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RESEARCH ARTICLE

A Bioanalytical Method for Quantification of Telmisartan in rat Plasma; Development, Validation and Application to Pharmacokinetic Study

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ABSTRACT:

The telmisartan was determined in a rat plasma using developed and validated a reversed-phase high performance liquid chromatographic (HPLC). The pre-treatment of the plasma sample involving liquid-liquid extraction using ethanol as the extracting solvent. The HPLC method validation has been shown a linear calibration curve over a plasma concentrations range of 0.7 to 10µg/mL with a correlation coefficient of 0.9979, the limit of detection and the limit of quantification were determined to be 0.025µg/ml and 0.07µg/ml, respectively. The precision and accuracy were in an acceptable limit. The pharmacokinetic parameters of telmisartan were adequately evaluated following a single oral dose (4mg/kg) in Sprague-Dawley rats. The results observed conclude that the developed bioanalytical HPLC method is appropriate and applicable as an analytical tool in the pharmacokinetic study of telmisartan.

KEYWORDS: Telmisartan, HPLC, Validation, Bioanalytical method, Rat plasma.

INTRODUCTION:

The bioanalytical method is employed for the quantitative estimation of drugs and their metabolites in biological media. It plays an important role in the estimation and interpretation of bioequivalence, pharmacokinetic and toxic kinetic studies[1-3]. The sensitivity and selectivity of bioanalytical methods are necessary in order to conduct preclinical and clinical studies successfully during the drug development process from the initial preclinical phase to the final clinical steps[4,5]. The chromatographic method is the most commonly used technology for the bioanalysis of small molecules[6].

Telmisartan (TEL) is chemically described as [1,1-biphenyl]-2-carboxylic acid, 4-[(1,4-dimethyl-2-propyl[2,6-bi-1H-benzimidazol]-1-yl)methyl][7], the chemical structure of TEL is shown in Fig.1[8].

It is an angiotensin II receptor antagonist (ARB) used in the treatment of hypertension. The high affinity of angiotensin II receptor blockers (ARBs) such as telmisartan to the angiotensin II type 1 (AT1) receptors leading to inhibition of the action of angiotensin II on vascular smooth muscle, which in turn result in a reduction in arterial blood pressure[9,10]. The present study describes a simple and reproducible HPLC method using liquid-liquid extraction to detect the plasma concentrations in male Sprague-Dawley rats. The applicability of pharmacokinetic is also demonstrated.

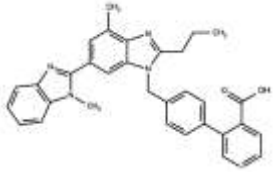

Figure 1: Chemical structure of TEL.

MATERIALS AND METHODS:

Chemicals and reagents:

Telmisartan (API) and pioglitazone were purchased from Hangzhou Hyper Chemicals Limited, China. Potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), absolute ethanol and absolute methanol were procured from Merck KGaA, Germany. Acetonitrile (HPLC grade), water (HPLC grade) was purchased from Fisher chemical, UK.

Instrumentation and Chromatographic Conditions:

HPLC system (HPLC 1100, Agilent) coupled with UV detector, autosampler and column oven, was used to study the concentration of TEL in rat plasma. A reversed-phase HPLC system was used that involves polar mobile phase and non-polar, hydrophobic stationary phase. Samples were separated using ZORBAX Eclipse Plus C18 4.6mm x 25cm column with a pore size of 5µm (Agilent Technologies) as a stationary phase, and the column temperature was ambient. The mobile phase consisted of a 50:50 (%v/v) mixture of acetonitrile (ACN) and 10mM phosphate buffer solution (pH 3.8). For all samples, the flow rate of the mobile phase was 1mL/min with a fixed total run time of 14 minutes and the injection volume of the sample was 10µL. The mobile phase was filtered using 0.45µm membrane filter paper and subjected to degassing in an ultrasonic cleaner before running the HPLC analysis. The detection was done by UV detector with measuring wavelength at λ_{max} (231nm), determined earlier by UV spectrophotometric method. The chromatograms data was obtained by using Lab Solution software (Agilent Corporation).

