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Development of a new rapid, economical and eco-friendly HPLC method for *in vitro* and *in vivo* determination of zolmitriptan

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Abstract: Multiple high-performance liquid chromatographic (HPLC) approaches have been briefly defined for the assessment of zolmitriptan (ZMT). These methods are either cumbersome or require a plentiful volume of organic solvents, thus offering extortionate procedures. The objective of this study was to establish and validate a new rapid, eco-friendly and cost-effective HPLC method for the analysis of ZMT. The calibration curve for ZMT was established using simulated salivary fluid (SSF) and rat plasma for *in-vitro* and *in-vivo* analysis, respectively. Chromatogram separation was performed using a CST column (250mm × 4.6mm, 5µm) as a stationary phase and maintained at a temperature of 40°C. The methods were authenticated for linearity, system suitability, accuracy, precision, reproducibility, limit of detection (LOD) and limit of quantification (LOQ). The results of the validation variables and stability studies indicated that the methods were established in accordance with the guidelines of ICH and the USFDA. The established technique was time-saving, precise, eco- friendly and economical compared with the reported technique. In addition, the developed method was sufficiently repeatable for *in vitro* and *in vivo* analysis of ZMT.

Keywords: ICH and USFDA guidelines, method development and validation, zolmitriptan, rizatriptan, RP-HPLC.

INTRODUCTION

The antimigraine drug zolmitriptan (ZMT) is a member of tryptamines and chemically recognized as (4S)-4-{[3-(2-dimethylaminoethyl)-1H-indol-5-yl] methyl}-1,3-oxazolidin-2-one (fig. 1). ZMT is a triptan type selective serotonin receptor agonist of 5-hydroxytryptamine (5-HT1B/ 1D) and developed for the management of acute migraine (Vijayakumar *et al*., 2010). It binds selectively with high affinity to human 1B and 1D 5-HT receptor subtypes, resulting constriction of cranial blood vessels. Clinical studies have shown that the therapeutic action of ZMT is due to its inhibitory effect on the peripheral trigeminovascular system and its ability to reach primary sites in the brainstem involved in headache management (Raza *et al*., 2007).

Various analytical approaches for the quantitative analysis of ZMT (in-vitro/in-vivo) have been described previously. These include UV-visible spectrophotometry (Raza *et al*., 2007) , reversed-phase HPLC method (Annapurna and Nanda, 2011), liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Chen *et al*., 2006), Ultra-high performance liquid chromatography-MS/MS (Reddy *et al*., 2013), HPLC with florescence detection method (Chen *et al*., 2004) and proton NMR spectroscopy (Rao *et al*., 2016). The HPLC analytical technique is still widely employed for the analysis of pharmaceutical constituents such as ZMT and published. However, the described methods in the literature suffer from one or more drawback such as a very narrow linearity range, reliable control of the variables under experimental investigation, poor sensitivity, lengthier retention time, higher volume of hazardous organic solvent and expensive machine (Annapurna and Nanda, 2011). In present work, a simple, cost-effective, reproducible and eco-friendly technique for the assessment of ZMT has been described. This method has been validated in accordance with ICH and USFDA guidelines and could be effectively employed for routine quantification (in-vitro/in-vivo) of the selected drug.

MATERIALS AND METHODS

Zolmitriptan test tablets (batch No: 010725) and capsules (batch No: 010806), zolmitriptan reference standard (99.7% purity) and rizatriptan benzoate reference standard (99.3% purity)

Zolmitriptan, purity 99%, bought from Energy Chemical Co., Ltd. Shanghai. Rizatriptan, purity 99% and phosphoric acid were received from Jiangsu Qian sheng Functional Chemical Co., Ltd, China. Trimethylamine (TEA), potassium hydroxide, potassium di-hydrogen phosphate, anhydrous sodium di-hydrogen phosphate and sodium chloride were obtained from Sino pharm group of chemical reagents, China. Gradient grade acetonitrile (ACN) and methanol (MEH) were bought from Fisher

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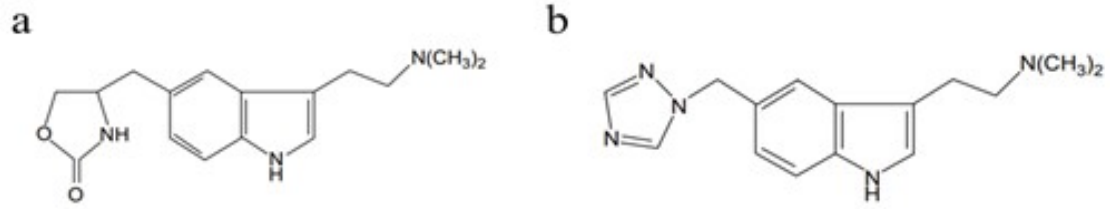

Fig. 1: Chemical structure of (a) zolmitriptan (ZMT) and (b) rizatriptan (RZT)

Scientific Worldwide, Shanghai, China. ZMT film-coated tablets ® (each tablet contained 2.5mg ZMT were purchased from Xunhui Pharmaceutical. Co., Ltd. Sichuan, China. Deionized water (DI) was purified before being used. Other chemicals were used as received.

