Rapid quantitative estimation of metformin and ertugliflozin in rat plasma by liquid chromatography-tandam mass spectroscopy and its application to pharmacokinetic studies

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Background
The development of sound bioanalytical liquid chromatography-mass spectroscopy (LC-MS) method(s) is of paramount importance during the process of drug discovery and development, eventually culminating in marketing approval. The use of oral antidiabetic agents has been increased significantly from past decades, and till now, no bioanalytical method is available for quantitation of metformin (MET) and ertugliflozin (ERT) in the biological matrix that can be applied in bioequivalence studies using LC-MS/MS.
Objective
To study the use of highly responsive simple liquid–liquid extraction method development using deuterated MET and deuterated ERT, LC-MS/MS method for gradation of MET and ERT in the rat plasma.
Materials and methods
The chromatographic condition involves isocratic mode using Waters XBridge C_{18} 3.5 µ (150×4.6 mm) column. Mobile phase was 0.1% orthophosphoric acid and acetonitrile in the ratio of 80 : 20 v/v. Detection was carried out on a triple quadrapole MS employing electrospray ionization technique, operating multiple reactions, monitoring with the transitions of m/z 258.2→174.1, m/z 250.1→210.2, m/z 258.2→174.1, and m/z 260.3→210.2 for MET, ERT, deuterated MET, and deuterated ERT, respectively, in the positive ion mode.
Results and conclusion
The method has been validated, and the linearity was observed in the range of 10–150 ng/ml and 0.1–1.5 ng/ml for MET and ERT, respectively. For intraday and interday %RSD, the values were found to be within the acceptable limits. Recovery studies for MET and ERT obtained, mean recovery of 99.5 and 98.6%, respectively. A battery of stability studies like bench-top stability, autosampler stability, freeze-thaw stability, and long-term stability were performed. Highly responsive simple LC-tandem MS assay method was developed and witnessed for the gradation of MET and ERT in the rat plasma; the developed method was applied to pharmacokinetic studies.

Keywords:
ertugliflozin, liquid chromatography-mass spectroscopy, metformin, method validation, pharmacokinetic study

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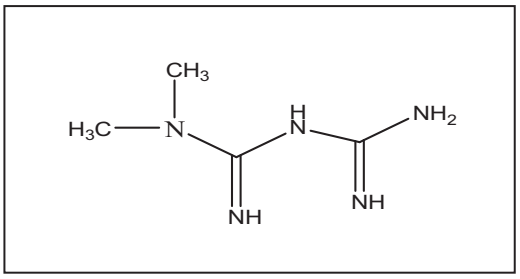
Introduction

An oral antidiabetic drug used for the treatment of type 2 diabetes is metformin (MET), and chemically, it is 3-(diaminomethylidene)-1, 1-dimethylaniline (Fig. 1). MET is an oral antihyperglycemic agent of the biguanide class and used for the treatment of type 2 diabetes. MET is the first drug of choice for the treatment of type 2 diabetes. So MET is considered as an antihyperglycemic agent because it lowers blood glucose concentration in type 2 diabetes without causing hypoglycemia. Control of high blood sugar levels helps to prevent kidney damage, nerve problems, blindness, loss of limbs, and sexual problems. MET helps restore body's proper response to the insulin as well as helps in the natural production of insulin. It also decreases the amount of sugar level made by the liver and that absorbed by the stomach and intestines [1–6].

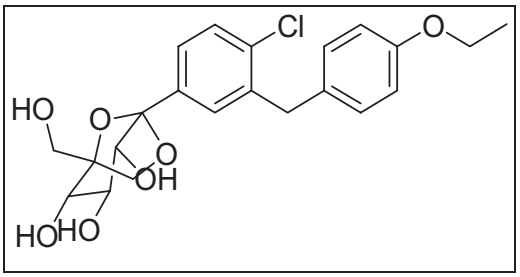
Ertugliflozin (ERT) is in a class of medication called sodium-glucose cotransporter 2 inhibitors, which belongs to gliflozin class and is used for the treatment of type 2 diabetes. It lowers blood sugar level by causing the kidneys to get rid of more glucose in the urine. Chemically, ERT is (1S,2S,3S,4R,5S)5-(4-chlorp-3-(4-ethoxybenzyl)phenyl)-1-(hydroxymethyl)-6,8-dioxabicyclo octane-2,3,4-triol, with (2S)-5oxopyrrolidine-2-carboxylic acid (Fig. 2). In the United states, it was approved by the FDA for use as monotherapy and as affixed dose combination with either sitagliptin or MET [7–10].

