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Development and Validation of Sensitive, Specific and Stability-Indicating LC-MS/MS Method for Simultaneous Quantification of Metformin and Becalutamide in Human Plasma

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Metformin, an oral anti-hyperglycaemic drug belongs to the biguanide class and is traditionally known as an insulin sensitizer which lowers the blood glucose levels by activating AMPK (adenosine monophosphate protein kinase). Metformin has shown anti-neoplastic effects in various tumour models, including prostate, ovarian, breast, colorectal and endometrial cancers, by activating AMPK, which in turn blocks the mTOR/S6 kinase pathway and inhibits insulin/insulin-like growth factor (IGF)-mediated cellular proliferation. Bicalutamide, is a non-steroidal anti-androgen class agent, binds to androgen receptors, thus blocking the effects of androgenic hormones such as testosterone and dihydrotestosterone. Previous reports on *in vivo* and *in vitro* studies suggests the combination of metformin and biclutamide dramatically decreases prostate cancer cell proliferation more than either drug alone. This work was undertaken to examine the effect of metformin on bicalutamide pharmacokinetic parameters, which may further aid in dose adjustment when administered concurrently to treat prostate cancer. So, for the simultaneous quantitation of metformin and becalutamide in spiked human plasma by LC-MS/MS method was used. The preconcentration of the analyte was done using Protein precipitation using acetonitrile. Separations were done on Agilent 1290 infinity II LC system paired with an Agilent 6470 triple quadrupole mass spectrometer with positive and negative ionization modes. The mobile phase comprised 0.1% acetic acid in MilliQ water as solvent A and 100% methanol as solvent B with gradient flow at 0. 6 mL/min. column, column oven and degasser with a Zorbax Eclipse plus C8 column (4.6 × 100 mm, 3.5 µm) was utilized. The retention times for metformin, bicalutamide and the IS (propranolol and tolbutamide) were 3.6 min, 4.7 min, 5.9 min and 6.1 min, respectively. For the samples, subsequent Q1/Q3 transitions: bicalutamide, ESI^- *m/z* 427.2 > 184.5 (10 eV); metformin, ESI^+ *m/z* 130.10 > 60.2 (12 eV); tolbutamide, ESI^- *m/z* 269.09 > 170 (16 eV); and propranolol, ESI^+ *m/z* 260.2 > 183.0 (32 eV). Data acquisition and instrument control were operated with Mass Hunter workstation (version 10.1). The established method was validated by following USFDA bioanalytical guidelines, Linear regression equations for metformin and biclutamide were 0.0822x + 0.00848 and 0.09588 x + 0.02635, respectively, with (1/x) weighting factor, the regression coefficients for metformin and biclutamide are 0.9963 and 0.9971, respectively. Mean extraction recoveries for metformin was 85.04% and for biclutamide was 86.23% all QCs. Solifenacin's stability in plasma was confirmed through rigorous testing, including bench-top exposure (17 h), injector residence, six freeze-defrost cycles and long-term storage at –20 ± 5 ºC for over 7 days. The compound exhibited consistent integrity under all conditions. The analytical method underwent full validation and met qualification standards, supporting its reliability for routine bioanalysis and pharmacokinetic profiling in biological matrices.

Keywords: Metformin, Biclutamide, LC-MS/MS, Human plasma, Pharmacokinetic study.

INTRODUCTION

Metformin (MET), an oral anti-hyperglycaemic drug belongs to the biguanide class and is traditionally known as an insulin sensitizer. It lowers blood glucose levels by inhibiting hepatic glycogenolysis and gluconeogenesis, primarily *via* stimulation of adenosine monophosphate protein kinase (AMPK). Metformin has demonstrated anti-neoplastic effects in various tumor models including prostate, ovarian, breast, colorectal, and endometrial cancers primarily by activating AMPK, which inhibits the mTOR/S6 kinase pathway and suppresses insulin/IGF-mediated cellular proliferation [1]. Bicalutamide (BCT), is a non-steroidal anti-androgen class agent, binds to androgen receptors, thus blocking the effects of androgenic hormones

such as testosterone and dihydrotestosterone. Bicalutamide exhibits high protein-binding capacity, undergoes oxidation and glucuronidation metabolism and is well absorbed orally with minimal impact from food intake [2,3]. Metabolites are elimi-nated *via* bile and urine, whereas the majority of drugs are eliminated through faeces. Bicalutamide has a relatively long plasma half-life in preclinical species including rodents, canines and humans [2].