Instrumentation and chromatographic conditions

ZMT concentrations were determined using a Shimadzu® (SPD-15c) HPLC system equipped with a Shimadzu® UV detector (u-2600). The mobile phase consisted of 0.2% (v/v) TEA in water (attuned to pH 3 using 85% ortho-phosphoric acid**)** and ACN (85:15, v/v). The flow rate was adjusted to 1.5mL.min^{-1} for a total run time of 10min. A CST (4.6 × 250mm, 5µm) column at 40°C was utilized. Detection was performed at a wavelength of 240nm. The injection volume was 20µL and Shimadzu® LCMS solution software was employed for data acquisition and processing.

Preparation of solutions

Simulated salivary fluid (SSF) was prepared by dissolving 0.348gm potassium phosphate dibasic, 0.111.6gm magnesium chloride hexa-hydrate, 0.842.4gm sodium chloride, 0.1911gm calcium chloride dehydrate and 1.2 gm potassium chloride in 1000mL purified water at an adjusted pH of 6.8 (Nalluri *et al*., 2013).

Standardized stock solution equivalent to 300µg.mL^{-1} ZMT was processed by liquefying 30 mg of the pure drug in 100mL SSF at pH 6.8. The solution was further diluted to produce standardized serial solutions of ZMT in the concentration range of 0.25-100µg.mL^{-1}.

Method validation

Specificity

The specificity of the established technique was confirmed by comparing the respective chromatograms of blank SSF samples and samples with a known concentration of 10µg.mL^{-1} ZMT.

Linearity

Working serial dilutions of ZMT ranging from 0.25-100 µg.mL^{-1} were prepared from the standardized stock solutions. 20µL of each dilution was manually injected into the HPLC machine and the corresponding chromatograms were detected. The mean peak areas of each concentration were calculated and a plot of the concentrations versus the peak areas was constructed.

Sensitivity

Sensitivity was evaluated in terms of limit of quantification (LOQ) and limit of detection (LOD) in a concentration range of 1-15ng.mL^{-1}. The LOD was determined to be the lowermost concentration giving a signal-to-noise ratio of three for ZMT. The LOQ was determined based on the criterion that the specimen response at LOQ must be ten folds of the baseline noise.

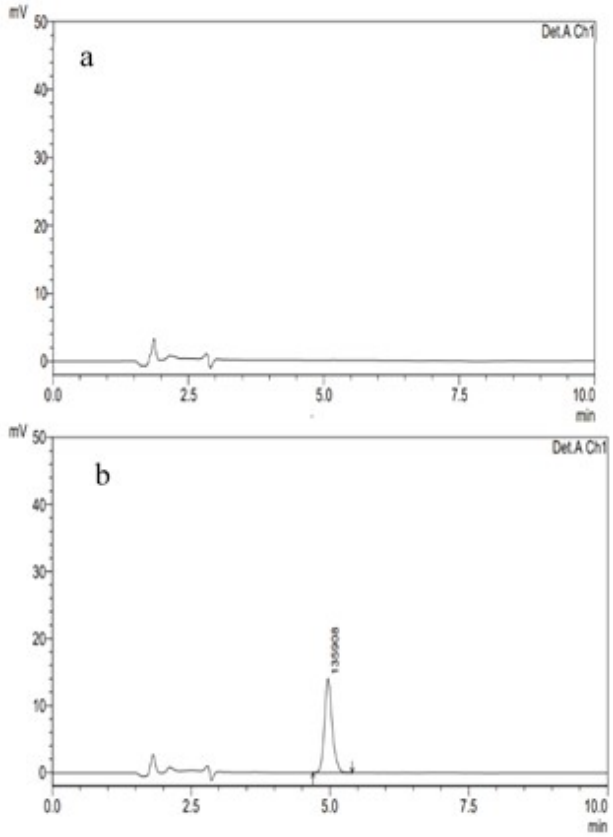

Fig. 2: Typical chromatograms of blank simulated salivary fluid (a), and 10 µg·mL^{-1} standard solution in simulated salivary fluids (b).

Accuracy and precision

Authentic samples were analyzed at three concentration levels of ZMT (10, 40 and 80µg.mL^{-1}) to evaluate accuracy and precision. The recovery (%) and relative standard deviation (RSD, %) were calculated from the amount of ZMT added and detected to determine the accuracy.