Figure 1



Chemical structures of metformin.

Figure 2



Chemical structures of ertugliflozin.

A strategy is discussed for the validation of chromatographic methods that are developed to quantify drugs in biological matrices [11]. According to the literature survey, several liquid chromatography (LC)-tandem mass spectroscopic (MS) methods have been reported for the determination of MET [12–29] and ERT [30] individually and in combination with other drugs in biological matrices. No methods have been reported for the estimation of MET and ERT in biological matrices by LC-MS/MS. In this work, an attempt has been made to develop a simple, rapid method for the simultaneous determination of MET and ERT in rat plasma by LC-MS/MS and application to the pharmacokinetic studies.

Materials and methods

Chemicals and reagents

Acetonitrile [high-performance liquid chromatography (HPLC) grade], orthophosphoric acid (OPA) (analytical grade), and water (HPLC grade) were purchased from Merck (India) Ltd (Worli and Mumbai, Maharashtra, India). All API's of MET and ERT as reference standards were procured from spectrum Pharma Research Solution Pvt Ltd (Hyderabad, India). The combination of the formulation was procured from the local market.

The experimental protocols were approved by the institutional Animal Ethics Committee (IAEC) constituted under Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) (Regd. No: 1736/PO/Re/S/14/CPCSEA).

Equipment

HPLC system (waters alliance E2695 model) with MS QTRAP 5500 triple quadruple instrument was used. Data processing was performed with Empower 2.0 software (Waters coperation, 34 maple street, Milford, MA, USA).

Chromatographic conditions

Chromatographic separation was carried out in an isocratic mode at room temperature using Waters XBridge C_{18} (150×4.6 mm, 3.5 µ) column. The mixture of 0.1% OPA and acetonitrile 80 : 20 v/v was used as mobile phase, and the flow rate was maintained at 1.0 ml/min. The injection volume was 10 µl, and eluents were monitored at 258 m/z using PDA detector. The run time was 5 min.

Preparation of standard and quality control samples

MET and ERT stock solutions were prepared for the calibration curve and quality control samples for validating the method and for patient sample analysis. MET and ERT stock solutions were prepared to obtain the concentrations of 1000 and 10 ng/ml, respectively. From the stock solutions, working standard and primary dilutions were prepared with diluent. Screening of blank rat plasma was carried out before spiking so that it was free from any endogenous interference at the retention time of MET and ERT. By spiking the blank plasma with appropriate amount of MET, seven-point standard curve and four quality control samples were prepared. Sample calibration was done at concentrations of 10, 25, 50, 75, 100, 125, and 150 ng/ml of MET and 0.1, 0.25, 0.5, 0.75, 1, 1.25, and 1.5 ng/ml of ERT.

Sample preparation

For the sample preparation, 200 µl of plasma sample, 300 µl of acetonitrile, 500 µl of internal standard, 500 µl of standard stock, and 500 µl of diluent to precipitate all the proteins were used and mixed in the vortex cyclo mixer. Centrifugation was done at 500 rpm for 30 min. Collection of the supernatant solution in the HPLC vial was done, followed by injection it into the chromatogram.

Method validation [31–34]

Selectivity

Analysis of six different rat plasma selectivity was performed for testing the interference of analytes at the retention times (Figs 3 and 4).

Matrix effect

Matrix effect for MET and ERT was evaluated by comparing the peak area ratio in the postextracted plasma sample from six different drugs' free blank plasma samples and neat reconstitution samples. This experiment is performed in low-quality control (LQC), medium-quality control (MQC), and high-quality control (HQC) levels in every three different preparations from the marketed formulation with six different lots of rat's plasma. Finally, the recovery is within the acceptable limit (%CV) of less than or equal to 15%.

Precision and accuracy

It was done in six different quality control samples (n=6) from marketed formulation at a lower limit of quantification (LLOQ), LQC, MQC, HQC levels are extracted to plasma. The %CV should be less than 15% for accuracy at LQC, MQC, and HQC, except LLOQ, which should be within 20%.

Recovery

The extraction efficiencies of MET and ERT were determined by analysis of six replicates at each quality control concentration. The percentage recovery was evaluated by comparing the peak area of extracted standards to the peak areas of unextracted standards.

