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DEVELOPMENT AND VALIDATION OF UV SPECTROSCOPIC METHOD FOR ESTIMATION OF EMPAGLIFLOZIN IN RAT PLASMA FOR PHARMACOKINETIC APPLICATIONS

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ABSTRACT

The objective of present study is to develop a simple, sensitive, rapid and reproducible validated UV method to quantify empagliflozin in rat plasma and applying the developed method for pharmacokinetic studies of empagliflozin. The method was developed to estimate empagliflozin at 223 nm in ethanol and water using UV spectrophotometer by extracting empagliflozin from rat plasma using liquid extraction with dicloromethane. The developed method was validated as per ICH guidelines. The calibration curve of empagliflozin was linear in the range of 1-30 µg/ml. Coefficient of Variation for intra and inter-day precision was found to be 0.28 and 0.45 respectively. % Recovery for accuracy study of empagliflozin was found to be between 99.46% to 99.78%. Limits of detection and quantification were found to be 0.10 and 0.33 respectively. The coefficient of variation for robustness and ruggedness was found to be 0.26 and 0.39. Recovery studies were found to be consistent and stability studies were also found to be within the limits. The developed UV method was validated and it was rapid, simple, sensitive and cost effective. The proposed method has been successfully adapted for pharmacokinetic studies of empagliflozin in rats after a single oral dose.

Keywords: Empagliflozin, Liquid-liquid extraction, Human plasma, UV-method, Validation

INTRODUCTION

Empagliflozin **(Figure 1)** chemically is (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl] oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol.]

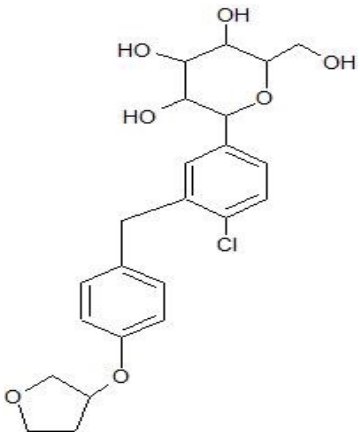

Figure 1: Structure of Empagliflozin

A bioanalytical method involves collecting, processing, storing and analysing the biological matrix for a chemical compound. The purpose of bio analytical method development is to define all the necessary conditions and limitations of the method for its intended purpose and to make sure that the method is optimized for validation **[1]**. Bioanalytical method validation involves all the procedures which make sure that the developed method is reliable and reproducible.

Sample preparation is the crucial step in bio analytical method development to obtain a good result. The main motto of sample preparation technique is to extract the analyte of interest from the biological matrix in concentrated form without any interference. There are many biological matrices, some of them are plasma, serum, whole blood, urine, nails, hair, saliva and sweat. Plasma is the commonly used biological matrix in analysis when compared to other biological matrices. The collected biological matrix cannot be used as such for analysis but it need to be processed (sample preparation) to separate the analyte. Basically there are three important sample preparation techniques.

SPE is a sample preparation method where the analyte is bound to a solid a support and the interferences are washed off and the analyte of interest is selectively eluted. It is based on adsorption principle. SPE involves a small plastic disposable column or cartridge which is packed with a sorbent, which is usually a reverse phase material like C_{18} silica. Solid phase extraction involves four steps they are: conditioning of packing, sample application, washing the packing (removal of interferences) and recovery of the analyte **[2]**.

Liquid–liquid extraction (LLE) involves the transfer of an analyte from one solvent to another. One solvent is the biological matrix like plasma and the other solvent is organic solvent. In this it consists a step of mixing the

liquids followed by shaking them followed by separating the two layers. Here the analyte partitions between the two phases depending upon its solubility and partition coefficient between it's the two phases **[3]**.

In protein precipitation, the proteins are precipitated by addition of an organic solvent like ethanol, methanol, acetone, acetonitrile **[4]**. Methanol is the favoured solvent among the other solvent for protein precipitation as it produces a clear supernatant **[5]**. Some solvents can also be used in ratio and are effective in removal of protein like use of methanol: acetonitrile in the ratio of 2:1 precipitates 98% of the protein from the plasma **[6]**. The other mechanisms by which protein precipitation is done is by adding a salt like ammonium sulphate which has high solubility and high ionic strength **[7]**, High salt concentration will cause the protein to aggregate and precipitate from the solution. By changing the p^H also protein precipitation can be achieved.