Precision studies were performed on the same day to determine intra-day variability and on three consecutive days to measure inter-day precision.

System suitability

To test the suitability of the system, a solution of known concentration (40µg.mL^{-1}) was introduced to HPLC six times in succession and values for area and RSD (%) were measured.

Stability

The stability study was conducted at low, intermediate and high levels (10, 40 and 80µg.mL^{-1}) of ZMT. For each concentration and storage condition, five replicates were analyzed in one analytical series. The stability of the samples on the laboratory bench was tested by keeping them at room temperature (RT) for 6h to determine bench-top stability. Freeze-thaw stability was investigated by freezing the samples at -20°C and then thawing them to RT (25ºC). For auto-sampler stability, the prepared samples were kept at 4°C temperature for 12h. After 3 freeze-thaw cycles, the samples were examined. The prepared samples were stored at -20°C for a period of 72 h to determine the long-term stability of ZMT in SSF.

Bioanalytical method development and validation

Chromatographic conditions

The concentration of ZMT in plasma was determined using an HPLC system equipped with a ultraviolet detector at a fixed wavelength of 254 nm. Separation was carried out using the CST (250mm × 4.6mm, 5µm) column maintained at 40°C. ACN and 0.2% TEA in water at a volumetric proportion of 10:90 v/v with adjusted pH of 3 were employed as the mobile phase. The fluid flow rate was held at 1.0 mL.min^{-1} and 20µL was injected into HPLC instrument (Kumar *et al.,* 2022).

Preparation of stock standard solutions

The primary stock solution (100µg.mL^{-1}) of ZMT was prepared and further diluted with normal saline to obtain a serial dilution in the concentration range of 0.25-10µg.mL^{-1}. Correspondingly, 10µg.mL^{-1} rizatriptan (RZT) stock solution was also prepared as an internal standard (IS).

Extraction procedure

All samples were extracted using the liquid-liquid extraction method. Approximately 0.4mL of blood was withdrawal in heparin tubes via the retro-orbital sinus. The samples were centrifuged at 5000 × g for 20 min and plasma was then collected in a tube. Aliquots of drug (20 µL) from each standard working solution were carefully added to the plasma (180µL). Approximately 1.8mL of dichloromethane (DCM) was added to achieve a known concentration of 0.25-10µg.mL^{-1} in rat plasma. After centrifugation, the supernatant organic layer was taken and dried with nitrogen stream at 40ºC. The residue obtained was reconstituted with 120µL of mobile phase and 10µL of RZT (10µg.mL^{-1}) as an IS and vortexed for 3 min. The suspension was centrifuged and 20µL supernatant was introduced into the HPLC apparatus.

Method validation

Specificity was tested by comparing chromatograms of blank rat plasma (without drugs) with chromatograms of plasma containing ZMT, RZT, or both ZMT/RZT.

Linearity was determined in the concentration range of 0.25-10µg.mL^{-1} and the calibration curve was generated by graphing the ZMT/RZT ratio versus plasma concentration.

Five replicates of each spiked sample were analyzed at lower, intermediate, and higher concentrations (0.25, 1, 5µg.mL-1) to measure accuracy and precision (Reddy *et al*., 2019).

The system suitability test was carried out by examining the highest concentration (10g.mL^{-1}) of the calibration curve. The produced samples were injected six times in a row and the variations in area ratio were noticed.

Stability

Stability study was conducted at low, medium and high concentrations (0.25, 1, 5µg.mL^{-1}) with five duplicates at each concentration.

The plasma samples were kept at RT (25ºC) for 6h after being combined with the drugs. Following that, the samples were examined to evaluate bench-top stability.

To test post-preparative or auto-sampler stability, processed plasma samples were maintained at 4°C for 12h and a 20 µL sample was cautiously injected into HPLC instrument.

Plasma samples spiked with ZMT were frozen (-20˚C) and thawed (RT, 25ºC). After three cycles (freezing and thawing), the samples were examined for their freeze-thaw stability.

Freshly prepared samples containing the ZMT and RZT were maintained at -20°C for a period of 72h to determine long-term stability. The acceptance criterion for the stability test was set at a deviation of less than 15% of each concentration (Reddy *et al*., 2019).

Application to clinical study

The pharmacokinetic (PK) study was performed using Sprague-Dawley rats (180-220g, Shanghai, China). The experimental procedures and ethical clearance protocol were approved by the Animal Care and Use Committee of Soochow University (Permit Number: SUDA20220 407A02). Five ZMT tablets (each containing 2.5 mg) were dissolved in 6.25mL of normal saline in a 10mL Eppendorf tube to prepare a 2mg.mL^{-1} ZMT-i.g (intragastric) suspension. Finally, 1mL of suspension was

administered i.g into the stomach of the rats via an oral applicator and the blood was collected in heparin tubes at 10, 30, 60, 120, 240, 480 and 720 min, respectively. Afterwards, the same method as described in the section (extraction procedure) was used.

