Development and Validation of HPLC Method for quantification of Zonisamide in Spiked Human Plasma

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ABSTRACT

In present work a simple, economic, accurate and precise HPLC method with UV detection was developed for the quantification of zonisamide (ZON) in spiked human plasma using trimethoprim (TRI) as an internal standard. Both ZON and TRI were well separated and resolved from each other on C18 column using mobile phase blend of methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) in an isocratic mode at flow rate of 1 mL/min with proportion of 25:5:70 %, *v/v/v*. The detector wavelength was set at 240 nm. Maximum recovery of ZON and TRI from plasma were obtained with dichloromethane (DCM) as extracting solvent. The calibration curve was found to be linear in the range of 3-60 µg/mL with regression coefficient (r^2) = 0.997. The LLOQ of the method was 3 µg/mL. Although, with acceptable r^2, heteroscedasticity of the calibration data was observed which further was reduced with use of weighted linear regression with weighting factor 1/*x*. Finally, the method was validated with respect to sensitivity, accuracy, precision, recovery, stability and for carry-over as per US-FDA guidance for Bioanalytical Method Validation, May 2018.

Keywords: Bioanalytical method validation; HPLC; Spiked human plasma; weighted linear regression; Zonisamide.

INTRODUCTION

Chemically zonisamide (ZON) is 1-(1,2-benzoxazol-3-yl)methanesulfonamide [1]. It is used as an anticonvulsant alone or in combination for the treatment of partial, generalized and atypical seizures. ZON blocks Na channels and voltage sensitive T type calcium channels thus stabilizing neuronal membrane and neuronal hyperpolarization. This helps in suppression of propagation of seizures [2].

There are several HPLC methods reported for the estimation of ZON in different biological fluids. These include, estimation of ZON in human breast milk and plasma where, solid phase extraction method was used for the extraction of ZON [3], estimation of ZON in human serum and its application to pharmacokinetic study [4], estimation of ZON in pharmaceuticals and in human plasma using C 18 column, where protein precipitation method was used to extract ZON [5], estimation of ZON in human plasma using microextraction by packed sorbent. In this method, Plackett-Burman Design was implemented to obtain optimized extraction conditions [6].

Considering the increase in the misuse of anti-epileptic agents in drug abuse, suicide, overdose and in drug facilitated crime, there are several analytical methods reported for the estimation of different anti-epileptic agents including ZON. These include, HPLC methods for estimation of ZON in different biological fluids along with other anti-epileptic agents, which includes, estimation of

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ZON with levetricitam in human plasma and serum[7], estimation of three anti-epileptic drugs including ZON in human plasma, where analytes were extracted by liquid-liquid extraction [8,9], estimation of seven anti-epileptic drugs including ZON in human serum [10], estimation of anti-epileptic drugs and their metabolites in human plasma [11–14]. In these methods, protein precipitation technique was used to extract analytes. Also an HPLC method was reported for estimation of few anti-epileptic drugs including ZON in dried plasma spots [15]. Further, there is an HPTLC method reported for the estimation of ZON in human plasma with other anti-epileptic drugs [16]. Along with these, there are several hyphenated techniques reported for the estimation of ZON alone including an LC-MS method [17], where ZON was extracted using liquid-liquid extraction and estimation of ZON along with other anti-epileptic drugs, these methods include, estimation of nine anti-epileptic drugs including ZON by LC-MS using solid phase extraction [18], estimation of twenty-two anti-epileptic drugs in blood, serum, and plasma using LC-MS/MS [19], a UPLC-MS/MS method [20], estimation of nine anti-epileptic drugs including ZON by LC-MS/MS in dried blood spots [21].

Although several analytical methods reported till date for the estimation of ZON, however, with these reported methods either the calibration curve samples and QC samples were not selected considering the Cmax of the drug [22] or the method was not validated as per US-FDA guidelines[26]. Further, solid phase extraction method was used in several papers for the extraction of analyte. Although it is most widely used method considering its high extraction efficiency, high selectivity and ability to separate wide variety of analytes from mixture (from polar to non-polar), its advantage over other extraction method for complex analytical mixtures is accepted. However, it increases the financial toll associated with the use of cartridges containing different sorbents and may not be economical and found time consuming for mixtures containing few analytes [23]. Also, few other methods implemented protein precipitation/ deproteination method for the extraction of analyte/s, although the technique is simple and economic, however it is insufficient and may block the LC column [24]. Even tough, the reported hyphenated methods were found more sensitive, however, these methods are expensive and the instruments used are not readily available in routine quality control laboratories.