Empagliflozin, a sodium-glucose co-transporter 2 (SGLT2) inhibitor is a drug of choice nowadays especially in individualized approaches to diabetic care. Although many methods are available for identification and quantification of empagliflozin, there is no standard official method available. A literature search has shown that there are no methods available for the quantification of empagliflozin in biological fluids using UV spectroscopy. Thus there is a need to develop a simple, sensitive, reproducible bionalytical method for estimation of empagliflozin in rat plasma using UV spectroscopy which can be adapted to pharmacokinetic studies.

MATERIALS AND METHODS

Equipment

The analytical method was developed and validated using Double beam UV spectrophotometer from ELICO.The data was obtained using Spectra Treats 3.11.01Rel 2b. Cyclomixer from REMI, Centrifuge from REMI and Analytical balance from Contech were also used.

Chemicals and reagents

Empagliflozin was obtained as a gift sample from Dr. Reddy's Laboratories, Hyderabad. Analytical grade dichloromethane and ethanol was obtained from SD fine chemicals, Mumbai.

Animals

Three male albino wistar rats weighing about 180 grams were procured from the CPCSEA certified vendor and subjected to quarantine before using it for the study. The animals were maintained at standard laboratory conditions and study was conducted as per the guidelines laid by the CPCSEA. The protocol was approved by Institutional

Animals Ethics Committee (IAEC) of RBVRR Women's College of Pharmacy.

Selection of wavelength

10mg of empagliflozin was accurately weighed and transferred into 10 ml of volumetric flask and the volume was made up to the mark with ethanol as diluent. Then from this 0.1 ml was pipetted out and transferred into another 10 ml volumetric flask and the volume was made up to the mark with water to give 10 µg/ml solution and this was scanned between 200 to 400 nm.

Preparation of Standard Solutions

100mg of empagliflozin drug was accurately weighed and transferred into 10ml of volumetric flask and the volume was made up to the mark with ethanol to give 10mg/ml solution. From this further dilutions were done with water to prepare working standards.

Preparation of Calibration standards and Quality control samples

The calibration standards of empagliflozin blank in plasma was prepared by spiking 0.9 ml blank of plasma samples with 0.1 ml of the previously prepared working standard solutions to achieve final concentrations of 1 – 30 µg/ml .

Appropriate volumes from stock solution of Empagliflozin were added to rat plasma to get low and high quality control samples and stored at −20°C. The quality control samples were taken out from storage for analysis to determine intra- and inter-day precision and accuracy.

Collection and Separation of Plasma from Blood

The blood was collected from the rats using retro-orbital puncture method in an EDTA tube and centrifuged at 3000rpm for 20 min to get plasma.

Spiking Procedure for Blank Rat Plasma

0.9ml of blank rat plasma was taken in a centrifuge tube and 0.1 ml of Standard empagliflozin solution was added.

Extraction Procedure for Spiked Rat Plasma

The above mixtures were shaken for 5 minutes and then vortexed for 5 minutes to allow complete mixing of plasma with drug. 5ml dichloromethane was added to the above mixture and vortexed for 15 minutes at 50 rpm and centrifuged for 10 minutes at 4000g.The upper organic layer was transferred into separate test tube, then the lower aqueous layer was re-extracted with another 5ml dichloromethane. The two organic layer were added and allowed to air drying a china dish. Little quantity of ethanol is added to dissolve it and then this is transferred into separate 10ml volumetric flask and the volume is made upto the mark

with ethanol. Then 1ml from the above solution is transferred into another separate 10ml volumetric flask and the volume is made upto the mark with water. The final preparation is scanned between 200-400nm to fix Absorption maxima.

