the calibration curve was used to back-calculate the measured concentrations for standards and QC samples, and the results were compared to the nominal concentrations to obtain the accuracy, expressed as a % bias from the nominal value.

3. Results and discussion

3.1. Method development

Metformin (*N*-1,1-dimethyl-biguanide) is a biguanide with high polarity (log *P* octanol/water = −2.64) which makes it difficult to

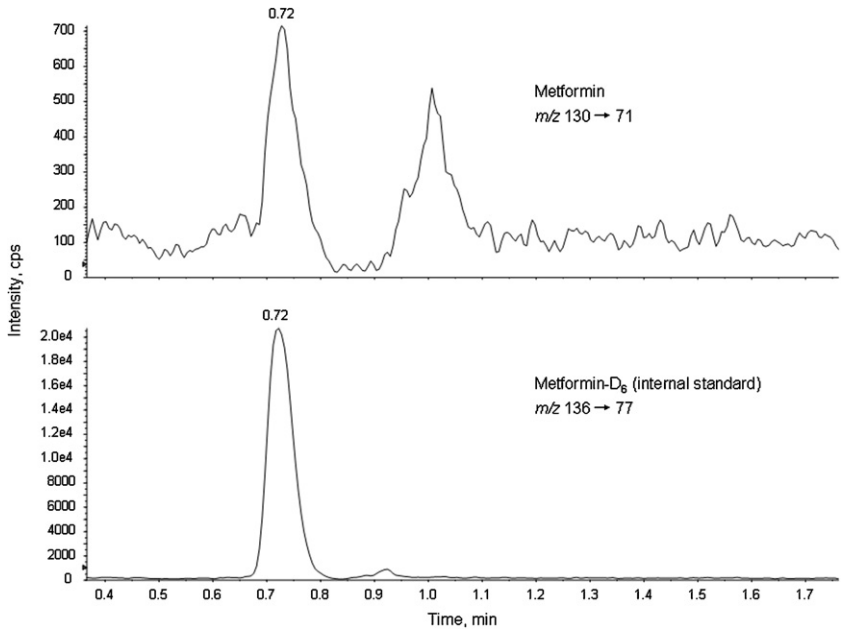

Fig. 2. Positive ion electrospray LC–MS/MS chromatograms of a LLOQ calibration standard containing metformin (0.5 ng/mL) and the internal standard.

analyze in biological samples. Various analytical techniques have been employed to solve this problem, including gas chromatography (GC) with derivatization [24], capillary electrophoresis (CE) [25] and high performance liquid chromatography (HPLC) [17]. HPLC (normal phase, reversed phase, cation exchange, ion pair and HILIC) with UV or mass spectrometric detection was the most commonly used method for metformin analysis [8–18,23]. However, these methods suffered from a number of disadvantages, such as low sensitivity, complex and time-consuming preparation procedures, the need for large sample volumes, and long chromatographic run times.

The high polarity of metformin makes it difficult to retain on analytical columns. Various polar columns, including GL Sciences Inertsil HILIC, Inertsil CN-3 Cyano, Phenomenex Synergi polar-RP and Luna Silica, were evaluated for the determination of metformin in human plasma. The HILIC column was found to give the best chromatography with adequate retention. Hydrophilic interaction liquid chromatography (HILIC), first introduced by Alpert in 1990, is a pseudo-normal phase chromatographic technique [19]. It is commonly believed that in HILIC, the mobile phase forms a layer of water molecules on the surface of the polar stationary phase particles creating a liquid–liquid partition system [20]. In the present study, the chromatographic separation was achieved using isocratic mobile phase of water/acetonitrile (30:70, v/v) with 0.1% formic acid.

Electrospray mass spectrometry in positive ion mode was chosen in this study for the detection of metformin and its internal standard, metformin-D_6. The purpose of the internal standard is to improve the precision of analytical method by controlling for variations in extraction, LC injection and ionization efficiency. A stable isotope-labeled analyte is an ideal internal standard since it is chemically similar to the analyte but does not occur naturally. The ion transitions of m/z 130–71 and m/z 136–77 were used for multiple reaction monitoring of metformin and metformin-D_6, respectively, since the protonated molecules were the most abundant precursor ions during MS and the product ions of m/z 71 and 77 formed by β-cleavage at the N–R bond with rearrangement of hydrogen atoms were the base peaks of the product ion mass spectra.

Metformin, being hydrophilic, makes its extraction from biological fluids difficult. To overcome this problem, various extraction procedures have been used, including liquid–liquid extraction [9], solid phase extraction [11], ion pair extraction [13], and protein precipitation with a wash step [8,26]. However, these methods are complex and time-consuming for applications in pharmacokinetics studies and general drug monitoring. In the present experiment, a simple one-step protein precipitation procedure was developed to reduce sample preparation time without sacrificing extraction efficiency. The protein precipitation solution consisted of acetonitrile, 0.5% formic acid and the internal standard at a concentration of 5 ng/mL.