Previous reports on *in vivo* and *in vitro* studies suggest that the combination of metformin and biclutamide dramatically decreases prostate cancer cell proliferation more than either drug alone [4,5]. This effect appeared to be achieved by a reduction in cell proliferation rates of androgen receptor-positive cells, but the combined therapy appeared to increase apoptosis in androgen receptor-negative cells. The current work has been conducted to assess the effect of metformin on bicalutamide pharmacokinetic parameters, which may further aid in dose adjustment when administered concurrently to treat prostate cancer.

Various methodologies based on LC-MS/MS had been reported for their individual quantification of metformin and bicalutamide [6-17]. However, an efficient and sensitive analytical methodology capable of simultaneously detecting and estimating metformin and bicalutamide in plasma is required. As per the searched things now, this study presents the first validated method to quantify metformin and bicalutamide in plasma simultaneously. The primary focus in the present research was to utilize (LC-MS/MS) as a quantitative tool to investigate preclinical pharmacokinetic interactions. LC-MS/MS has been acknowledged as a highly sensitive, stable and reliable technique for quantitative bioanalytical studies. The LC-MS/MS technique for simultaneous quantitation of metformin and bicalutamide in spiked plasma has been developed and validated as per guidelines successfully. This method makes use of simple protein precipitation approach for the sample preparation. One of the prime advantages of the present developed method is its capability to overcome endogenous matrix interference, allowing for accurate simultaneous quantification of metformin and bicalutamide in biological substrates. Furthermore, the developed bioanalytical method holds potential for pharmacokinetic studies of metformin and bicalutamide in human plasma.

EXPERIMENTAL

Metformin (MET, 99%), bicalutamide (BCT, 99%), internal standards propranolol (99%) and tolbutamide (99%) were received from Cipla-(India) Pvt. Ltd, Hyderabad, India as a gift sample. LC-MS grade methanol and acetonitrile were acquired from Merck, Mumbai, India), whereas Fluka Analytical-Sigma Aldrich supp-lied MS-grade acetic acid.

Mass spectrometric analysis: The BCT and MET were analyzed using both negative and positive mode of ionization using an Agilent Infinity II HPLC paired with an Agilent 6470 triple quadrupole mass spectrometer. The mobile phase comprised 0.1% acetic acid in MilliQ water as solvent A and 100% methanol as solvent B. The separation has been achieved at a flow of 0.6 mL/min using the gradient % B (time) of 45% (0.00 min), 80% (0.0-10 min), 80-45% (10.00-10.1 min), 45-45% (10.1-15 min). A Zorbax Eclipse plus C8 column (4.6 × 100 mm, 3.5 μm) was utilized for the optimized separation of metformin and biclutamide with injection volume of 2 μL and the temperature of autosampler was kept at 10 ºC. The retention times for MET, BCT and the IS (propranolol and tolbutamide) were 3.6 min, 4.7 min, 5.9 min and 6.1 min, respectively. The source gas temperature was maintained at 280 ºC with a gas flow rate of 10 L/min, the nebulizer was kept at 45 psi, while the sheath gas was kept at 250 ºC, the sheath gas flow at 10 L/min, capillary voltage was operated at ± 3500 V and nozzle voltage at 500 V were maintained throughout the analysis. A positive mode and a negative mode of the ESI interface, in addition to the multiple reaction monitoring (MRM) modes were employed to detect the compounds. For the samples, subsequent Q1/Q3 transitions: BCT, ESI^- *m/z* 427.2 > 184.5 (10 eV); MET, ESI^+ *m/z* 130.10 > 60.2 (12 eV); tolbutamide, ESI^- *m/z* 269.09 > 170 (16 eV); and propranolol, ESI^+ *m/z* 260.2 > 183.0 (32 eV). Data acquisition and instrument control were operated using a MassHunter workstation (version 10.1), whereas qualitative data analysis was performed using the MassHunter Workstation qualitative data application manager (version 10.1).