Pharmacokinetic Study

Before administration of drug to the rats were fasted for 12 hours. 20 mg of pure empagliflozin was accurately weighed and dissolved in 0.5ml of water and it was administered to the rat orally. Blood was collected from the rats using retro orbital puncture method in a EDTA tube at 0.5 hour, 1 hour, 1.5 hour,2 hour,4 hour,6 hour and 8 hour of administration. The plasma obtained from the 3 rats collected at different time points is taken individually in different centrifuge tubes and empagliflozin extracted separately using dicloromethane. The final preparations absorbance is measured at 223nm and the absorbance was converted to concentration by using the regression equation obtained from the linearity calibration plot. As per the single compartmental pharmacokinetic model, different pharmacokinetic parameters like peak serum concentration (*C*Max), time to reach peak concentration (*T*Max), area under the curve (AUC0-t and AUC0-∞), half life (*t*1/2), elimination rate constant (K), and volume distribution (Vd) were calculated using pK solver software.

RESULTS AND DISCUSSION

Method Development

Empagliflozin solubility was analysed in different solvents like ethanol, methanol and water. As empagliflozin was found to be soluble and stable at room temperature using ethanol and water, for further work the drug was solubilised in ethanol and water. Extraction of empagliflozin was maximum with dicloromethane compared to other LLE solvents (Chloroform, Diethyl ether) tried for the extraction. The absorption maxima of empagliflozin was found to be 223 nm **(Figure 2)**.

The objective of the International Conference on Harmonization **[8]** for validation of an analytical procedure is to demonstrate that the developed method should be suitable for its intended purpose. The developed method exhibited an excellent performance in terms of sensitivity, speed and simplicity.

Linearity

Calibration curve was constructed from spiked rat plasma with known amount of Empagliflozin to obtain 1, 5, 10, 15, 20, 25 and 30 µg/ml. Empagliflozin was linear in the range of 1-30 µg/ml in rat plasma **(Figure 3)**. The regression equation for this plot was found to be y=0.0583x+0.0564 and R^2 value

was found to be 0.9986. Methanol and water were the solvents used here and the linearity range attained using these solvents were broad compared to the reported methods.

Precision

Spiked Plasma sample of empagliflozin at a concentration of 10µg/ml was used for precision study. This procedure is repeated 6 times and absorbance of all was measured at 223 nm to determine intraday precision. 10µg/ml Spiked Plasma sample of empagliflozin was analyzed on five different days for interday precision determination **(Table 1)**.

Intra-day and interday precision study were performed and CV was found to be 0.2835 and 0.4588 respectively.

Accuracy

Known concentration of empagliflozin was (10µg/ml) was prepared in plasma and was spiked with lower, medium and high concentrations of standards to give 20, 25, 30 µg/ml solutions. Accuracy study was performed and % mean recovery was found to 99.46 to 99.78% **(Table 2)**.

Percentage recovery of the analyte was relatively high making the method more accurate. The precision and accuracy were almost equal to reported methods using chromatographic techniques **[10]**.

Limit of Detection

The and LOD of the standard drug in the developed methods were determined by using standard deviation of the response and slope approach as defined in International Conference on Harmonization (ICH) guidelines. LOD value was calculated using the relation,

$$\mathbf{LOD = 3.3*\sigma / S}$$

where σ = the standard deviation of the response S = the slope of the calibration curve

Limit of Quantification

The and LOQ of the standard drug in the developed methods were determined (Table 3) by using standard deviation of the response and slope approach as defined in International Conference on Harmonization (ICH) guidelines. LOQ value was calculated using the relation,

$$\mathbf{LOQ = 10*\sigma / S}$$

where σ = the standard deviation of the response S = the slope of the calibration curve.

The reported LOD and LOQ were relatively high compared to this method **[9]**. This proves the relative sensitivity of this method.

Ruggedness

Ruggedness is the ability to reproduce an analytical method in different laboratories or in different circumstances without the occurrence of unexpected differences in the obtained results. Ruggedness study was

performed and its % RSD was found to be 0.0523 **(Table 4)**.

Stability

Stability of an analyte in the sample matrix is dependent on storage conditions and duration, chemical properties of an analyte and the matrix. Stability of empagliflozin in sample matrix was determined in triplicate, by assay of QC samples at 2 levels. In bench-top stability study, 3 aliquots of Low Quality Control sample and High Quality Contro samples were prepared from spiked rat plasma and kept at room temperatures for 6.5 hours and its absorbance was measured at 223nm. % obtained and % degraded was calculated.3 aliquots of LQC and HQC samples were frozen for 12 hours and thawed at room temperature ,after it is completely thawed ,it is frozen again for 12 hours and thawed again at room temperature and this freeze -thaw cycle is repeated 2 more times and the sample absorbance is measured 223nm at the third cycle. Standard stock solution was kept in room temperature for 6 hours and after which LQC and HQC samples were prepared their absorbance was measured and compared with the freshly prepared solutions and % obtained and % degraded was calculated **(Table 5)**.