To our knowledge, the HILIC–MS/MS method described has a higher sensitivity than previously published procedures [8–18,23,26]. The combination of HILIC chromatography and electrospray ionization MS detection offers an increase in sensitivity over reversed phase chromatography because of the higher concentration of organic mobile phase which is more volatile and provides greater ionization efficiency for metformin. The LLOQ for plasma metformin for the assay is 0.5 ng/mL (0.1 pg on-column) using a plasma volume of 50 μL. Representative LC–MS/MS chromatograms of an LLOQ calibration standard are shown in Fig. 2.

3.2. Method validation

This analytical method was fully validated according to Matrix BioAnalytical Laboratories SOPs which conform to the U.S. FDA's Good Laboratory Practice (GLP) regulations (21 CFR part 58). The validation parameters and criteria included the selectivity, the lower limit of quantification, calibration curve linearity, intra-day and inter-day accuracy and precision, assay recovery, and stability.

3.2.1. Selectivity and matrix effects

Selectivity is the ability of an analytical method to measure and differentiate the analyte in the presence of endogenous components in biological matrix. Potential interference from endogenous components was investigated by analyzing double blank samples prepared from six different sources of human plasma (three from male subjects and three from female subjects). The acceptance

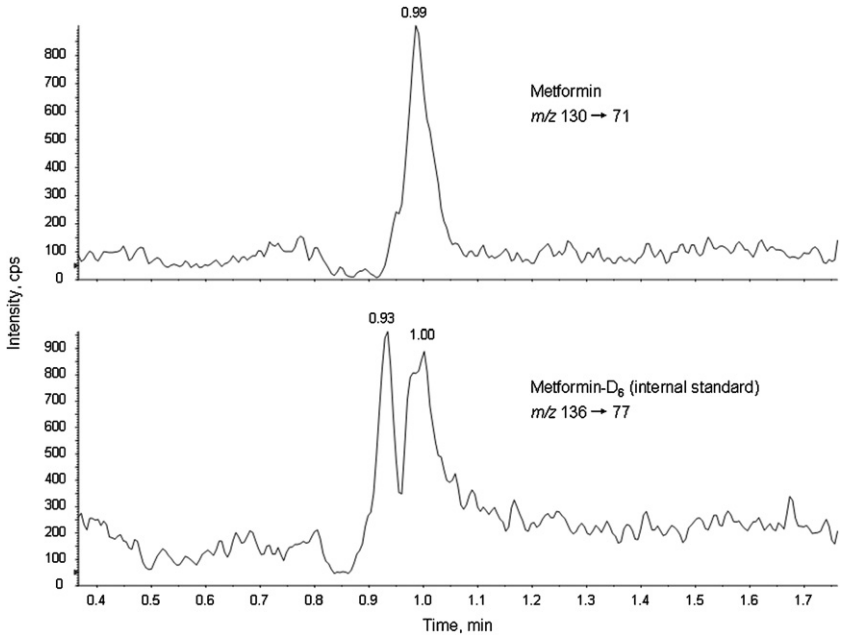

Fig. 3. LC–MS/MS analyses of double blank plasma showing the absence of endogenous interference at the retention time of 0.72 min.

Table 1
Matrix effects on LLOQ and ULOQ.

Lot number[a]	LLOQ (0.5 ng/mL)		ULOQ (500 ng/mL)	
	Measured value (ng/mL)	% Bias	Measured value (ng/mL)	% Bias
Lot-1	0.5050	1.00	484.37	−3.13
Lot-2	0.5087	1.74	497.94	−0.41
Lot-3	0.4930	−1.40	491.49	−1.70
Lot-4	0.5087	1.73	500.34	0.07
Lot-5	0.5229	4.57	513.19	2.64
Lot-6	0.5547	10.94	496.26	−0.75

[a] Matrix effects on LLOQ and ULOQ were evaluated using six individual lots of K_2EDTA human plasma (Lots 1–3 from male subjects and Lots 4–5 from female subjects).

Table 2
Intra-day accuracy and precision.