Stability

The stability of the samples is comparing the area response of area response versus sample prepared from the freshly prepared sample solution. Stability studies were performed at the LQC and HQC concentration levels using six replicates at each level in plasma. In bench-top method, the stability of spiked rat plasma samples stored at room temperature (bench-top stability) was evaluated for 24 h. Autosampler stability sample are spiked with rat plasma at LQC, QC, and HQC, and they are stored at 2–8°C in an autosampler (autosampler stability) and were evaluated for 24 h. The autosampler stability was determined by comparing the extract plasma samples that were injected immediately, with samples that were reinjected after storing in the autosampler at 2–8°C for 24 h. The reproducibility was determined by comparing the extracted plasma samples that were injected immediately, with the samples that were reinjected after storing autosampler at 2–8°C for 24 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at 30°C and thawed three times with freshly spiked quality control sample. Six aliquots at each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. Moreover, the studied drug showed stability in rat plasma when storing the sample at 200°C for 1 month as long-term stability when compared with the freshly prepared sample as per US FDA guidelines; all stability condition samples were stable below 15%.

Results and discussion

In this method, electrospray ionization having maximum response over atmospheric pressure chemical ionization mode has been selected. Method optimization of instrument was done to give sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at both polarities at a flow rate of 10 µl/min. MET and ETR gave more positive response in ion mode when compared with negative ion mode.

Trails have been performed to obtain the best chromatographic conditions with different columns such as C_{18}, C_8, and CN-propyl, and mobile phases which are composed of 0.1% OPA and acetonitrile were tested. Best chromatographic separation was occurred on XBridge C_{18} column by using the mobile phase 0.1% OPA and acetonitrile in 80 : 20 ratios at a flow rate 1 ml/min, and detection was carried out at 258 m/z by PDA.

Selectivity and sensitivity

Blank plasma spiked with lower limit quantification was obtained as a representative chromatogram. Between six different lots of rat plasma, the percentage mean interference observed at the retention time of analytes, which included hemolyzed and lipedemic plasma containing K_2EDTA as an anticoagulant, was 0.00 and 0.00% for MET and ERT, respectively, and was within the acceptance levels. At LLOQ level from the six replicates of extracted samples, one of the plasma samples having the least interference at the retention time of MET and ERT has been prepared and analyzed. The six replicates %CV area ratios of samples were observed as 1.1% for MET and 1.5% ERT, respectively.

Matrix effect

At MQC level, the percentage of coefficient of variation of ion suppression/enhancement in the signal was found to be 1.0% for MET and ERT, indicating that the matrix effect on the ionization of analyte is within acceptable range under these conditions.

Linearity

The peak area ratios of calibration standards in each assay over the nominal concentration range of 10–150 and0.1–1.5 ng/ml for MET and ERT were observed,

Table 1 Linearity data for metformin and ertugliflozin

Linearity	Metformin		Ertugliflozin	
	Concentration (ng/ml)	Peak response	Concentration (ng/ml)	Peak response
1	10	0.68	0.01	0.30
2	25	2.02	0.1	0.84
3	50	4.05	0.25	1.51
4	75	6.03	0.5	2.35
5	100	8.05	0.75	3.05
6	125	10.08	1	3.86
7	150	14.12	1.25	4.93
Slope	1.147211		3.821560	
Intercept	0.51909		0.611222	
Correlation coefficient	0.9999		0.9997	

Table 2 Within-run and between-run precision and accuracy for metformin

Nominal concentration (ng/ml)	Within-run			Between-run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
5	5.2452	0.53	99.7	5.1268	0.75	101.4
50	50.2369	0.67	97.6	50.2487	0.64	99.5
100	100.2635	0.72	100.2	100.2514	0.81	98.7
150	150.1578	1.69	98.2	150.1036	0.39	100.5

Table 3 Within-run and between-run precision and accuracy ertugliflozin

Nominal concentration (ng/ml)	Within-run			Between-run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
0.05	0.0568	0.95	100.5	0.05263	0.87	101.6
0.5	0.5247	0.65	100.7	0.5295	0.79	100.8
1	1.1658	0.52	99.8	1.1528	0.52	99.4
1.5	1.5263	0.46	99.5	1.5189	0.49	97.4

respectively (Table 1). Linearity of calibration was described well by least square regression lines (Figs 5, 6 and Table 1); the correlation coefficient was more than or equal to 0.9999 for MET and ERT, respectively.

Precision and accuracy

Polling of all individual assay results of replicate of five separate batch runs has been analyzed on four different days for inter-run precision and accuracy determination. The inter-run precision (%CV) was less than 5% and inter-run accuracy was between 95 and 105% for MET and ERT, respectively (Tables 2 and 3).