Stability studies were performed and the % degraded was found to be between 1.45% to 11.50 %.

Pharmacokinetic Study

The developed bioanalytical method was applied to rat plasma after a single oral dose of empagliflozin. The plasma concentrations of empagliflozin Vs Time graph was obtained **(Figure 4)** from PK Solver software and various pharmacokinetic parameters for computed by PK solver by assuming one compartmental modelling **(Table 6)**. The pharmacokinetic parameters estimated using this method was also in line with previous findings **[11]**.

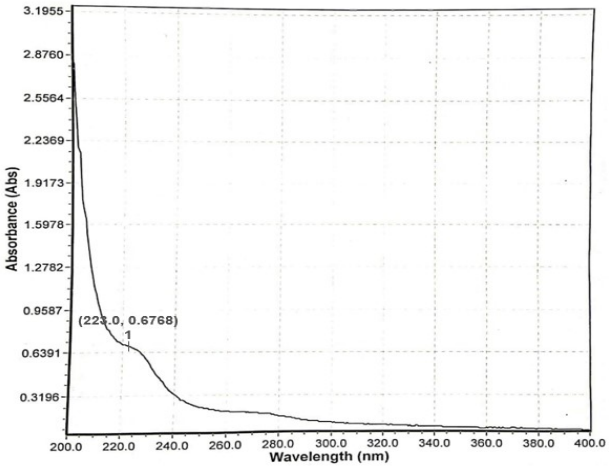

Figure 2: UV spectrum of extracted empagliflozin (spiked)

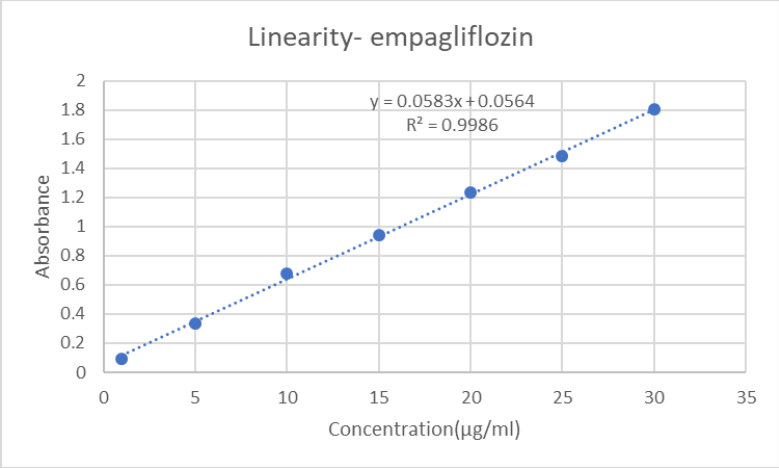

Figure 3: Calibration plot of empagliflozin

Table 1: Intraday precision and Inter day precision

Intraday Precision		Interday Precision	
Sample no.	**Absorbance**	**Day**	**Absorbance**
1	**0.6768**	**Day 1**	**0.6768**
2	**0.6719**	**Day 2**	**0.6730**
3	**0.6745**	**Day 3**	**0.6718**
4	**0.6731**	**Day 4**	**0.6699**
5	**0.6729**	**Day 5**	**0.6689**
6	**0.6760**		
Mean	**0.6742**	**Mean**	**0.67208**
SD	**0.001912**	**SD**	**0.003084**
	0.2835	**CV**	**0.4588**

Table 2: Accuracy study

S. No.	Level	Concentration in µg/ml		Mean % recovery	% RSD
		Spiked	Sample		
1.	**50%**	**5**	**15**	**0.13**	**99.59**
2.	**100%**	**10**	**15**	**0.39**	**99.46**
3.	**150%**	**15**	**15**	**0.56**	**99.78**