		Low QC (1.5 ng/mL)	Mid QC (40 ng/mL)	High QC (400 ng/mL)
Day 1 (n = 6)	Mean (ng/mL)	1.50	41.82	401.96
	% Bias	−0.08	4.55	0.49
	% CV	5.59	1.63	1.50
Day 2 (n = 6)	Mean (ng/mL)	1.50	41.07	404.47
	% Bias	0.07	2.68	1.11
	% CV	6.72	0.94	1.31
Day 3 (n = 6)	Mean (ng/mL)	1.52	41.94	400.87
	% Bias	1.60	4.83	0.23
	% CV	5.37	1.07	2.11
Day 4 (n = 6)	Mean (ng/mL)	1.46	42.16	423.88
	% Bias	−2.38	5.38	5.97
	% CV	5.35	1.06	2.52
Day 5 (n = 6)	Mean (ng/mL)	1.66	42.30	416.84
	% Bias	10.98	5.77	4.22
	% CV	4.19	2.61	1.82
Day 6 (n = 6)	Mean (ng/mL)	1.57	38.29	377.20
	% Bias	4.79	−4.28	−5.70
	% CV	4.72	1.63	1.13
Day 7 (n = 6)	Mean (ng/mL)	1.57	41.53	397.93
	% Bias	4.70	3.80	−0.51
	% CV	4.03	2.30	0.73

Table 3
Inter-day accuracy and precision.

	Nominal value (ng/mL)	Mean (ng/mL)	% Bias	% CV
LLOQ (n = 14)	0.5	0.50	0.64	7.18
Low QC (n = 42)	1.5	1.54	2.81	6.26
Mid QC (n = 42)	40	41.30	3.25	3.54
High QC (n = 42)	400	403.31	0.83	3.80
ULOQ (n = 14)	500	487.35	−2.53	3.19

Table 4
Dilutional linearity.

	Nominal value (ng/mL)	Dilution factor[a]	Mean (ng/mL)	% Bias	% CV
Dilution QC_Low (n = 3)	2	5000	2.11	5.30	3.53
Dilution QC_Mid (n = 3)	50	200	52.76	5.53	0.36
Dilution QC_High (n = 3)	450	22	442.29	−1.73	2.76

[a] Dilution QCs were prepared by diluting an ultrahigh dilution QC (10,000 ng/mL) to three concentrations near the Low QC, Mid QC and High QC of the assay.

criterion for metformin requires the interfering signal to be less than 20% of the signal at the LLOQ, and the internal standard, metformin-D_6, requires the interfering signal to be less than 5% of the signal at a working concentration of 5 ng/mL. No endogenous peaks that would interfere with metformin measurement were detected in the double blank samples (Fig. 3). Potential matrix effects on the quantitative analyses of the LLOQ (0.5 ng/mL) and the ULOQ (500 ng/mL) were evaluated using six different plasma sources. No significant ion suppression or ion enhancement was observed for both the LLOQ and the ULOQ during method validation (Table 1).

3.2.2. Calibration curve

Calibration curves were obtained from seven consecutively prepared batches and calculated using a linear least-squares regression of the analyte/internal standard peak area ratio to the nominal concentration, with a weighting factor of $1/x^2$. The results indicate that the seven calibration curves are virtually identical. The average slope and intercept of regression equations were 0.0421 and 0.0109, respectively. Excellent linearity (average correlation coefficient, 0.999) was found over a concentration range of 0.5–500 ng/mL for metformin. The lower limit of quantification (LLOQ) was determined based on acceptance criteria of five times the response compared to the blank response, as well as a precision of 20% and accuracy of 80–120%. The LLOQ of this assay was 0.5 ng/mL with an injection volume of 1 μL, which is lower than any previously reported values.

3.2.3. Accuracy and precision

Three sets of QC samples corresponding to the low (1.5 ng/mL), middle (40 ng/mL), and high (400 ng/mL) regions of the calibration curve were prepared and analyzed six times each with two calibration curves on seven consecutive days to assess accuracy and precision. Accuracy was determined by calculating the difference between measured values and nominal values (% bias) for the spiked samples and precision by calculating the coefficient of variation (CV, %) for the repeated measurements. The intra-day and inter-day accuracy and precision data for metformin are presented in Tables 2 and 3. Carryover that might influence the accuracy and precision of the assay was evaluated by injecting a double blank following the highest calibration standard (ULOQ, 500 ng/mL) and comparing the signal of this blank with the signal of the preceding LLOQ standard. No significant carryover or contamination was detected within the calibration range. Results indicate a bias of ±10.98% and the coefficient of variation between 0.73% and 7.18% for all concentration levels (LLOQ, Low QC, Mid QC, High QC, ULOQ) during seven validation runs.

3.2.4. Dilution

An ultrahigh dilution QC (10,000 ng/mL) was prepared and diluted to three concentrations near the Low QC, Mid QC and High QC of the assay. The set of dilution QCs (2 ng/mL, 50 ng/mL and 450 ng/mL) was analyzed as a part of method validation and the data are shown in Table 4. The results indicate that samples which

Table 5
Stability tests for metformin in human plasma and stock solution.