Ethical approval

The Institutional Animal Care and Use Committee of Soochow University, Suzhou, China, reviewed and authorized all protocols relating the use of laboratory animals (Permit Number SUDA20220407A02).

STATISTICAL ANALYSIS

The statistical analysis of the different validation and stability parameters were studied using Microsoft Excel 2016 software and all results are presented as mean ± S.D. The PK parameters were calculated with Win Nonlin 6.1 computer software (Certara, Princeton, NJ).

RESULTS

Method development and optimization

Specificity

A simple, accurate, precise and reproducible HPLC method for the analysis of ZMT was established and validated. The representative chromatograms of the blank SSF and known concentration of 10µg.mL^{-1} ZMT in SSF at pH 6.8 are shown in fig. 2. The results showed the absence of a peak in the retention time of ZMT or overlapping of peaks demonstrating the high specificity of the developed method.

Linearity

The linearity study was conducted over the range of 0.25-100 µg.mL^{-1} in SSF. Calibration curve was created by plotting the instrumental signal against concentration. The curve followed the following equation: y=11234x + 2789.3. The correlation coefficient (R^2) was 1, indicating a high probability of correlation between the variables (area and concentration).

Sensitivity

The sensitivity of the proposed method in terms of the LOQ and LOD were 17 and 3 ng.mL^{-1}, respectively, for ZMT.

System suitability

The low RSD value of 0.7% as shown in table 1 indicated a good performance of the system that could lead to accurate and precise results according to the standard specifications.

Accuracy

The results showed that the recovery (%) and RSD (%) of the five replicate injections were 98-102% and 1.01-1.47 (table 2) which were within the limits specified in the ICH guidelines.

Precision

Three distinct ZMT concentrations were evaluated in five duplicates for precision assessment. Following the specified precision constraints, the RSD (%) results for both intra-day and inter-day precision were less than 2%, as shown in table 3 (Dighe *et al.*, 2017).

Stability

Stability studies were performed using three concentrations (10, 40 and 80 µg.mL^{-1}). The recovery rate (%) of the samples for bench-top stability showed that the analyzed samples were stable at RT (25ºC) for at least 6 h. The test for freeze-thaw stability showed that the analyzed specimens were stable after three frozen (-20°C) and thawed (RT, 25ºC) cycles. The prepared samples for long term stability specified consistent stability at -20°C for at least three days**.** Post-preparative studies revealed constant stability behavior for a minimum 12 hours at 4°C. The overall findings of the stability experiments revealed that the sample recoveries (%) were all within the acceptable range of 85-115% as shown in table 4 (Reddy *et al.*, 2019).

Bioanalytical method development and validation

Specificity

The chromatographs of blank rat plasma and plasma spiked with ZMT and RZT were compared to confirm specificity. At the retention duration of ZMT and RZT, no endogenous interfering elements were found in the chromatograms of plasma samples spiked with ZMT and RZT (fig. 5). Furthermore, no possible interactions or peak overlaps between the peaks of the blank plasma, internal standard, and model drug were found (fig. 3).

Linearity

The linearity range of ZMT in rat plasma was determined to be between 0.25 and 10µg.mL^{-1}. A calibration curve was created by graphing the ZMT/RZT ratio against plasma concentration and showed a good linear association (R^2=0.9999) between the specified concentration range.

Accuracy

Five duplicates were used to assess the accuracy and precision (intra and inter assays). As listed in table 6, the accuracy test recovery and RSD values were 100.09-107.69% and 2.19-5.20%, respectively, following USFDA requirements for bio-analysis (Reddy *et al*., 2019).

Precision

The RSD (%) values for intra-day tests and inter-day assays varied from 2.27-7.02% and 2.47-8.76%, respectively, as shown in table 7. The low RSD values of the proposed method were in line with USFDA validation criteria, indicating that the developed approach was accurate and precise for quantitative determination of ZMT in rat blood.