Hence, considering the need for simple, economic still accurate, precise and selective method for estimation of ZON in human plasma, an attempt was made to develop an HPLC method for estimation of ZON in human plasma. ZON was extracted from plasma by liquid-liquid extraction using a suitable organic solvent. The calibration curve range and QC samples [Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC)] for analysis were selected considering the Cmax of the drug. The heteroscedasticty observed in the calibration data was minimized using weighted linear regression with suitable weighting factor [22,25]. Further, the method was validated as per US-FDA guidelines for Bioanalytical Method Validation [26].

MATERIALS AND METHODS

Chemicals and reagents

Pharmaceutical grade ZON (Zonisamide USP) was generously supplied by Sun Pharmaceutical Industries Ltd, Mumbai, Maharashtra, India as a gift sample and certified to contain 99.1 % *w/w* on anhydrous basis. Blank human plasma from different sources were obtained as a gift sample from Dr. Vasatrao Pawar Medical College Hospital and Research Centre, Nashik, Maharashtra, India and the pooled sample was prepared by rigorously mixing the obtained samples of plasma. The acetonitrile and methanol were of HPLC grade and the rest of the chemicals used were of analytical reagent grade. All chemicals were purchased from Merk Life Sciences Pvt. Ltd., Mumbai, Maharashtra, India. Freshly prepared double distilled water used in analysis was prepared using All Glass Distillation Assembly, purchased from Borosil India Pvt. Ltd., Mumbai, Maharashtra, India and further filtered using 0.45 µ × 47

mm membrane filter paper purchased from Milipore India Pvt. Ltd., Bengaluru, Karnataka, India.

Instrumentation and chromatographic conditions

The HPLC analysis was performed using JASCO HPLC system having dual PU-2080 *plus* pumps, UV-2075 multichannel detector equipped with Rheodyne (7725) injection system and a 20 µL sample loop. The obtained analytical data was processed using Borwin Chromatography Software (version 1.50).

Weighing was performed on AUX 220 digital weighing balance, Shimadzu Corporation, Tokyo, Japan. C-24 BL, cooling centrifuge used in analysis was purchased from Remi Sales and Engineering Ltd., Mumbai, Maharashtra, India.

ZON and internal standard (IS), TRI were separated and resolved from each other and from the plasma interferents using Hyperclone C18 column (250 × 4.6 mm, 5 µ) purchased from Phenomenex India Pvt. Ltd., Hyderabad, Telangana, India using blend of methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) (25: 5: 70 %, *v/v/v*) as a mobile phase in an isocratic mode with flow rate of 1 mL/min. All eluents were detected at 240 nm; the absorbance maxima of ZON.

Preparation of standard stock solutions

The standard stock solution of 1 mg/mL of ZON and TRI were prepared by dissolving 10 mg ZON and TRI individually in 10 mL volumetric flask using methanol, respectively. The prepared standard stock solution of ZON was further diluted with methanol to obtain 10 different working standard solutions of concentrations 30, 60, 90, 150, 250, 300, 400, 450, 550 and 600 µg/mL. Also, the standard stock solution of TRI was diluted appropriately with methanol to obtain a concentration of 180 µg/mL.

Liquid-liquid extraction (LLE) experiment

In LLE experiment, an aliquot of 1 mL of pooled plasma was taken in a stoppered glass tube of size 20 mL. In it, 100 µL of 100 µg/mL of ZON and 100 µL of 100 µg/mL of TRI (IS) was added and the solution was vortex mixed for 3 min. Further, an aliquot of 5 mL of dichloromethane (DCM) was added in it and the sample in the tube was vortex mixed again for 5 min. The tube was then centrifuged at 3000 rpm for 10 min at 4 º C in a cooling centrifuge. The separated organic layer was added to an Eppendorf tube and was evaporated to dryness under the stream of nitrogen. The residue obtained after this was then reconstituted with 500 µL of mobile phase. Finally, an aliquot of 20 µL of this solution was injected into the HPLC system.

