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Bioanalytically Validated LC-UV Method for Estimation of Hypoxia-Inducible Factor-2 alpha (HIF-2α) Inhibitor in Spiked Human Plasma

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Abstract

Belzutifan is a hypoxia-inducible factor-2 alpha (HIF-2α) inhibitor used to treat von Hippel–Lindau disease-associated renal cell carcinoma. Simple, accurate, precise, and specific HPLC method was developed to estimate Belzutifan (BZT) in human plasma serving Emtricitabine (ETC) as reference (ISTD). The analyte and ISTD were separated on a Kromasil C18 (250x4.6mmx5µ) column using a mobile phase composition. The buffer was composed of Acetonitrile (60:40). The RTs of the analyte and ISTD were found to be 3.446 and 2.363 min, respectively with a flow of 1ml/min. Further, the reported method validated as per USFDA, and was found to be well within the acceptable range for all parameters with concentrations of LLOQ 0.075mcg/ml, LQC 0.225mcg/ml, MQC 1.5mcg/ml, HQC 2.4mcg/ml, and ULOQ 3.0 mcg/ml. The matrix effect at HQC and LQC was 100.19 and 99.83%; the sensitivity at LLOQ was 99.63%; the precision and accuracy at HQC, MQC, LQC, and LLOQ was between 98.56 and 100.11%. The linearity concentration is in the range of 0.75-3mcg/ml Belzutifan with a correlation coefficient of r^2 = 0.999 with good stability. The proposed HPLC method as easy, fast, and particular for the calculation of Belzutifan in human plasma. Efficient sample preparation and rapid chromatographic analysis are crucial for high-throughput detection and quantification of target analytes in complex matrices such biological matrices. Thus, the reported HPLC method can be applied to the bioequivalence and pharmacokinetic studies of Belzutifan in human plasma samples and is appropriate for therapeutic drug monitoring in clinical laboratories.

Keywords: Belzutifan; Recovery; Precision; Therapeutic drug monitoring

Introduction

Belzutifan (MK-6482) is a suppressor of hypoxia-inducible factor-2 alpha (HIF-2α), which is a sequence specific DNA binding factor involved in the maintenance of oxygen homeostasis and cell metabolism. i.e, regulating the body's response to hypoxia levels. By inhibiting HIF-2α, Belzutifan can potentially prevent the growth and spread of tumours by blocking the signalling pathways that promote cancer cell survival and proliferation.

Belzutifan contains a fused bicyclic ring system and several functional groups, including an amide, an N-oxide and carboxylic acid. Its molecular formula is $C_{17}H_{14}N_4O_3$. Belzutifan is being investigated for its potential use in the treatment of various types of cancer, such as renal cell carcinoma. In clinical trials, Belzutifan has shown promising results in treating these types of cancer by inhibiting the growth and proliferation of tumour cells (1-13) (Figure 1).

A review of the existing literature revealed that, while a several HPLC (14-16) methods have been documented for quantifying Belzutifan in pharmaceutical formulations, no techniques have been established for measuring this drug in biological fluids using RP-HPLC. In the current study, we developed a specific RP-HPLC method to quantify Belzutifan in

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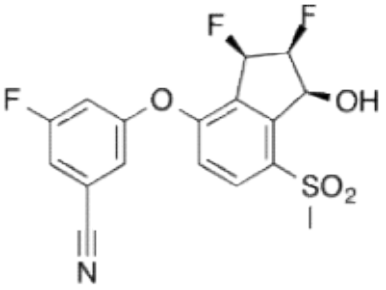

Figure 1: Structure of Belzutifan

human plasma. This study introduces a new, sensitive, quick, precise, and accurate HPLC-based approach for determining Belzutifan levels in human plasma samples.

Materials and Methods

Instrument

The developed method uses a Waters HPLC 2695 system equipped automated sample injector, temperature adjustable column, degassing unit and PDA Detector, integrated with Empower-2 software, to perform chromatography.