Standard solutions and quality control sample: Separate standard stock solutions of BCT and MET were prepared using methanol as the diluent at a concentration of 1 mg/mL. Similarly, prepare 1 mg/mL concentration of stock solution of tolbutamide (IS) and propranolol (IS) with methanol. Standard stock solutions were diluted to concentrations of 10-1500 ng/mL for using as working stock solutions. The blank plasma was used for spiking the working stock solution to prepare the calibration samples. The range of linearity was 1-150 ng/mL for both BCT and MET combinations. Four-quality control (QC) samples have been prepared at concentrations of 1 ng/mL as LLOQ (lower limit of quantification), 3 ng/mL as low QC (low quality control), 50 ng/mL as medium QC (medium quality control) and 120 ng/mL as high QC (high quality control) in the same as that of manner as calibration standards. Prior to usage, all solutions were kept at 2 to 8 ºC.

Sample preparation: Metformin and its respective internal standard both are extremely polar molecules, thus it was difficult to extract from the plasma matrices. Protein precipitation is preferred for MET, BCT and their internal standards. To the thawed plasma, a known quantity of standard solution of both drugs was spiked, followed by spiking of the internal standard and chilled acetonitrile was included to precipitate out the proteins. The samples were uniformly mixed and perform centrifugation for 10 min at 10,000 rpm. Then clear supernatant was isolated and concentrated under vaccum. After removal from the concentrator, reconstitute with 100 μL methanol and loaded into the system.

Method validation: The bioanalytical method development approach was followed and validated as per the FDA guidelines. All validation parameters were examined for variability relative to the allowed ranges using bioanalytical standards.

Selectivity and carryover: For identifying the method's selectivity, concerning factors like endogenous chemicals, metabolites and known degradation products evaluation is essential. The drug at the lower limit of quantification (LLOQ) was

spiked into blank plasma aliquots and compared to the peak areas of six distinct types of blank plasma to test the procedure. Carryover effect is performed with simultaneous injections of ULOQ followed by blank plasma.

Linearity and range: To characterize linearity, several samples were employed, including a blank sample (which lacked IS), zero sample (which contained IS) and eight non-zero samples (IS with known analyte concentrations). Linear regression analysis was employed to assess linearity, while the analyte concentrations were determined by constructing the calibration curve that graphed the ratio of each analyte of peak area to IS against the standard concentrations. The methods sensitivity was evaluated by calculating the LLOQ (1 ng/mL). The acceptable accuracy range for the LLOQ was ± 20% and that for the non-zero calibrators was ± 15% of the nominal concentration.

Accuracy and precision: Six replicates were run for each analyte to assess accuracy and precision (intra-day and inter-day) across the four concentration levels of LLOQ, low QC, medium QC and high QC. Accuracy and relative standard deviation percentages were calculated and used to express these values. The criteria for LLOQ were ± 20% and for other QC concentrations was ± 15% of the theoretical concentration for determining the accuracy. The correlation of variance (CV) was ± 15% for L, M and high QC, except for ± 20 for LLOQ% to calculate the precision.

Recovery: The analyte extraction efficiency has been calculated from the ratios of the analytes and IS peak areas acquired from the quality control samples (low QC, medium QC and high QC) were compared to those obtained from post-extraction spiked samples (n = 6). There must be uniformity, accuracy and repeatability in the variability across distinct samples.

Matrix effect: The study of the matrix effect involved the use of six different batches of plasma. Every batch of blank plasma samples was spiked with analytes after the extraction and the peak area of the resulting mixture was compared to that of the pure analyte solution. To ensure accuracy, the relative standard deviation (RSD) must not exceed ± 15%.