Preparation of Standard Stock Solutions:

Primary stock solutions of TEL and internal standard (IS) pioglitazone were prepared individually in methanol. An accurately weighed samples of 50mg of TEL and 50mg of pioglitazone were dissolved in 50ml of methanol in two separate 50ml volumetric flasks and sonicated for 5 minutes to produce standard stock solutions of 1000µg/ml.

Preparation of Working Solutions:

The working solutions of different concentrations for TEL were prepared from the stock solution (1000µg/ml) by adding an appropriate volume of mobile phase to a suitable volume of stock solution. They were used for the preparation of the calibration curve (CC) and quality control (QC) samples by diluting the individual solutions. The Working solution for pioglitazone (IS) was also prepared in the same way (Table 1).

Preparation of Calibration Standards and Quality Control Samples:

The 10µL of individual working solutions were spiked to 80µL of the blank rat plasma to prepare calibration curve with the concentrations of 0.07, 0.1, 0.5, 1, 2, 5 and 10 µg/ml for TEL in rat plasma (Table 2). Three different Quality control standards were prepared separately by spiking 10µL TEL solution to 80µL of blank rat plasma to achieve the final concentrations of 1.5 (Low QC), 7.5 (Mid QC), and 15 (High QC)µg/ml of TEL in rat plasma (Table 3). 10µl of internal standard solution (50µg/ml) was added to prepare a final concentration in plasma (5 µg/ml). All the calibration and QC samples were freshly prepared prior to the analysis.

Table 1: working solutions used in bioanalytical method

Type of working solution	Concentration (µg/ml)
Working solutions of TEL for (CC) samples	0.7, 1, 5, 10, 20, 50, and 100 µg/ml
Working solutions of TEL for (QC) samples	15, 75, and 150 µg/ml
A working solution of IS	50 µg/ml

Table 2: Telmisartan concentrations in plasma for standard curve

Stock TEL conc. (µg/ml)	Stock TEL spiked (µL)	Blank plasma + IS (µL)	TEL conc. in plasma (µg/ml)
0.7	10	90	0.07
1	10	90	0.1
5	10	90	0.5
10	10	90	1
20	10	90	2
50	10	90	5
100	10	90	10

Table 3: Telmisartan concentrations in plasma for QC samples

Stock TEL conc. (µg/ml)	Stock TEL spiked (µL)	Blank plasma + IS (µL)	TEL conc. in plasma (µg/ml)
15	10	90	1.5
75	10	90	7.5
150	10	90	15

The sample extraction procedure:

Sample preparation technique used for the study plays a significant role with respect to bioanalytical samples. Sample preparation is applied to remove the matrix of interfering biological compounds. It is essential to reduce the effect of the matrix formed due to biological and buffer components[1]. The liquid-liquid extraction procedure was used to extract telmisartan from plasma using ethanol as the extracting solvent. Samples were prepared by adding 10 µl of each working solution of TEL to 80 µl of blank plasma and vortexed for 1 minute. Then 10 µl of internal standard solution (IS) of pioglitazone (50 µg/ml) equivalent to 5 µg/ml in plasma was added to the plasma and vortexed for 1 minute. Then the sample was extracted with 1.5 ml ethanol. After the addition of extracting solvent, the sample was vortexed for 5 minutes and centrifuged at 3000 rpm for 10 minutes. The organic layer was separated and evaporated at 40 ºC to dryness by using a gentle stream of nitrogen. The residue was reconstituted in 200 µL of the mobile phase and vortexed for 1 minute. Finally, the sample was filtered using a 0.45 µm syringe filter, and 10 µL was injected into the HPLC system.

Bioanalytical method validation:
The developed HPLC conditions were validated as per the ICH guideline for bioanalytical method validation. The developed method was validated for its specificity and selectivity, the Limit of Detection (LOD) and Limit of Quantitation (LOQ), calibration and linearity, precision and accuracy as per the bioanalytical method validation guideline suggested by ICH.