Preparation of calibration curve (CC) standard and quality control (QC) samples

Considering the Cmax of the ZON 30 µg/mL[27], the CC standards and QC samples were prepared as per the US-FDA guidelines for Bioanalytical Method Validation. Hence, the CC standards were prepared in the range of 3-60 µg/mL. Also, QC samples, which includes, LLOQ – 3 µg/mL (10 % of Cmax), LQC – 9 µg/mL (3 times the LLOQ), MQC – 30 µg/mL (30 – 50 % of the calibration range), HQC – 45 µg/mL (near to the upper limit of CC range) were prepared.

The CC standards were prepared by taking 1 mL of aliquot of pooled plasma in 10 different stoppered glass tubes of size 20 mL. Individually in each tube, 100 µL of prepared working standard solutions of ZON and 100 µL of 180 µg/mL of TRI was added to obtain CC standards of 3, 6, 9, 15, 25, 30, 40, 45, 55, and 60 µg/mL of ZON, respectively.

All CC standard solutions were processed as per the procedure depicted in LLE experiment section and finally injected in the HPLC system under mentioned chromatographic conditions.

The QC samples of concentrations 9 µg/mL (LQC), 30 µg/mL (MQC) and 45 µg/mL (HQC) were prepared along with CC standard similarly.

Selection of internal standard

Different analytes with similar chromatographic behavior with that of ZON were tried as an internal

standard. The analyte which showed good resolution from the ZON and plasma interferences and with acceptable system suitability was selected as an internal standard. Further, to select the concentration of the IS, different concentrations of selected IS were injected in the HPLC system with the highest concentration of ZON (i.e. 60 µg/mL) and the IS concentration which gave 30-60 % peak area to that of highest concentration of ZON was selected.

Calibration curve and selection of calibration model

All CC standard were injected in six replicates. The obtained chromatograms of all CC standards were integrated and peak area ratio for ZON to TRI were calculated. The obtained peak area ratio for each CC standard was plotted against respective concentration to construct a calibration curve.

Further, the obtained data from the CC standards was subjected to unweighted and weighted linear regression. Different weighting factors, $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$ were evaluated and the calibration model with minimum % relative error (% RE) and with uniform scatter of points in residual plot was selected and used in further calculations.

Method validation

The developed method was validated as per the US-FDA guidelines for Bioanalytical Method Validation [26].

Selectivity was evaluated at lower limit of quantitation (LLOQ) at concentration of 3 µg/mL (10 % of Cmax), where the sample of LLOQ was analyzed, peak area was noted and compared with the response obtained for the blank plasma sample at the retention time of ZON. The experiment was performed for six times, for each source of plasma sample. The accuracy and precision of the method were accessed by recording the % RE and % RSD, respectively for five replicates of LQC, MQC and HQC samples for five successive days. The recovery study was performed by comparing the peak areas of the processed QC samples with the standard dilutions representing 100% recovery in five replicates. Stability of the samples at room temperature, at -20 °C, bench-top stability, freeze-thaw stability and long-term stability were studied. For each type of stability study, the % nominal and % RSD values were calculated. To evaluate the carryover between samples, a series of samples were injected in the HPLC system and the residue of the previous samples were observed in the subsequent sample.

RESULTS AND DISCUSSION

Optimized chromatographic condition

To obtain adequate retention with acceptable system suitability, different strengths of mobile phases were tried. The mobile phase with composition of methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) in proportion of 25:5: 70 % *v/v/v* gave adequate separation and resolution when used in an isocratic mode at flow rate of 1 mL/min. The separation was achieved on Phenomenex Hyperclone C18 (250 × 4.6 mm, 5µ). All eluents were detected at 240 nm. The retention time for ZON and TRI was found 7.40 min and 4.64 min, respectively.

Optimization of LLE experiment

Different organic solvents were investigated for the extraction of ZON and TRI from plasma. As depicted in Table 1, no extraction for ZON and TRI was observed in n-hexane, tetrahydrofuran and in toluene. However, good extraction of 89.72 % and 90.21 % was obtained in dichloromethane for ZON and TRI, respectively. The representative chromatogram of blank plasma is presented in Figure 1 and the representative chromatogram of ZON and TRI extracted in dichloromethane is presented in Figure 2. Further, 5 mL of organic solvent and 3000 rpm speed for centrifuge was found optimum.

Selection of Internal Standard

Out of the different analytes injected for the selection of internal standard, TRI gave adequate resolution from the plasma interferents and from ZON with acceptable system suitability. Hence, selected as an internal standard in this study. Also, when different concentrations of TRI were injected in HPLC with highest concentration of ZON, it was found that a concentration of 18 µg/mL gave acceptable peak area with that of ZON.