Dilution integrity: To verify the integrity of the dilution, analytes were added to the blank matrix at a high-quality control (high QC) concentration and then diluted with the blank matrix. The accuracy and precision of dilution were not affected. Dilution integrity was considered acceptable if the accuracy and precision were within ± 15%.

Stability samples: The analyte stability in plasma samples was evaluated using stability assays performed under varying conditions. Six replicates per level were tested at low QC and high QC levels.

Benchtop stability: Six replicates of low QC and high QC levels in the plasma matrix were prepared and placed on benchtop for 24 h. Fresh samples consisting of low QC and high QC standards were processed and analyzed. The stability and fresh samples used for comparisons were processed and analyzed together.

Freeze-defrost stability: Prepared six replicates of low QC and high QC levels in the plasma matrix placed them at -20 ºC and allowed them to freeze. The samples were thawed and the sample cycle has been repeated three times. The samples were processed and analyzed. Fresh samples consisting of low QC, medium QC and high QC standards were processed and analyzed. The processed and fresh samples were compared.

Autosampler stability: The processed samples at low QC and high QC were freshly placed in the autosampler and analyzed after 24 h. The stability of the sample was compared with that of a fresh sample and the accuracy was calculated.

RESULTS AND DISCUSSION

Method validation

Selectivity and carryover: Analyzing and comparing chromatographs of plasma blanks and plasma with quantities equal to the LLOQ of MET and BCT demonstrates the selectivity and sensitivity of the method (Fig. 1). The suggested approach is highly sensitive and selective for both analytes and the IS. When the blank sample was injected after the ULOQ samples, no measurable response was observed, indicating that the timing of the injections did not affect the accuracy of the results. Therefore, it can be concluded that the proposed approach does not produce any residual effects.

Calibration and sensitivity: The MET and BCT and calibration curves were linear from 1 to 150 ng/mL. The linear regression equations for MET and BCT were 0.0822x + 0.00848 and 0.09588x + 0.02635, respectively, with a weighting factor of (1/x). The correlation coefficients of MET and BCT were 0.9963 and 0.9971, respectively.

Accuracy and precision: The precision and accuracy of both intra- and inter-day measurements were consistent and reproducible. The results indicate that all values were within the accepted range, as mentioned in Table-1.

Recovery and matrix effect: The mean percentage recoveries for MET and BCT were calculated at the three concentrations. For MET, the recoveries of high QC, medium QC and low QC were found to be 86.10 ± 1.2%, 86.86 ± 3.7% and 82.81 ± 1.7%, with % RSD values of 1.47%, 4.31% and 2.11%, respectively. For BCT, the recoveries of high QC, medium QC and low QC were found to be 87.41 ± 3.4%, 88.75 ± 5.3% and 82.58 ± 2.23%, with % RSD values of 3.89%, 5.99% and 2.70%, respectively. The analyte recovery was reliable and reproducible. None of the six batches of quality control samples exhibited any matrix influence. The matrix effect results suggested that the co-eluting compounds did not affect the ionization of the analytes and internal standards. The matrix factor (Table-2) was calculated and reported for MET, BCT and the internal standards at all QC levels. The matrix effect (ME) was quantified by using the following formula:

$$ME = \frac{\text{Response of analyte in post extracted spiked matrix}}{\text{Response in solvent}} - 1$$

Stability samples: The drug's stability in plasma was investigated across a wide variety of environments that could be encountered during storage. All of the analytes were determined to be stable when subjected to benchtop, autosampler and freeze-thaw stability at low QC, medium QC and high QC levels as mentioned in Table-3 and all the results are within accepted limits.