Table 1: System suitability data of HPLC method (n=6)

No.	Area	Conc. (µg·mL^{-1})	Mean±SD	RSD (%)
1	514128	40.30	40.54±0.28	0.70
2	522009	40.93		
3	521188	40.87		
4	514118	40.30		
5	515978	40.45		
6	515478	40.41		

Table 2: Accuracy of HPLC method for ZMT quantification (n=5)

Added (µg·mL^{-1})	Detected (µg·mL^{-1})	Recovery (%)	RSD (%)
10	9.86±0.14	98.55±1.45	1.47
40	40.22±0.40	100.55±1.01	1.01
80	79.00±1.09	98.75±1.36	1.37

Table 3: Response of ZMT in term of Intra- and inter-day precision (n=5)

Added (µg.mL-1)	Intra-day		Inter-day	
	Mean ±SD	RSD (%)	Mean ±SD	RSD (%)
10	9.67±0.13	1.30	9.87±0.15	1.54
40	39.79±0.48	1.21	40.20±0.44	1.11
80	78.91±0.53	0.68	78.68±1.08	1.37

Table 4: Stability of ZMT samples determined at three different levels (n = 5)

	10 (µg·mL^{-1})		40.00 (µg·mL^{-1})		80.00 (µg·mL^{-1})	
	Recovery (%)	RSD(%)	Recovery (%)	RSD(%)	Recovery(%)	RSD(%)
Bench top stability (25 ºC)	98.08±2.18	2.22	98.41±2.10	2.14	98.17±3.10	3.16
Freeze and thaw stability (3 cycles)	98.20±3.02	3.08	99.90±2.60	2.60	99.63±1.83	1.84
Long-term stability (-20 ºC)	99.48±2.08	2.09	99.67±2.06	2.07	100.16±1.34	1.33
Post-preparative stability (4 ºC)	99.84±2.01	2.01	100.52±2.26	2.24	99.24±2.11	2.12

Table 5: Comparison between current HPLC method and reported methods for in-vitro study

Parameter	Method I	Method II	Method III	New method
Reference	(Vijayakumar *et al*., 2010)	(Rao *et al*., 2005)	(Dighe *et al*., 2017)	Current study
Analyte	Zolmitriptan	Zolmitriptan	Zolmitriptan	Zolmitriptan
Linear range of CAB (ppm)	0.05-1	25-150	1-6	0.25-10
Retention time	11.0	10	10.5	5
Flow rate (mL.min^{-1})	1.0	HPLC gradient program was set as: time/% solution B: 0/0, 10/0, 30/55, 35/55 and 36/0	1.0	1.0
Mobile phase (v/v)	0.02 M ammonium formate containing 0.1% n-propylamine and acetonitrile in (80:20 v/v)	A:(buffer):methanol:acetonitrile (70:20:10, v/v/v) B: buffer:acetonitrile (30:70 v/v)	Methanol: acetonitrile: water (35:38:27, v/v)	0.2% triethylamine in water: acetonitrile (85:15, v/v)
Column	Waters XTerra (250 × 4.6 mm × 5 µm)	Waters 250 mm × 4.6 mm × Terra RP18 column 5µm	C18 column 25 x 0.6cm × 5 µm	CST column (250 mm × 4.6 mm × 5 µm)
Wavelength (nm)	225	225	236	240
AGREE score	Dr. Naveed	Dr. Naveed	Dr. Naveed	Dr. Naveed

Table 6: Accuracy data of ZMT quantification in rat plasma (n=5)

Added ($\mu g \cdot mL^{-1}$)	Detected ($\mu g \cdot mL^{-1}$)	Recovery (%)	RSD (%)
0.25	0.27±0.01	107.69±5.60	5.20
1.00	1.00±0.04	100.09±4.44	4.43
5.00	5.19±0.11	103.77±2.27	2.19

Table 7: Intra-day and inter-day precision data (n=5)

Conc. ($\mu g \cdot mL^{-1}$)	Intra-day		Inter-day	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
0.25	0.29±0.02	7.02	0.27±0.01	5.20
1.00	1.00±0.04	4.43	1.06±0.09	8.76
5.00	5.24±0.12	2.27	5.22±0.13	2.47

Table 8: System suitability assay of ZMT in rat plasma (n=6)

No.	ZMT area	RZT area	Ratio(ZMT/RZT)	Conc.($\mu g \cdot mL^{-1}$)	$\bar{}$Mean±SD	RSD (%)
1	109925	100060	1.10	10.15	10.13±0.12	1.20
2	106494	98744	1.08	9.96		
3	104067	95267	1.09	10.09		
4	107359	96313	1.11	10.30		
5	107759	97817	1.10	10.18		
6	107657	101920	1.06	9.76		

Table 9: Stability study data of ZMT in rat plasma at different quality control (QC) levels (n=5)

	0.25 ($\mu g \cdot mL^{-1}$)		1.00 ($\mu g \cdot mL^{-1}$)		5.00 ($\mu g \cdot mL^{-1}$)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Short-term stability	91.64±9.28	10.12	92.53±5.05	5.46	93.46±4.74	5.07
Freeze and thaw stability	92.75±0.69	0.74	96.80±6.35	6.56	95.78±6.32	6.59
Long-term stability	88.22±7.64	8.66	95.02±5.04	5.30	94.89±4.25	4.48
Post-preparative stability	94.10±4.02	4.27	98.73±3.40	3.44	98.34±2.23	2.27