Recovery

Low-quality, medium-quality, and high-quality concentration levels of MET and ERT of six aqueous (sample spiked reconstitution-solution) were prepared for recovery for determination; the area obtained for extracted samples was analyzed with the same batch run on the same day. For MET and ERT, mean recovery was 99.79% and precision was 0.52%, which indicates the extraction efficiency for MET and ERT.

Reinjection reproducibility

Reproducibility of the samples was checked by performing back calculated concentration for reinjected samples and change was less than 2.0% at LQC and HQC concentrations. Sample was prepared to be reinjected after 24 h, and they also showed percentage changes less than 2.0% at LQC and HQC concentration levels.

Stabilities

MET and ERT stock solution stability was performed by preparing in stock solutions with diluents and storing at 2–8°C in a refrigerator. Aforementioned stock solutions were compared with the stock solutions prepared 24 h before. For MET and ERT, the percentage change observed was 1.27 and 0.75%, respectively, which indicates the stock solutions were stable for at least 24 h. Bench-top and autosampler stability for MET and ERT was investigated at LQC and HQC levels.

Stability of MET and ERT was not affected and was confirmed by repeated freezing and thawing of spiked

Table 4 Stability study of the metformin

Stability experiments	Spiked plasma concentration (*n*=6, ng/ml)	Concentration measured (*n*=6, ng/ml)	%CV (*n*=6)
Bench-top stability			
LQC	50	50.2364	0.87
HQC	150	150.1254	1.24
Autosampler stability			
LQC	50	50.2687	0.91
HQC	150	150.1269	1.35
Long-term stability			
LQC	50	50.1598	0.95
HQC	150	150.4258	1.40
Freeze-thaw stability			
LQC	50	50.1257	0.88
HQC	150	150.3574	1.45

HQC, high-quality control; LQC, low-quality control.

Table 5 Stability study of ertugliflozin

Stability experiments	Spiked plasma concentration (*n*=6, ng/ml)	Concentration measured (*n*=6, ng/ml)	%CV (*n*=6)
Bench-top stability			
LQC	0.5	0.5124	0.95
HQC	1.5	1.5236	1.69
Autosampler stability			
LQC	0.5	0.5241	0.86
HQC	1.5	1.5142	1.51
Long-term stability			
LQC	0.5	0.5269	0.72
HQC	1.5	1.5278	1.52
Freeze-thaw stability			
LQC	0.5	0.5298	0.64
HQC	1.5	1.5364	1.62

HQC, high-quality control; LQC, low-quality control.

Table 6 Mean pharmacokinetic parameters of metformin and ertugliflozin

Pharmacokinetic parameters	Metformin	Ertugliflozin
$AUC_{0\text{-}t(}$ng h/ml)	752	12
C_{max} (ng/ml)	81.2	1.1
$AUC_{0\text{-}\infty}$ (ngh/ml)	814	16
$AUC_{t\text{-}\infty}$ (ngh/ml)	62	4
T_{max} (h)	6	16

plasma sample at LQC and HQC levels; they were stable in plasma in for at least 24 h at room temperature as well in an autosampler at 20°C. In case of long-term stability studies for MET and ERT, they were stable in the matrix for 24 h at a temperature of –30°C (Tables 4 and 5).