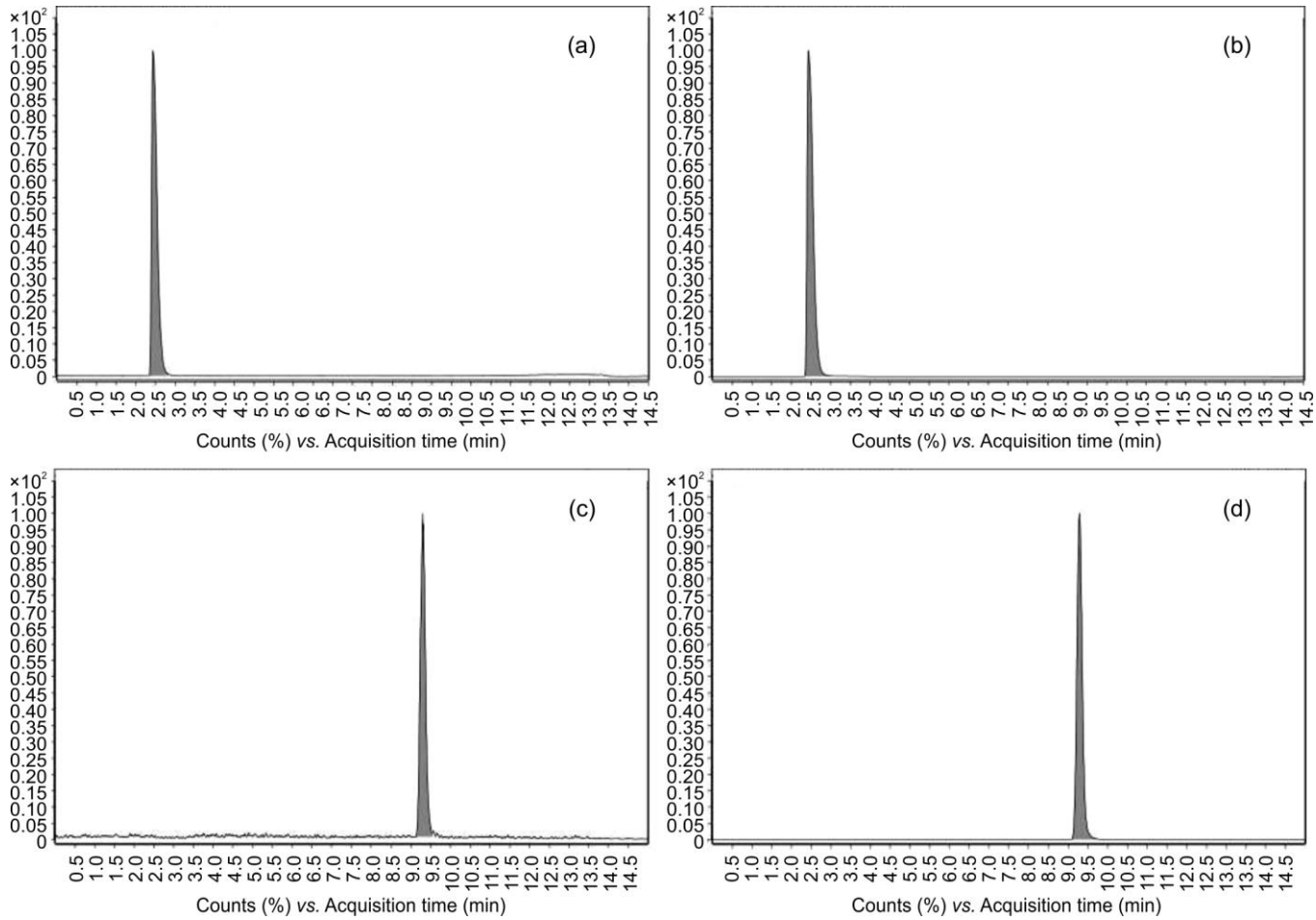

Fig. 1. Representative chromatograms of MRM chromatogram of (a) Metformin at LLOQ (1 ng/mL) (b) Metformin-1 h post spiked with bicalutamide) (c) Bicalutamide at LLOQ (1 ng/mL) and (d) Bicalutamide-1 h post spiked with metformin)