Table 10: Comparison between current HPLC method and reported methods for in-vivo study

Parameter	Method I	Method II	New method
Reference	(Kılıç *et al*., 2007)	(Zhang *et al*., 2004)	Current method
Analyte	Zolmitriptan	Zolmitriptan	Zolmitriptan
Internal standard (IS)	Paroxetine	Rizatriptan	Rizatriptan
IS volume (µL)	50	15	10
Reconstitution solvent	Mobile phase	Mobile phase	Mobile phase
Blood volume (µL)	Not mentioned	Not mentioned	400
Plasma volume (µL)	Not mentioned	1000	180
Extraction solvent and volume	Saturated ethyl acetate: dichloromethane (4:1, v/v)	DCM: ethyl acetate mixture (20:80, v/v)	DCM
Extraction solvent volume	5 mL	5	1.8
Flow rate ($mL.min^{-1}$)	0.25	0.2	1
Mobile phase (v/v)	Acetonitrile: 5 mM ammonium acetate: formic acid (50:50:0.053, v/v/v)	Methanol:water (78:22, v/v)	0.2% triethylamine in water: acetonitrile (90:10, v/v)
Mass spectroscopy	Quattro Micromass LC triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source	Quadrupole mass spectrometer with an ESI interface.	------
Column	XTerra RP18 column (3.5 lm, 100.3.0 mm i.d.)	Lichrospher CN (5µm, 150 mm × 2.0 mm)	CST (250 mm × 4.6 mm, 5 µm)
Retention time (min)	1.87	5.4	12.5
Accessibility	Limited availability	Limited availability	Eassy availability
Consumption of organic solvent	High	High	Low
Cost	Costly	Costly	Cost effective
AGREE score	Dr. Naveed	Dr. Naveed	Dr. Naveed

Table 11: Pharmacokinetics parameters of ZMT after intragastric administration at a dose of 10 mg.kg^{-1} to rats.

S. No.	PK-Parameters	ZMT-marketed product
1	Cmax (ng.mL^{-1})	1676 ±160.1
2	Tmax (h)	0.5
3	AUC (0-t) (ng.h/mL)	2662.6 ± 481.1
4	MRT (h)	2.1 ± 0.2

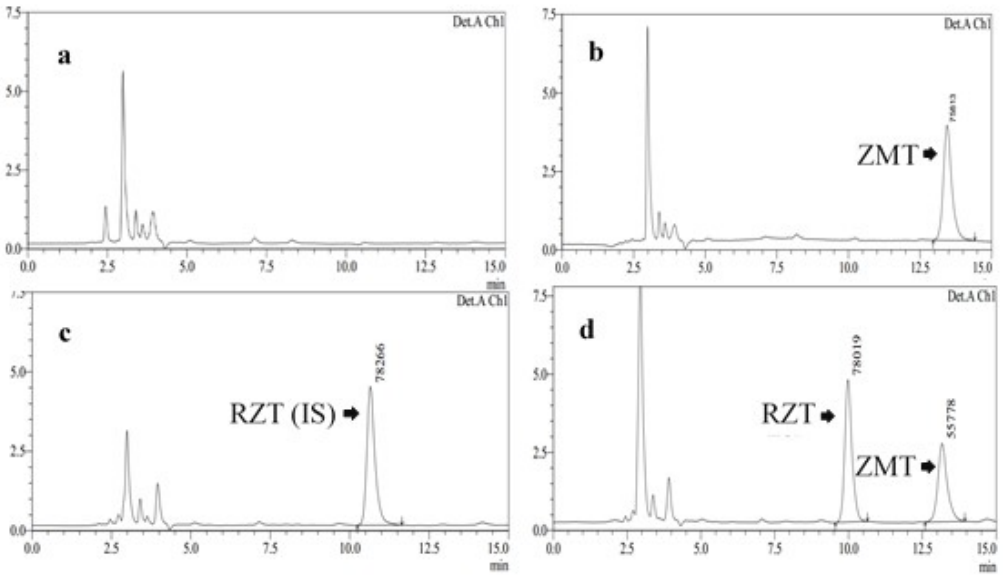

Fig. 3: HPLC chromatogram of blank rat plasma (a), plasma spiked with zolmitriptan (b), plasma spiked with rizatriptan as an internal standard (c), plasma spiked with zolmitriptan and rizatriptan (d).

System suitability

The system suitability assessment was accomplished using six duplicates at a single concentration of 10 µg.mL^{-1}. The results indicated a good system suitability, with a lower RSD value of 1.20 (%) (table 8).