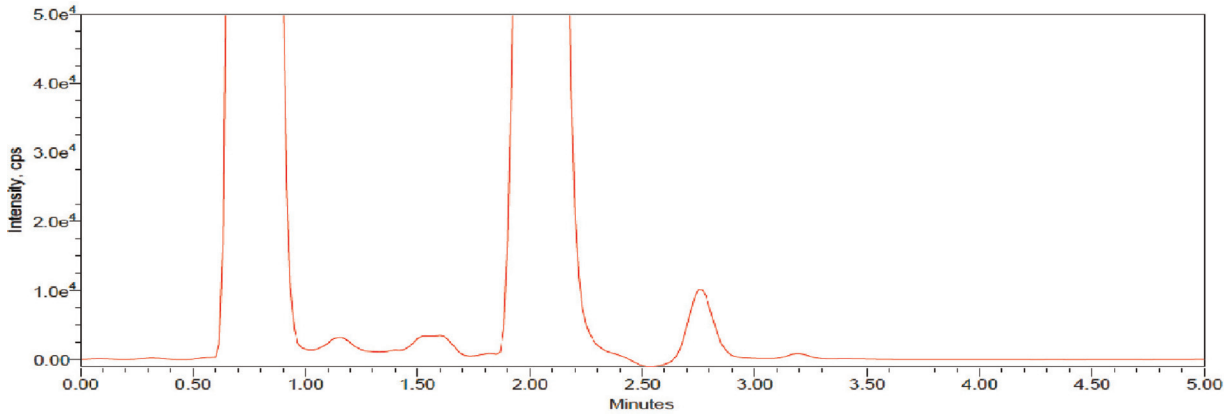
Application to pharmacokinetic study

LC-MS/MS method was developed and has been applied for the analysis of MET and ERT in plasma samples obtained from rats and also applied to the pharmacokinetic studies. MET and ERT were coadministered by oral gavage at a dose 2.5 and 0.0375 mg/kg, respectively. The test/reference ratios for C_{max}, $AUC_{0\text{-}t}$, and $AUC_{0\text{–}\infty}$ were within 80–125% for all analytes. The 90% confidence interval of C_{max}, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$ for MET and ERT are expressed. The detailed pharmacokinetic parameters (C_{max}, T_{max}, $AUC_{0\text{-}t}$, and $AUC_{0\text{–}\infty}$) of MET and ERT are presented in Table 6.

Conclusion

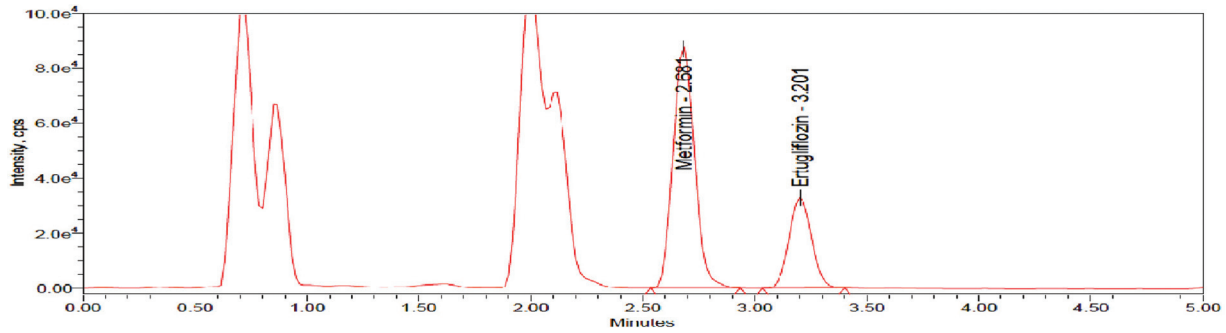
Validated highly sensitive HPLC-ESI-MS/MS has been developed for the determination of MET and ERT simultaneously in rat plasma. A fast, rugged, reproducible simple bioanalytical method has been developed that can be used in pharmacokinetic studies along with the monitoring of the investigated analyte in the body fluids. High recovery with liquid–liquid extraction method and lesser retention time is time saving when compared with other reported methods. The specified method was simple, specific, and rapid and allows for easy application in laboratories; moreover, it is a valuable tool for bioavailability, bioequivalence, and pharmacokinetic studies.

Figure 3



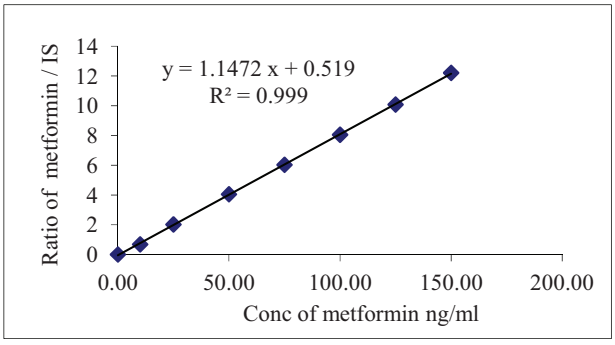
Chromatogram of blank rat plasma.

Figure 4



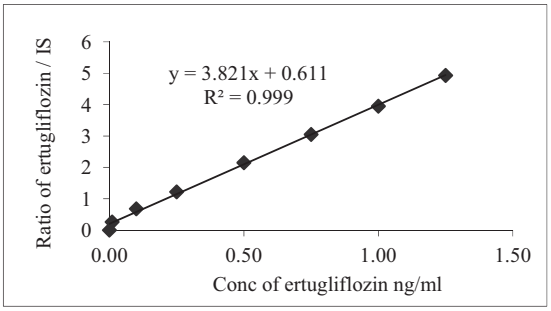
Blank plasma spiked with analyte.

Figure 5



Calibration plot for metformin.

Figure 6



Calibration plot for ertugliflozin.

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Conflicts of interest

There are no conflicts of interest.

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