	Storage condition	Duration	Sample[a]	% Bias	% CV
Short-term stability	Room temperature	24 h	Low QC	−0.27	2.54
			High QC	3.10	1.16
			Stock	−7.96	5.89
Long-term stability	−20 °C	38 days	Low QC	−3.09	5.56
			High QC	−11.53	1.83
	−80 °C	38 days	Low QC	6.73	8.16
			High QC	−4.20	3.54
	4 °C	47 days	Stock	1.41	0.93
Post-preparative stability	Autosampler set at 4 °C	88 h	Low QC	−4.07	6.40
			High QC	1.22	1.26
Freeze–thaw stability	−20 °C	4 Cycles	Low QC	0.97	6.36
			High QC	−2.80	0.38
	−80 °C	4 Cycles	Low QC	6.03	5.49
			High QC	−2.32	1.85

[a] Multiple sets of Low QC (1.5 ng/mL) and High QC (400 ng/mL) samples were prepared using K_2EDTA human plasma for the evaluation of storage stability. Stock solution of metformin was prepared by dissolving the weighed reference standard in water/acetonitrile (20:80, v/v), and diluted to an appropriate concentration immediately before analysis.

contain more metformin than the ULOQ may be diluted and re-assayed.

3.2.5. Recovery

A simple and rapid protein precipitation method was developed and used in the present study to extract metformin from K_2EDTA human plasma. The extraction efficiency was determined by extracting spiked QC samples and comparing the response obtained from extracted QCs with non-extracted neat solutions at the corresponding level. The absolute extraction recovery was 81% for metformin, which supports sensitive and accurate measurement of metformin in human plasma.

3.2.6. Stability

Multiple sets of Low QC (1.5 ng/mL), High QC (400 ng/mL) and stock solution were prepared and tested under various conditions which were chosen based on sample collection, handling, storage, and assay processes. The acceptance criterion for metformin stability conforms to Matrix BioAnalytical Laboratories SOPs and states that the measured concentrations of stability QCs must be within ±15% of their nominal concentrations and the coefficient of variation of replicate measurements must be less than 15%. The stability results are summarized in Table 5.

Short-term temperature stability of the analyte in biological matrix (K_2EDTA human plasma) and in stock solution was evaluated at room temperature for up to 24 h, based on the expected maximum time that samples and stock solutions would be maintained at this temperature during assay processes. The samples were found to be stable during 24 h at room temperature, with a % bias less than ±3.10% and ±7.96% for QC samples and stock solution, respectively.

Long-term storage stability of the analyte in biological matrix was evaluated at −20 and −80 °C and long-term stock solution stability was evaluated at 4 °C. After a period of storage at reduced temperature, three sets of QC samples were thawed and extracted using the validated protein precipitation method and six replicates of stock solution were diluted using pure acetonitrile to yield an appropriate concentration and then extracted in the same manner. The processed samples were then analyzed on the basis of newly prepared calibration curve. Long-term stability of metformin in human plasma was established for 38 days at −20 and −80 °C and a stock solution of metformin was found to be stable over 47 days at 4 °C.

Post-preparative stability, or the stability of processed samples in the refrigerated autosampler during injection, was determined as follows. A set of processed standards and QCs was injected onto the LC–MS/MS system, and then the same QCs were re-injected after a measured period of storage in the autosampler. The concentrations of originally injected QCs and re-injected QCs were analyzed on the basis of the original calibration curve. A post-preparative stability period of 88 h was established in an autosampler set at 4 °C, with a % bias of less than ±4.07%.

Freeze–thaw stability was tested at −20 and −80 °C for four freeze–thaw cycles to simulate conditions that would occur during sample analysis. QC samples were frozen initially for at least 24 h. Each subsequent freeze/thaw cycle consisted of unassisted thawing and remaining at room temperature for 6 h, and following storage at the appropriate reduced temperature for at least 12 h before being removed for another thaw. QC samples for evaluation of freeze–thaw stability were analyzed along with calibration standards and QCs that underwent only one freeze/thaw cycle, and the % differences for four cycles were within ±2.80% and ±6.03% at −20 and −80 °C, respectively.

4. Conclusions

The use of HILIC technique associated with a high level of organic mobile phase solved the problem of poor retention of metformin on an analytical column and increased the sensitivity of the assay by improving ionization efficiency for electrospray ionization mass spectrometry. The LLOQ established for metformin was 0.5 ng/mL, which is lower than other published methods. A one-step protein precipitation method was developed and used for metformin extraction. This extraction method significantly reduced sample preparation time and allowed the quantitation of metformin for a concentration ranging from 0.5 to 500 ng/mL using 50 μL of plasma. This HILIC–MS/MS method has been fully validated for the quantitative analysis of metformin in human plasma in support of clinical pharmacokinetic studies. The method offers significant advantages in terms of sensitivity, selectivity, accuracy, reproducibility, ease of sample preparation, and short run time.

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