Specificity and Selectivity:
These parameters were conducted on rat plasma after it was prepared for analysis by liquid-liquid extraction. The unspiked sample was run and analysed to determine if there is any interference by peaks coming from blank / non-spiked plasma sample at the retention time of TEL and IS.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):
A calibration curve with five points at low concentration levels for TEL in plasma was drawn by plotting the peak area ratio (TEL: IS) against concentration 0.07, 0.1, 0.5, 1 and 2 µg/ml. The linear regression equation was investigated along with its correlation coefficient for Beer's Law agreement. From the curve, the LOD and LOQ in terms of the concentration of the TEL were estimated by calculation using the following equations.

$$LOD = \frac{3.3SD}{S} \quad \text{(Eq 1)}$$

$$LOQ = \frac{10SD}{S} \quad \text{(Eq 2)}$$

Where SD the standard deviation between area ratios of six replications of low concentration standard and S is the slope of the regression line. The obtained LOD and LOQ values were then prepared in plasma and injected into the system for six replicate measurements. The relative standard deviation (% RSD) for LOD and LOQ were calculated by the following equation.

$$\% RSD = \frac{SD}{M} \times 100\% \quad \text{(Eq 3)}$$

Where SD is the standard deviation and M is the mean value of six replicate measurements. The acceptance criteria for LOD and LOQ is % RSD less than 33% and 10%, respectively[11].

Calibration and Linearity:
The seven points of calibration curve were constructed by plotting peak area ratio of TEL to the internal standard pioglitazone versus TEL concentration in plasma. Linearity was tested for TEL in the plasma concentrations of 0.07, 0.1, 0.5, 1, 2, 5, and 10µg/ml. The linearity of the method was determined by the estimation of the regression coefficient (R^2) value. The desirable value of R^2 should not be less than 0.999[12].

Precision and Accuracy:
Precision and accuracy were determined by analyzing three replicates at three different concentration levels. The three quality control samples were LQC, MQC and HQC. The low QC (LQC) was 1.5µg/ml, mid QC (MQC) was 7.5µg/ml, and the high QC (HQC) was 15 µg/ml. The samples for all quality control levels were prepared by the same method used for preparing the samples for linearity. All concentrations were spiked into plasma with internal standard and subjected to all the steps mentioned above of sample preparation. The concentration of quality control samples was calculated by using the linearity equation. The precision was expressed as % RSD between the accuracy values at each QC concentration levels. Accuracy was calculated using the following equation.

$$\text{Accuracy (\% recovery)} = \frac{\text{measured concentration}}{\text{added concentration}} \times 100\% \quad \text{(Eq 4)}$$

Pharmacokinetic study:
The applicability of the developed HPLC method for TEL in rat plasma was demonstrated by the results obtained from pharmacokinetic studies conducted on three male Sprague-Dawley rats weighing 280 ± 20g and aged 9-10 weeks were purchased from Sapphire Enterprise, Selangor, Malaysia. Before the experiment, the rats had free access to normal food and water. The three rats were housed in one cage and acclimatized with laboratory environment for one week on a standard 12 hours light/dark cycle at a room temperature of 25 ± 3ºC and relative humidity of 50 ± 10%. The rats were kept in fasting condition overnight (12 h) before the experiment was carried out, but the water access was allowed. The dose of telmisartan was 4mg/kg, which was administered through gastric gavage. Blood samples were collected at different time points after dosing. The study protocol was approved by Institutional Animal Care and Use Committee (IACUC-IIUM) in the meeting No. 2/2017 on the 28 November 2017. The rats were anesthetized by pentobarbital intraperitoneal injection (50mg/kg, in volume 0.83ml/kg). 0.5ml of blood samples were collected from the retro-orbital vein of rat in EDTA tubes as an anticoagulant at 0.5, 1, 2, 4, 6 hours after dosing. They are mixed and immediately centrifuged at 3000rpm for 10 minutes. The plasma was separated and stored at -80ºC until drug analysis was carried out using HPLC method. Pharmacokinetic parameters of TEL were calculated using non-compartmental methods. The maximum concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were obtained directly by visual inspection of the data from the plasma concentration versus time plot. Areas under the plasma-concentration-time curve from time zero to the last measurable TEL sample time and to infinity (AUC_{0-t} and

$AUC_{0\text{-}inf}$) were calculated by trapezoid rule. The elimination rate constant (K_{el}) was derived from the slope of the log plasma concentration versus time plot, and elimination half-life ($T_{1/2}$) was calculated by formula $T_{1/2} = 0.693/K_{el}$ (Nair et al., 2014) for the period of 0 to 6 h.