Materials

Pure Belzutifan sample was procured from Merck & co, India. Formic acid, all HPLC-grade chemicals, including acetonitrile, were received from the Merck, Mumbai. Ultrapure water, purified using a Milli-Q system, was utilized throughout the study. K2 EDTA control Human plasma was purchased from Deccan Pathological Labs, Gulzar Houz and Hyderabad.

Biological matrix Samples Preparation

Protein precipitation was the chosen analyte extraction technique. First, 25 µL of belzutifan (150 mcg/mL) was added to 75 µL of plasma in a 5 mL polypropylene tube. The spiked plasma underwent 2 minutes of vertexing, then was extracted using 100 µL of a chilled acetonitrile: methanol solution containing 50 mcg/mL of emtricitabine as the internal standard. The solution was spinned for 10 min at 10,000 rpm, and subsequently collect the supernatant. A 10 µL aliquot was then injected to the LC–UV instrument for analysis. This analytical method was thoroughly applied throughout the study.

Procedure for CC and QC Standards

Methanolic stock solutions having Belzutifan and the internal standard were prepared at concentrations of 150 mcg/mL and 500 mcg/mL, respectively and serially diluted for calibration curve standards. The working calibration concentrations for non-zero calibrators were 0.75, 1.5, 2.25, 6.00, 15.00, 18.00, 24.00, and 30.00 mcg/mL. Non-zero calibrators were created by the addition of 25 µL of every working calibration solution to 75 µL of blank plasma. Calibration standards for Belzutifan were set at 0.075, 0.15, 0.225, 0.600, 1.800, 2.400, and 3.000 mcg/mL. QC sample concentrations were determined based on the LLOQ and ULOQ: LQC at three times the LLOQ (0.075 mcg/mL), MQC at 40–50% of the calibration scale, and HQC at 75% of the calibration scale. The QC samples were prepared at 0.225 mcg/mL (LQC), 1.500 mcg/mL (MQC), and 2.400 mcg/mL (HQC) for assessment of recovery, stability, and dilution integrity. Three aliquotes of each QC level were injected between sets of test samples, comprising 5% of the unknown samples.

Method Development

The establishment of the RP-HPLC method entailed conducting various trials, the optimal mobile phase was determined to be acetonitrile and 0.01M potassium orthophosphate buffer in a 60:40 (v/v) ratio, meeting all necessary peak parameters such as theoretical plates, tailing factor, and resolution. After scanning individual drugs in the 200–400 nm range, 268 nm was chosen as the ideal wavelength for drug detection with sufficient sensitivity. A Kromasil C18 column with a mobile phase flow of 1ml/min was selected to improve component resolution. Emtricitabine served as the internal standard (ISTD) to address variations in sample extractions recovered using protein precipitation. Belzutifan and Emtricitabine peaks appeared at 3.454 min and 2.398 min, respectively, during a 10-minute run.

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Table 1: Matrix effect of Belzutifan

S. No.	Plasma Lot number	High QC	Low QC
		Spiked Concentration (mcg/ml)	
		2.400	0.225
		Spiked Concentration Range (mcg/ml)	
		(2.040-2.760)	(1.9125-2.5875)
		Measured Concentration (mcg/ml)(n=3)	
1	LOT-1	2.409	0.224
2	LOT-2	2.402	0.224
3	LOT-3	2.402	0.225
4	LOT-4	2.403	0.224
5	LOT-5	2.403	0.225
6	LOT-6	2.405	0.225
N		18	18
Mean		2.404	0.224
SD		0.005181	0.001838
% CV		0.22	0.82
% Mean Accuracy		100.19	99.55
No. of QC Failed		0	0

Method Validation

This analytical method was thoroughly validated, to comply with FDA guidelines and meet the acceptance criteria.