Calibration curve study and selection of regression model

When the obtained CC data (Table 2) was subjected to unweighted linear regression, acceptable r^2 of 0.9974 was observed with CC equation of $y = 3.38 \times 10^{-5}x + 0.0026$. However, when the CC data was subjected to test of homoscedasticity, the F calculated was found greater than F theoretical. This suggests the need for weighted linear regression. Hence, the CC data was subjected to weighted linear regression with different weighting factors, $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$. The regression analysis of ZON with different weighting factors is shown in Table 3. Thus, it was found that weighted linear regression with weighting factor $1/x$ showed minimum % RE and was further used for calculations.

Method validation

When the selectivity was evaluated at the LLOQ concentration of 3 µg/mL of ZON and compared with the peak areas of blank plasma at the retention time of ZON, no significant peaks in the chromatograms of blank plasma were observed at the retention time of ZON. The response of blank plasma samples was found less than 20 % to the LLOQ. The response of each plasma sample with that of ZON is depicted in Table 4.

The results of accuracy and precision studies are shown in Table 5. The minimum % RE and % RSD at each QC level proved the accuracy and precision of the method within the selected CC range.

The recovery data presented in Table 6, proved the acceptable recovery of ZON and TRI within the given experimental conditions.

The results of stability studies are presented in Table 7 – 9. From this data, it can be concluded that the % nominal values were between 85-115% and the % RSD values were less than 15% for all the stability samples. This proved that the drug remained stable after the completion of the stability cycles.

Carryover study was conducted as per the mentioned sequence (Table 10). No residue of ZON with previous samples were observed in the subsequent runs. Thus, it can be concluded that no carry-over effect was seen in the developed method.

Although few methods have been reported for the estimation of ZON in biological fluids in previous literature. However, these methods have been reported for estimation of ZON in human breast milk[3], which is not readily available. In another reported method, human serum matrix was used[4], which is expensive.

CONCLUSIONS

- In the present work, a simple, rapid, accurate, precise and selective HPLC method was described for the quantification of ZON in human plasma.
- Liquid -liquid extraction provided the good recovery with clear extract of ZON and TRI (internal standard) from plasma using dichlromethane.
- ZON and TRI were well separated and resolved from each other and from plasma interferents on C18 column using methanol: acetonitrile: 20 mM phosphate buffer (pH 3.0) (25:5: 70 % *v/v/v*) in an isocratic mode at a flow rate of 1 mL/min. The method proved to be economic as total run time per sample was less than 10 min. All eluents were detected at 240 nm.
- When the calibration data was subjected to linear regression, in spite of acceptable r^2 of 0.9974, the calibration data was found susceptible to heteroscedasticity, which may lead to error at higher concentration level.
- To reduce the heteroscedasticity, weighted linear regression models were implemented with different weighing factors and the weighing factor of $1/x$ proved to give acceptable results with minimal % RE.
- The developed method was validated as per the US-FDA guidelines for Bioanalytical Method Validation, May 2018, acceptable selectivity, accuracy, precision, recovery, sample stability and carryover was obtained within the studied calibration range.

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Table 1: The recovery of ZON and IS in different organic solvents

Sr. No.	Organic Solvent used for LLE	% Recovery of ZON	% Recovery of TRI
1	n-Hexane	-	-
2	Tetrahydrofuran	-	-
3	Toluene	-	-
4	Chloroform	38.43	59.34
5	Chloroform +1% Formic acid	42.43	63.41
6	TBME (tert-butyl methyl ether)	52.34	53.87
7	Ethyl Acetate	55.25	56.18
8	TBME+5%Formic acid	71.49	78.33
9	TBME+1%Formic acid	72.25	78.12
10	TBME+Ethyl acetate+1%Formic acid	80.51	84.06
11	**Dichloromethane**	**89.72**	**90.21**

Table 2: Calibration curve (CC) data for ZON

Sr. No.	Concentration (µg/mL)	Area Ratio (Mean ± SD) n = 6	% RSD
1	3	0.1230 ± 0.00261	2.121951
2	6	0.2141 ± 0.00436	2.036432
3	9	0.3228 ± 0.00488	1.511772
4	15	0.4648 ± 0.00354	0.761618
5	25	0.8155 ± 0.00373	0.457388
6	30	1.0530 ± 0.00261	0.247863
7	40	1.3248 ± 0.00383	0.2891
8	45	1.5825 ± 0.00243	0.153555
9	55	1.8941 ± 0.00515	0.271897
10	60	1.9920 ± 1.00610	0.306225