Table 3: LOD and LOQ value for empagliflozin

Name of the drug	LOD (ppm)	LOQ (ppm)
Empagliflozin	**0.10**	**0.33**

Table 4: Ruggedness

Analyst	Absorbance	Analyst	Absorbance
Analyst 1	**0.6768**	**Analyst 2**	**0.6758**
	0.6757		**0.6766**
	0.6745		**0.6760**
Mean	**0.6756**	**Mean**	**0.6761**
Mean of mean	**0.67585**		
SD	**0.0003535**		
%RSD	**0.0523**		

Table 5: Stability study

	Bench top stability		Freeze-thaw stability		Stock solution stability	
Quality control sample	% Obtained	% degraded	% Obtained	% degraded	% Obtained	% degraded
LQC	92.09	7.91	88.63	11.37	96.53	3.47
	89.61	10.39	88.52	11.48	96.10	3.90
	91.23	8.77	88.20	11.80	95.88	4.12
Mean ±SD	90.97±1.25	9.02±1.05	88.45± 0.22	11.55±0.22	96.17±0.33	3.83±0.33
HQC	95.25	4.75	91.83	8.17	98.55	1.45
	95.13	4.87	91.85	8.15	98.46	1.54
	95.23	4.77	91.94	8.06	98.30	1.70
Mean ±SD	95.20±0.06	4.90± 0.06	91.87±0.05	8.13±0.06	98.43±0.12	1.60± 0.13

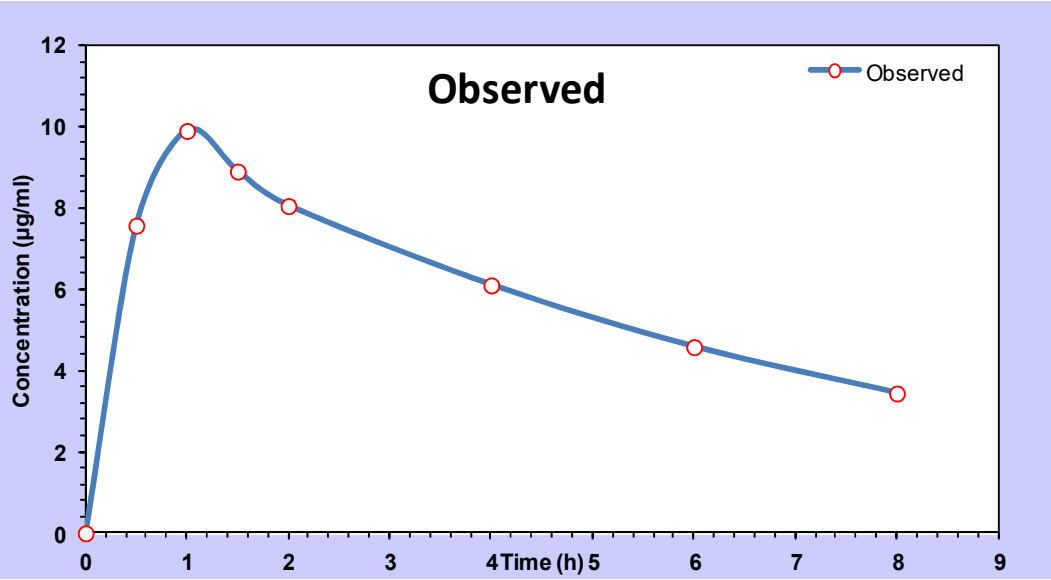

Figure 4: Time vs plasma concentration curve

Table 6: Pharmacokinetic parameters obtained through the software

Pharmacokinetic Parameters	Value
$t_{1/2}k_a$ (h)	0.247
$t_{1/2}k_{10}$ (h)	4.421
V/F (mg)/(µg/ml)	1.827
CL/F (mg)/(µg/ml)/h	0.286
T_{max} (H)	1.09
C_{max} (µg/ml)	9.23
AUC $_{0-t}$ (µg/ml*h)	48.73
AUC $_{0-inf}$ (µg/ml*h)	69.84
AUMC (µg/ml*h^2)	470.4
MRT (h)	6.74

CONCLUSION

In conclusion a rapid, simple, accurate, precise and sensitive validated UV method has been developed for estimation of Empagliflozin in rat plasma. The developed method can be used for pharmacokinetic studies of the empagliflogin

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