RESULTS AND DISCUSSION:

Bioanalytical Method validation:

HPLC method and extraction of drug from plasma:

The developed HPLC method was very simple, involving an equal volume ratio of buffer and acetonitrile. Total chromatographic run time was 14 min. Plasma extraction method developed was a simple liquid-liquid extraction. TEL analysis in plasma sample was previously reported following liquid-liquid extraction involving dichloromethane as extracting organic solvent[13]. In this research, ethanol was used as an extracting solvent, but the quantity required of ethanol is very small, only 1.5ml per sample.

Selectivity and Specificity:

Specificity and selectivity were studied for the examination of the presence of interfering components[14]. The developed HPLC method was found to be selective and specific as it was able to differentiate and quantify TEL in the presence of other plasma component and the IS. IS was selected as internal standard because of its adequate resolution with TEL, satisfactory peak shapes, stability and consistency in area count during the analysis. During the validation process, no peaks were found at TEL and IS retention times in mobile phase and plasma as illustrated in Fig.1. The chromatogram of the same showed good separation with low background noise. The retention time for IS and TEL were about 7.5 min and 12.0 min, respectively. The total chromatographic run time was 14.0 min.

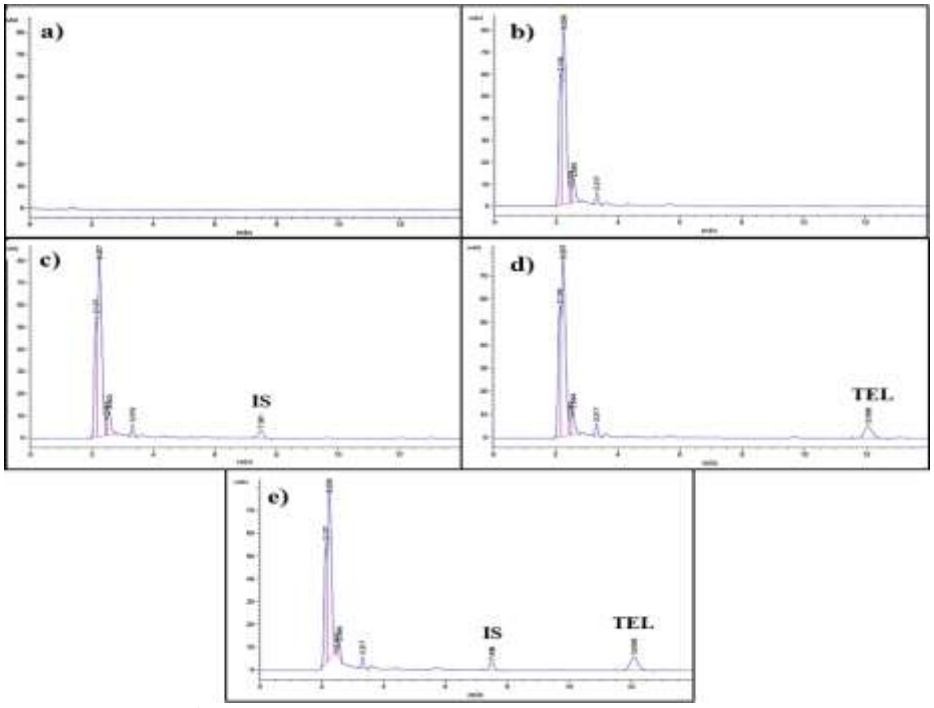

Fig 1. Typical HPLC chromatogram of a) blank mobile phase, b) blank rat plasma, c) pioglitazone (IS) (5µg/ml) spiked in rat plasma, d) telmisartan (5µg/ml) spiked in rat plasma, and e) telmisartan (5µg/ml) spiked in rat plasma with IS.