Stability

The recovery (%) for all evaluated samples were in the range of 88.22±7.64 to 98.73±3.40, respectively. The results revealed that ZMT was stable in rat plasma under different experimental conditions as shown in table 9.

Application to pharmacokinetic study

The proposed ZMT technique was utilized to assess the pharmacokinetic (Pk) parameters of ZMT following a single dose of intragastric administration to rats.

Table 11 showed that the maximum ZMT plasma concentration (Cmax) and the time to achieve maximum plasma concentration (Tmax) were 1676±160ng.mL^{-1} and 0.5±0 h, respectively. The mean residence time (MRT) of ZMT was 2.1±0.2h, indicated the retention time of ZMT in-vivo. The area under drug concentration curve (AUC (0-t) ng.h/mL) was 2662.6±481.1ng.mL^{-1} after i.g administration of ZMT in rats. The plasma concentration profile of ZMT after i.g suspension in rat plasma was plotted against time as shown in fig. 4.

DISCUSSION

In preliminary tests, we investigated a variety of aqueous mobile phases containing buffer solutions with alter pH values mixed with various modifiers composed of MEH, CAN and TEA in varied proportions. Based on the most satisfactory findings in terms of ZMT peak and retention time, 0.2% (v/v) TEA in water (pH=3) and ACN in a volumetric proportion of 85:15 (v/v) with a 1.5 mL.min^{-1} flow rate was used as a mobile phase. In this study, we optimized a mobile phase composition that has considerable advantages over earlier published processes in terms of availability, hazardous organic solvent, cost, and analysis time of ZMT (Rao *et al.*, 2005). In the specificity test, the blank samples showed no significant interference in the retention time of ZMT, indicating that the method was unambiguous (fig. 2) and can be used pragmatically for the quantification of ZMT.

The system suitability test showed good performance of the system and was able to consider in accurate and precise results according to the standard specifications (table 1). Furthermore, the precision and accuracy results were consistent with the ICH recommendations (Raza *et al.*, 2022). The good recovery rate (%) determination further supported the accuracy and validity of the proposed method (table 2). The sensitivity method

directed that the method can be used for the detection of such lower concentrations of ZMT. The precision data directed that the RSD (%) values were less than 2%, following specified precision limits (table 3). The inclusive results of the stability studies indicated that all samples were stable and their recoveries (%) were within the acceptance range of 90-110% (table 4).

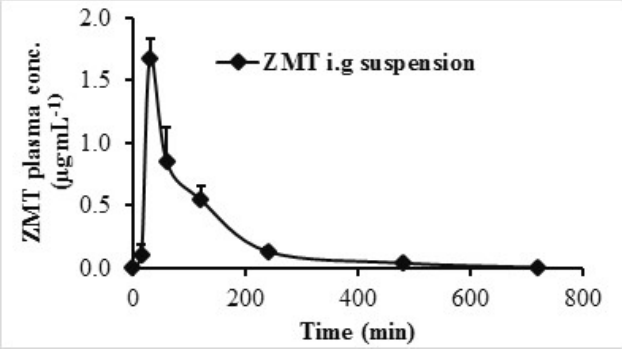

Fig. 4: Pharmacokinetics profile of ZMT after oral administration of intragastric suspension at a dose of 10 mg.kg^{-1} to rats. Data are given as mean ± S.D., (n= 3).

The analysis of drugs requires organic solvents and the two notoriously hazardous solvents (MEH and ACN) have been widely used as a mobile phase due to their exceptional combination which are promising for HPLC applications. They are entirely miscible with water, readily available in high purity, and have minimal chemical interactions with majority chemical compounds, as well as HPLC column and apparatus. Despite their remarkable properties, these solvents have some problems in terms of environmental impact, health safety and waste disposal. Regrettably, an uninterruptedly operating HPLC equipped with a conventional HPLC column (length; 15-25 cm, diameter 4.6mm, particle 5µm) and an eluent flow at a rate of 1 mL·min^{-1} produces about 1500mL of waste per day, which translates into about 500,000mL of liquid waste per year. Since, it is difficult to avoid the use of organic solvents in HPLC method but it's better to make this technique eco-friendlier. One of the straightforward strategy that can successfully pursued to achieve eco-friendlier method is the volume reduction of organic solvent. Therefore, we developed and validated a new analytical method with abridged amount of organic solvent that subsequently reduced the overall generated harmful solvent compared to reported methods. Sankar *et al*., and Dighe *et al*., established and authenticated methods for the analysis of ZMT. In comparison to the current investigation, the organic volume in the mobile phase was found to be substantially larger (Sankar *et al.*, 2009, Dighe *et al.*, 2017). Other methods reported an extended retention time of 15 min compared to the 5 min of the current study (Vijayakumar *et al*., 2010, Rao *et al*., 2005). In contrast to the current study, the increased retention time makes it time-consuming or quite expensive to do a quantitative analysis due to machinery or high solvent supply. Furthermore, extensive in-vitro/in-vivo stability investigations of ZMT, as well as technique development and validation, have been rarely described.