Table 3: Weighted linear regression of ZON with different weighting factors

Sr. No.	Weighing Factor	Intercept (a)	Slope (b)	r^2	% RE
1	1	0.004900433	0.00003381	0.997	88.701
2	**$1/x$**	**0.010899824**	**0.00003345**	**0.998**	**64.054**
3	$1/x^2$	0.017234891	0.00003288	0.998	73.720
4	$1/\sqrt{x}$	0.007124143	0.00003370	0.998	83.363

Sr. No.	Weighing Factor	Intercept (a)	Slope (b)	r^2	% RE
5	1/y	0.009318948	0.00003345	0.998	88.433
6	$1/y^2$	0.015485202	0.00003279	0.998	116.923
7	$1/\sqrt{y}$	0.006283860	0.00003366	0.998	94.667

Table 4: Blank response and peak areas of ZON at LLOQ

Sr. No.	Blank response (µV. sec)	Peak areas at LLOQ (µV. sec)	% peak area in blank
1	4972.16	441119.25	1.12%
2	5323.44	437215.64	1.21%
3	5194.26	445018.86	1.16%
4	6437.38	429986.93	1.49%
5	6198.91	450128.16	1.37%
6	7011.21	435625.45	1.60%

Table 5: Results of assessment of the accuracy and precision studies of ZON

QC Level	Conc. added (µg/mL)	Inter day (n=5) mean Conc. found (µg/ml)	%RE	%RSD	Intraday(n=5) mean conc found (µg/ml)	% RE	% RSD
LQC	9	9.025	0.283	0.646	9.090	1.003	0.214
MQC	30	30.581	1.938	1.657	31.012	3.375	0.021
HQC	45	44.798	-0.448	5.334	46.069	-1.187	0.024

Table 6: Recovery of ZON and TRI

Samples	Peak area of ZON			Peak area of TRI (µV.sec)
	QC Levels			
	LQC	MQC	HQC	
Unextracted	169253.4133	582099.9450	782553.2783	566705.6783
Extracted	152310.4867	518536.0750	710582.7183	520469.2517
%Recovery	89.986%	89.087%	90.807%	91.839%

Table 7: The stability of ZON at room temperature and at -20 ° C

QC level	Stability at room temperature						Stability at -20 ° C					
	% Nominal			% RSD			% Nominal			% RSD		
	2h	4h	6h	2h	4h	6h	10 days	20 days	30 days	10 days	20 days	30 days
LQC	98.8	98.4	98.7	1.91	1.17	1.51	99.7	99.1	99.7	1.01	1.12	3.04
HQC	101	100	101	2.33	1.49	1.60	100	101	101	1.50	1.27	1.71

Table 8: Freeze thaw stability of ZON

	Freeze thaw stability					
	%Nominal			%RSD		
QC Level	**FT1**	**FT2**	**FT3**	**FT1**	**FT2**	**FT3**
LQC	99.826	99.347	99.448	0.16464	0.2779	0.32526
HQC	101.281	98.230	100.984	0.0415	0.04066	0.04012

Table 9: The bench top and long-term stability study results

QC Level	Bench top		Long term	
	%Nominal	%RSD	%Nominal	%RSD
	8h	8h	30 days	30 days
LQC	99.800	0.101	100.419	0.066
HQC	100.041	0.087	101.430	0.019

Table 10: Results for carryover Study

SR. NO.	Sample	Area (µV.Sec)	
		ZON	TRI
1	Blank solution	0	0
2	Unextracted ULOQ	467486.79	559303.58
3	Blank solution	0	0
4	Unextracted ULOQ	459387.84	568700.87
5	Blank solution	0	0
6	Extracted blank plasma	0	0
7	Extracted ULOQ	416063.25	505964.47
8	Extracted blank plasma	0	0
9	Extracted ULOQ	409893.75	512348.50
10	Extracted blank plasma	0	0