TABLE-1
WITHIN A DAY AND BETWEEN THE DAYS ACCURACY AND PRECISION OF METFORMIN AND BICALUTAMIDE IN PLASMA

Drug		Within a day precision and accuracy				Between the days precision and accuracy			
	QC sample	LLOQ	Low QC	Medium QC	High QC	LLOQ	Low QC	Medium QC	High QC
	True concentration (ng/mL)	1	3	50	120	1	3	50	120
MET	Mean actual concentration (ng/mL) ± SD	1.04 ± 0.09	3.03 ± 0.37	50.9 ± 1.96	127.6 ± 13.65	1.02 ± 0.07	3.10 ± 0.21	50.07 ± 1.43	131.01 ± 13.93
	Precision (CV, %)	8.51	3.06	2.15	9.31	8.16	5.83	3.44	10.14
	Accuracy (%) ± SD	101 ± 5.57	101 ± 3.10	102 ± 2.19	106.39 ± 8.97	98.68 ± 6.14	101.26 ± 7.05	101.21 ± 13.49	108.58 ± 11.12
BCT	Mean actual concentration (ng/mL) ± SD	0.99 ± 0.14	3.10 ± 0.18	51.29 ± 1.08	121.07 ± 3.99	0.97 ± 0.10	3.23 ± 0.26	52.64 ± 2.34	117.74 ± 13.43
	Precision (CV, %)	13.78	5.66	3.51	3.05	10.08	8.11	4.82	11.32
	Accuracy (%) ± SD	98.64 ± 13.59	103.37 ± 5.85	105.06 ± 3.62	101.01 ± 3.08	97.29 ± 9.80	107.51 ± 8.72	105.13 ± 5.07	98.23 ± 11.12

TABLE-2
MATRIX EFFECT OF MET AND BCT AT
LOW QC, MEDIUM QC AND HIGH QC LEVELS

MET			BCT		
Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
1.16	1.11	1.10	0.86	0.87	0.95
1.03	0.95	1.05	0.77	0.89	1.06
1.21	1.31	1.15	1.17	0.98	0.92

Dilution integrity: A concentration higher than the ULOQ which is 300 ng/mL concentrations was prepared by spiking the analyte to the blank plasma. Further, this solution was diluted two-fold with the blank plasma, processed and analyzed. In a study with six replicates and two-fold dilutions, the percentage recovery of MET ranged from 86.62 to 90.12% and the percentage recovery of BCT was determined to be 88.38 to 92.38%. The %relative standard deviation (RSD) was found to be less than 10% for both analytes.

TABLE-3
STABILITY OF METFORMIN AND BICLUTAMIDE IN PLASMA UNDER DIFFERENT TEMPARATURE CONDITIONS, (n = 6, MEAN ± SD)

Stability	QC samples	Theoretical concentration (ng/mL)	Mean estimated concentration (ng/mL) ± SD		Precision (% CV)	
			MET	BCT	MET	BCT
Bench top stability	Low QC	3	2.95 ± 0.09	2.97 ± 0.09	3.18	3.18
	High QC	120	118 ± 12.97	118 ± 12.96	10.92	10.91
Auto sampler stability	Low QC	3	3.13 ± 0.39	2.96 ± 0.06	13.02	2.11
	High QC	120	120.01 ± 4.75	109.34 ± 10.45	3.44	10.84
Freeze-thaw stability	Low QC	3	2.96 ± 0.06	3.03 ± 0.09	2.11	3.06
	High QC	120	115.34 ± 14.45	128.69 ± 13.65	12.84	10.31

Conclusion

To measure the concentration of metformin and bicalutamide from plasma, a quick LC-ESI-MS-based method has been developed. The advantage of this method is high sensitivity, the ability to analyze both analytes in a single run and the ease with which proteins can be extracted and precipitated. The analysis time for each run was 15 min, allowing for a rapid turnaround to fulfill the needs of preclinical pharmacokinetic research. The developed LC-MS/MS technique meets the need for low detection of analytes in plasma, with an LLOQ of 1 ng/mL for both metformin and bicalutamide. The assay was validated following the bioanalytical requirements established by the US-FDA and the method was effectively employed to explore the kinetic interaction research of the aforementioned medications in plasma. As per the literature knowledge, this is the first LC-MS/MS method for the concurrent assessment of metformin and bicalutamide in plasma was developed and validated.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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