Moreover, the model drug was quantified in rat plasma by employing HPLC system equipped with a UV detector. The HPLC apparatus and chromatographic column were identical to those described earlier. The wavelength, flow rate and mobile phase composition differed somewhat from those reported in the *in-vitro* analysis. The influence of pH, buffer molarity and a number of organic solvents were also considerate to improve the peak shape and reduce retention time of the drugs.

The results showed satisfactory virtuous peak shape and retention time by employing a mixture of 0.2 % TEA in water and ACN at a volumetric ratio of 90:10, v/v as a mobile phase with a flow rate of 1.0 mL·min^{-1}. The obtained results confirmed no interference between the peaks of the model drug and the IS using CST column (fig. 3). The chosen IS (RZT) was appropriate due to chemical structure similarities (fig 1b), retention time, ionization with the analyte and the lower endogenous interferences at rizatriptan [M + H]+, m/z 270.10 that did not impair the measurement of ZMT in rat plasma (fig. 3) (Zhang *et al*., 2004). All the samples were extracted by liquid-liquid extraction technique because this technique cannot only decontaminate but also concentrate the plasma sample and had a determined recovery rate (%) for ZMT compared to other methods (Reddy *et al*., 2019). We intended to establish and validate a convenient analytical method that can determine ZMT/RZT in the concentration range required for the detection of clinical plasma samples. Several analytical methods have been described for the determination of ZMT in plasma samples, but they are either expensive or cumbersome. For instant, Vishwanathan *et al*., reported a liquid chromatography/ tandem mass spectrometry technique for ZMT analysis (Vishwanathan *et al*., 2000). However, the proposed method was fairly expensive and is not readily offered in most experimental research laboratories. Similarly, a rapid, sensitive and precise LC–MS–MS method with electrospray ionization (ESI) was validated for the quantification of ZMT in human plasma (Kılıç *et al*., 2007). The described method was expensive and consumed relatively higher volume of organic phase compared to the current study.

In order to offer more directions and analyze the pharmacokinetic parameters in rats, a cost effective, eco-friendly, and reproducible HPLC with UV detection method was established in this study. The developed method showed excellent linear response and a remarkable correlation amongst the peak area and analyte concentration. The resulted linear regression coefficient was used to determine the unknown concentration of ZMT in rat plasma. Representative chromatograms showed no interference of endogenous components of rat

plasma with the measurement of ZMT or their respective IS (fig. 5 a,b,c and d). The validation data for ZMT indicated that the developed method for the quantitative analysis of ZMT in rat blood was accurate and precise in the concentration ranges studied (table 5, 6 and 7). The stability of ZMT in short-term (6h at 25ºC), freeze-thaw (three cycles), long-term (-20°C for 3 days) and post preparative (12h at 4°C) studies was within acceptable ranges and are consistent with the earlier work (Reddy *et al*., 2019, Chen *et al*., 2004). In addition, the results of the stability studies revealed that the selected drugs didn't degraded in the plasma samples, indicating that plasma samples can be handled without particular care. The applicability of the established method was demonstrated by studying ZMT concentrations after a single dose in rat plasma. The pharmacokinetic parameters (Cmax, Tmax (h), AUC (0-t) and MRT (h) were evaluated after detection of ZMT in rat plasma samples using the proposed method, and the parameter values are consistent with those previously published (table 9) (El-Nabarawy *et al*., 2019). The current approach can be considered appropriate for the quantification of ZMT in plasma samples.

CONCLUSION

The suggested HPLC technique for quantifying ZMT was simple, accurate, reproducible, and eco-friendly, and it was validated in accordance with ICH recommendations. In contrast, this approach is free of stringent assay conditions and is distinguished by its simplicity, cost-effectiveness, reasonable sensitivity, and use of readily available chemicals compared to reported assays of ZMT.

Furthermore, the proposed approach was appropriate for assessing ZMT in plasma samples, such as pharmacokinetic or bioequivalence studies. The bioanalytical method was validated and the results of all validation parameters and stability data were within acceptable limits as specified in the USFDA guideline for bioanalytical study. The developed approach was successfully employed for quantifying ZMT in rat plasma. The proposed approach, however, was only used for a brief pharmacokinetic research in a rat model. Therefore, a thorough pharmacokinetic research in human patients is required in the future.

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