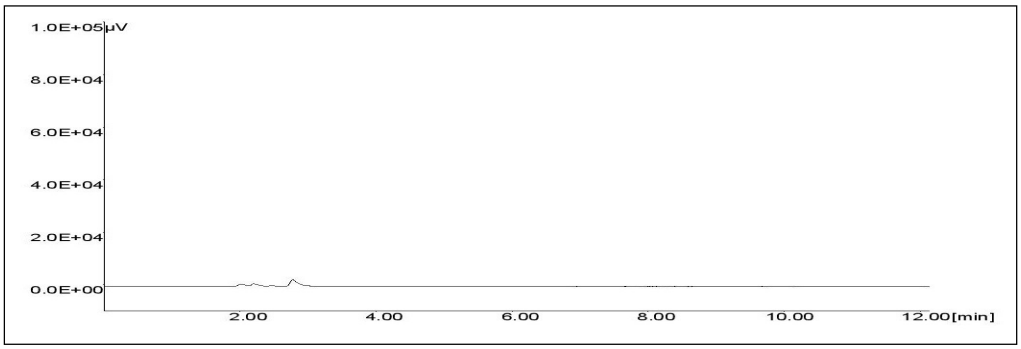

Figure 1. Representative chromatogram of blank plasma extracted with dichloromethane.

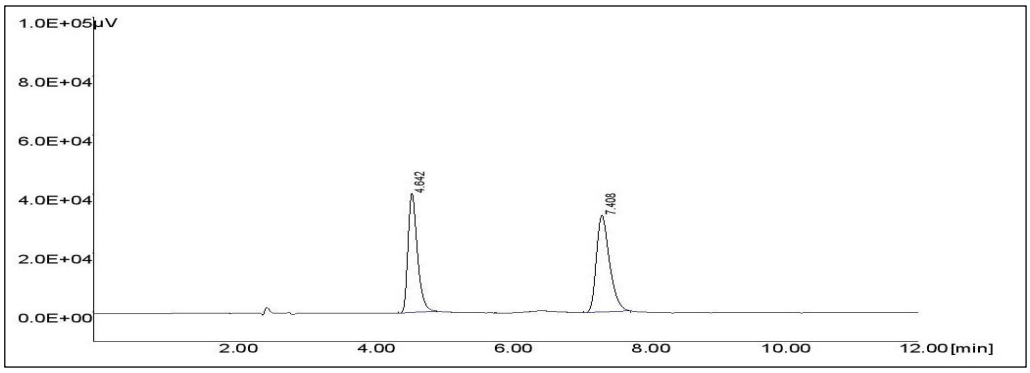

Figure 2. Representative chromatogram of ZON and TRI (IS) extracted in dichloromethane. (ZON RT: 7.40 min; IS RT: 4.64 min)

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طريقة HPLC لتقدير كمية Zonisamide في البلازما البشرية المسننة

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ملخص

في العمل الحالي ، تم تطوير طريقة HPLC بسيطة ودقيقة ودقيقة مع الكشف عن الأشعة فوق البنفسجية من أجل قياس كمية zonisamide (ZON) في البلازما البشرية المسننة باستخدام تريميثوبريم (TRI)كمعيار داخلي. تم فصل كل من ZONو TRIجيدًا وحلهما عن بعضهما البعض في عمود C18 باستخدام مزيج الطور المتحرك للميثانول : acetonitrile: 20 mM phosphate buffer (pH 3.0) في الوضع الأيزوقي بمعدل تدفق 1 مل / دقيقة بنسبة 25: 5 : 70٪ ، حجم / حجم / حجم .تم الحصول على أقصى قدر من استعادة ZON و TRIمن البلازما باستخدام ثنائي كلورو ميثان (DCM)كمستخلص مذيب. تم العثور على منحنى المعايرة ليكون خطي في نطاق 3–60 ميكروغرام / مل مع معامل الانحدار .0.997 = (r2)على الرغم من أن r2 مقبول ، لوحظ مرونة غير متجانسة لبيانات المعايرة والتي تم تقليلها أيضًا باستخدام الانحدار الخطي الموزون مع عامل الترجيح 1 .x/أخيرًا ، تم التحقق من صحة الطريقة فيما يتعلق بالحساسية ، والدقة ، والدقة ، والاستعادة ، والاستقرار ، والترحيل وفقًا لتوجيهات إدارة الأغذية والعقاقير الأمريكية للتحقق من صحة الطريقة التحليلية الحيوية ، مايو 2018.

الكلمات الدالة: التحقق من صحة الطريقة التحليلية ؛ HPLC ؛ ارتفعت البلازما البشرية الانحدار الخطي المرجح Zonisamide.

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