Results and Discussion

Chromatographic elution of Belzutifan was refined through multiple trials using a C18 column with mobile phase adjusting composition of potassium dihydrogen orthophosphate buffer and acetonitrile at several pH levels. Optimal conditions were achieved with a 60:40 (v/v) ratio of potassium dihydrogen orthophosphate buffer (pH 4.8) to acetonitrile. Sufficient sensitivity was obtained at 268 nm wavelength. A flow rate of 1 ml/minute provided optimal separation. The analyte and internal standard were detected at retention times 3.685 and 2.326 min, respectively.

Specificity and selectivity

In essence, specificity is a critical component of method validation to ensure the accurate and reliable measurement of Belzutifan in complex biological matrices, free from interference. Six distinct blank plasma samples collected from six different lots of blood samples were injected to assess the methods selectivity Analysis of all samples revealed no intervention at the analytes retention time in blank samples.

Matrix effect

In the present study, the matrix effect was assessed by analysing six different plasma lots having different matrix components spiked at two QC concentration levels (HQC, LQC).The matrix effect for High QC and Low QC levels of Belzutifan were represented in Table 1.

Linearity

The method's linearity was evaluated across multiple concentration levels, including the lower limit of quantitation. A linear curve was observed spanning the range of 0.075–3 mcg/mL with 0.999 as coefficient of correlation. The linear curves were generated through three independent runs, each involving triplicate analyses of spiked blank plasma samples. As shown in Figure 2, the graph depicts a linear relationship. All back-calculated standard concentrations were within acceptable limits, as presented in Table 2.

Accuracy &Precision

The precision of the developed method was conducted with QC samples includes HQC, MQC, LQC & LLOQC in 6 replicates individually and estimated against a set of standards of calibration curve

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Table 2: Linearity data of Belzutifan

S. No.	Concentration (mcg/ml)	Back calculated Concentration (mcg /ml)			Average	%CV	%Mean Accuracy
		1	2	3			
1	0.075	0.07419	0.07502	0.07462	0.07461	0.556347	99.48
2	0.15	0.14803	0.14904	0.15042	0.149163	0.804329	99.44
3	0.225	0.22507	0.22509	0.22606	0.225407	0.251054	100.18
4	0.6	0.60048	0.60052	0.60053	0.60051	0.004406	100.08
5	1.5	1.50270	1.50974	1.50675	1.506397	0.234551	100.42
6	1.8	1.80198	1.79812	1.81452	1.804873	0.475064	100.27
7	2.4	2.40647	2.40165	2.40074	2.402953	0.128147	100.12
8	3	3.00287	3.0017	3.00120	3.002035	0.039336	100.06

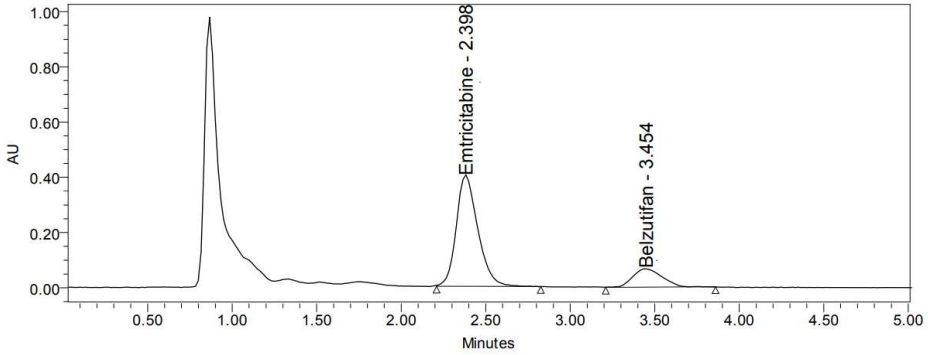

Figure 2: Displays the optimized chromatogram

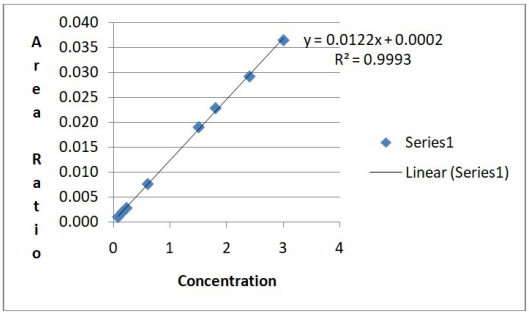

Figure 3: Calibration curve of Belzutifan

(Figure 3). The percent mean recoveries ranging from 99.84 to 100.17 for interday and 99.56 to100.11 for intraday were observed respectively. The results were shown in Table 3.