Linearity and Range:

The linearity of an analytical method is its ability to elicit that test results are proportional to the concentration of analyte in samples within a given range (15). Linearity was plotted over 7-points concentration ranges: 0.07, 0.1, 0.5, 1, 2, 5, and 10µg/ml for TEL in plasma. The Linear calibration curve (Fig. 2) with a correlation coefficient (R^2) 0.9986 was obtained. The R^2 indicated an excellent linear correlation between peak area ratios (TEL to IS) and concentration of telmisartan. The linearity equation was y = 0.5908x + 0.0652.

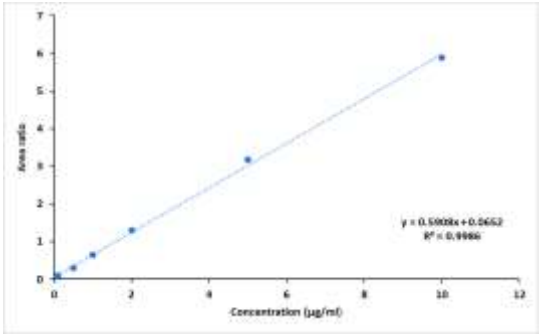

Figure 2: Linear calibration curve by HPLC method for TEL spiked plasma samples

LOD and LOQ:

The limit of detection (LOD) is the smallest concentration of the analyte that gives the measurable response while the limit of quantification (LOQ) is the smallest concentration of the analyte, which gives response that can be accurately quantified[16]. A five-points of low concentrations of telmisartan in plasma ranges 0.07, 0.1, 0.5, 1, and 2µg/ml were plotted in area ratio of telmisartan to IS versus concentration of TEL which shows acceptable linearity in low concentration range (Fig. 3). The minimum detectable concentration of telmisartan (LOD) was found to be 0.025µg/ml, whereas the quantitative limit (LOQ) was 0.07µg/ml. Confirmation tests with LOD and LOQ showed acceptable values of % RSD (5.16% for LOD and 4.2% for LOQ) between area ratios of six concentrations each. Low detectable and quantifiable concentration indicate the sensitivity of the HPLC methods. One of the previously reported bioanalytical methods involving HPLC-UV detection for TEL in plasma sample resulted in LOQ of 1µg/ml[17]. In this research, 0.07µg/ml TEL concentration as LOQ is indicative of higher sensitivity compared to that reported methods. The high sensitive method is suitable enough to quantify low drug concentration in a pharmacokinetic study.

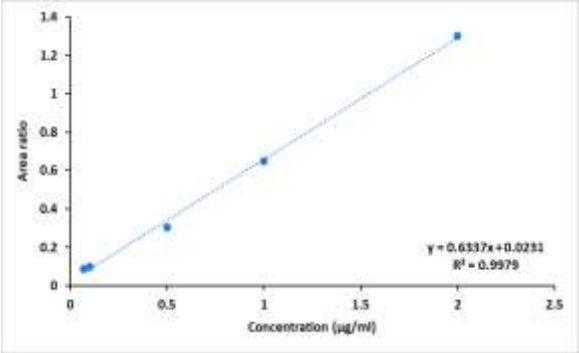

Figure 3: Low concentration calibration curve of TEL in plasma (0.07 – 2 µg/ml)

Precision and Accuracy:

The accuracy of an analytical method describes the closeness of test results to the actual concentration of analytes. In contrast, the precision is a measure of the degree of repeatability and reproducibility of the analytical method[18]. In this research, only intra-day precision was evaluated as part of the partial method validation study. The accuracy and precision of the method were assessed by analysing the three Quality control (QC) samples (1.5 µg/ml LQC, 7.5 µg/ml MQC, and 15 µg/ml HQC). The blank plasma was spiked with these QC samples according to the procedure described in the methodology. Results are shown in Table 4. The accuracy of this bioanalytical method for all QC samples was ranged within 85.17% to 107.97 %. The acceptable limit for accuracy values in case of plasma sample was stated as 85 -115% whereas the acceptable precision value calculated as % RSD should be within ± 15%[19,20]. Therefore, the developed method was satisfactory in terms of accuracy and precision. Apart from the analyst's skill, in bioanalytical methods, accuracy and precision depends mainly on the consistency of plasma extraction, tailing free peak shapes, consistency of the analytical system that is used in the study. The method employed in this research bears a simple and rapid liquid-liquid extraction with excellent recovery, sharp peak shapes and consistent performance of the HPLC system[21]. The mixed standard containing TEL and IS were injected before and after each set of analysis. The area ratios were compared between six consecutive injections, which was within 2% indicating consistent running of the chromatographic system. All of these factors contributed to the acceptable precision and accuracy values. Therefore, the accuracy and precision of the method evaluated at three quality control level met the acceptance criteria. This bioanalytical method was used in the pharmacokinetic studies to quantify the telmisartan in the plasma of rats.

Table 4: Precision and accuracy of the bioanalytical method for telmisartan in rat plasma

QC samples	Added conc.	Measured conc.	Mean conc.	SD	Precision % RSD	Accuracy % recovery
LQC (µg/ml)	1.5	1.466				97.71
	1.5	1.278	1.35	0.10	7.66	85.17
	1.5	1.298				86.55
MQC (µg/ml)	7.5	6.962				92.83
	7.5	6.932	7.33	0.66	9.07	92.43
	7.5	8.098				107.97
HQC (µg/ml)	15	14.019				93.46
	15	13.792	13.63	0.49	3.60	91.94
	15	13.079				87.19

Pharmacokinetic Parameters:

The established HPLC method was sensitive enough to assess the pharmacokinetic parameters following a single-dose of TEL in experimental rats. The mean plasma drug concentration-time profile of TEL is shown in Figure 4. The pharmacokinetic parameters values such as maximum plasma concentration (C_{max}), time required for maximum plasma concentration (T_{max}), area under the curve (plasma concentration) from the initial time to 6 h (AUC_{0-t}), area under the curve (plasma concentration) from time zero to infinity ($AUC_{0-\infty}$), the elimination rate constant (K_{el}), and terminal half-life ($t_{1/2}$) have been summarized in Table 5.

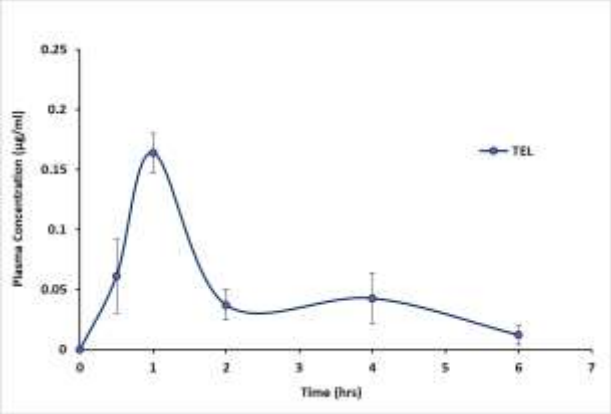

Figure 4. *In- vivo* pharmacokinetic profile of TEL, Error bar represents SD (n=3).

Table 5: Pharmacokinetic parameters of TEL after oral administration of TEL to rats.

Parameters	Mean ± SD (n=3)
C_{max} (µg/ml)	0.164 ± 0.016
T_{max} (h)	1 ± 0.000
AUC_{0-t} (µg h/ml)	0.306 ± 0.080
$AUC_{0-\infty}$ (µg h/ml)	0.333 ± 0.078
K_{el} (h^{-1})	0.448 ± 0.074
$T_{1/2}$ (h)	1.575 ± 0.256

CONCLUSIONS:

The HPLC method developed and validated was simple, rapid, specific, sensitive and reproducible for the quantification the TEL and studying the pharmacokinetic parameters in the rat model.

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