Recovery of analyte

As defined by FDA (2001), recovery is the detector's response to the quantity of analyte introduced & extracted from a biological matrix. For Belzutifan, the extraction recovery was determined to be 50.45, 57.40, and 58.21 at the HQC, MQC, and LQC levels, respectively. The internal standard showed a recovery of 74.76. These findings align with the results. Tables 4 and 5 present the recovery study outcomes, which were within the acceptable range. The acceptance criteria stipulated that the % CV of recovery for each QC level and IS should not exceed 15.00%. Additionally, the overall mean recovery % CV across all QC levels was required to be no greater than 20.00%.

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Table 3: Intraday & Interday precision data of Belzutifan				
	High QC	Medium QC	Low QC	Lower Limit of QC
	Spiked Concentration (mcg/mL)			
	2.400	1.500	0.225	0.075
Interday Precision (n=18)				
Mean	2.402	1.502	0.225	0.075
SD	0.004	0.002	0.001	0.001
%CV	0.183	0.167	0.494	0.800
% Mean Accuracy	100.066	100.111	100.022	99.681
Intra Precision and Accuracy (n=18)				
Mean	2.401	1.501	0.225	0.0746
SD	0.0043	0.0025	0.0012	0.006
%CV	0.18	0.17	0.56	0.81
% Mean Accuracy	100.06	100.11	100.05	99.56

Table 4: Recovery of Belzutifan						
Sample	High QC		Medium QC		Low QC	
	Non-extracted outcome	Extracted outcome	Non-extracted outcome	Extracted outcome	Non-extracted outcome	Extracted outcome
Mean(n=6)	159739	158209	99723	99393	14889	14746
SD	4106.03	1530.07	199.07	120.81	69.33	158.15
% CV	2.57	0.97	0.20	0.12	0.47	1.07
%Mean Recovery	99.04		99.67		99.04	
Overall % Mean Recovery	99.251					
Overall SD	0.3624					
Overall % CV	0.37					

Table 5: Recovery of Emtricitabine (Internal standard)		
S. No.	Non-extracted outcome	Extracted outcome
Mean(n=6)	5291750.0	5266667.0
SD	14357.21	3480.86
% CV	0.27	0.07
% Mean Recovery	99.53	

Table 6: Stability data of Belzutifan			
QC level	Spiked conc (mcg/mL)	Mean measured conc (mcg/mL) ±SD.(n=6)	% CV
Stability of day zero			Drug
HQC	2.400	2.402	0.14
LQC	0.225	0.226	0.19
Stability of long-term storage at 28°C			
HQC	2.400	2.402	0.15
LQC	0.225	0.226	0.85
Stability at -80°C			
HQC	2.400	2.403	0.82
LQC	0.225	0.225	0.52

Stability studies

The analyte's stability was evaluated by analyzing replicate samples of high-quality controls (HQC), middle-quality controls (MQC) and low-quality controls (LQC) under various time points and storage conditions, including zero hours and long-term storage at -28°C and -80°C. Results showed that LQC, MQC, and HQC exceeded 95%, indicating stability throughout the analytical process. Refer to Table 6 for a summary of the findings.

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Conclusion

The proposed HPLC method for determining Belzutifan in spiked human plasma using a UV detector is simple and accurate. It uses protein precipitation with acetonitrile. The isocratic elution, short elution time of less than 10 min, and UV detection make the method cost-effective. This analytical method was thoroughly validated, rendering it suitable for routine bioanalysis of Belzutifan using HPLC-UV detection.

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Conflict of Interest

The authors declare that no conflict of interest.

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