WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.453
Volume 13, Issue 23, 1115-1128. Research Article ISSN 2277–7105

SIMULTANEOUS DETERMINATION OF ARGATROBAN AND ZONISAMIDE BY RP – HPLC METHOD

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Article Received on
05 October 2024,

Revised on 05 Nov. 2024,
Accepted on 26 Nov. 2024

DOI: 10.20959/wjpr202423-34837

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ABSTRACT

UV and HPLC Method was developed and validated for Argatroban, anticoagulant and anticonvulsant drug zonisamide used to treat Alzheimer's Disease. High-performance Liquid Chromatography method as per ICH Q_2R_1 guidelines has been developed for determination of Zonisamide and Argatroban in synthetic mixture. Chromatographic separation was archived with Phenomax Kinetex ® (C18, 5µ, 250 x 4.5 mm) column. As a mobile phase mixture of Acetonitrile, Methanol and Water in the ratio of 60:25+15 v/v/v with pH 6.8 adjusted with OPA was used. Drugs were analysed at 277 nm wavelength with 1 mL/min flow rate. HPLC method was developed and all the results of linearity, accuracy, precision proved to be within limits with LOD and LOQ. Robustness of method was confirmed as no significant difference was detected on analysis by subjecting the method to slight change in the method condition. % Assay was 101.00 ± 0.015 and 100.76 ± 0.097 for Zonisamide and Argatroban respectively.

KEYWORDS: Zonisamide, Argatroban, HPLC method.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative condition primarily impacting older adults globally, marked by a gradual deterioration in cognitive abilities.[1] It is the most common type of dementia, with estimates suggesting that by 2050, over 100 million people around the globe will be affected. Therefore, AD is categorized by the World Health Organization (WHO) as a disease of public health priority.[2,3] The earliest Greek and roman philosophers

and physicians linked old age with increasing dementia. It was not until 1901 that germen psychiatrist Alois Alzheimer's identified the first case of what became known as Alzheimer's disease, named after him, in 50-year-old women he called Auguste D. Alois Alzheimer followed her case until she died in 1906, when he first described publicly on it. The Alzheimer's disease was first described in 1907 by Alois Alzheimer. The pathological hallmarks of Alzheimer's disease are the presence of amyloid plaques and neurofibrillary tangles (NFTs). This condition is characterized by diffuse atrophy of the cerebral cortex and subsequent dilation of the ventricles. Deposits are primarily found in the hippocampus, temporal cortex, and the nucleus basalis of Meynert. The resulting neuronal loss due to these pathological changes leads to reduced levels of neurotransmitters, particularly acetylcholine, which contributes to cognitive deficits in affected individuals. Amyloid, senile plaques and neurofibrillary tangles these are the pathological hallmark of the disease.[4] In clinical settings, the diagnosis of Alzheimer's disease (AD) primarily relies on medical history, physical and neurological examinations, and neuropsychological evaluations, along with selective ancillary testing to exclude other potential causes. The clinical diagnosis of AD has an accuracy of 70-90% compared to pathological diagnoses, with even higher accuracy often achieved in specialized settings like memory disorder clinics.[5] There is no single test to detect for Alzheimer's disease. The doctor may carry out the following test to detect the Alzheimer's disease. Advanced medical imaging with computed tomography (CT-Scan), Magnetic resonance imaging (MRI), Single photon emission computed tomography (SPECT), Positron emission tomography (PET) and Genetics testing. There is no any identified cure for Alzheimer's disease. In Alzheimer's disease, the death of brain cells cannot be reversed. There is a growing interest in developing multi-target drugs that can address various aspects of AD pathology, including anti-Aβ deposition, tau protein phosphorylation, oxidative stress and mitochondrial autophagy dysfunction.[6,7]

Zonisamide ($C_8H_8N_2O_3S$, Chemically known as 1-(1,2-benzoxazol-3-yl) methansulfonamide) is sulfonamide derivative unrelated to other antiseizure agent. Zonisamide block sodium and T-type of calcium channels (Voltage sensitive calcium channels), which is leads to the suppresses neuronal depolarization and neuronal hyper synchronization. Zonisamide also inhibit weak carbonic anhydrase inhibitors it is equally to the anticonvulsant topiramate. Zonisamide is also regulate glutamatergic neurotransmission and GABAergic.[8-9]

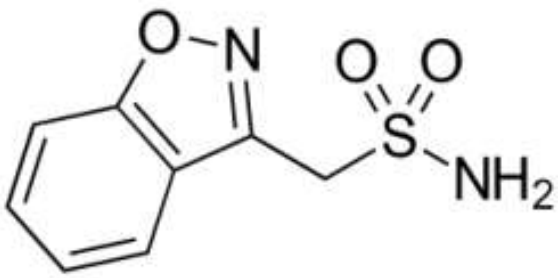

Figure 1: Structure of Zonisamide.

Argatroban [$C_{23}H_{36}N_6O_5S.H_2O$, Chemically known as (2R,4R)-1-[(2S)-5-(diaminomethylideneamino)-2[(3-methyl-1,2,3,4-tetrahydroquinolin-8-yl)sulfonylamino]penta noyl]-4-methylpiperidine-2-carboxylic acid] drug is anticoagulant class of drug. Chemically it is a synthetic derivative of L-arginine with antithrombotic movement. Synthetic direct thrombin inhibitor used for inhibition and treatment of thrombosis associated to heparin use. For prophylaxis or treatment of thrombosis in patients with heparin induced thrombocytopenia drug used Argatroban was licensed by the FDA in the year of 2000.[10-11]

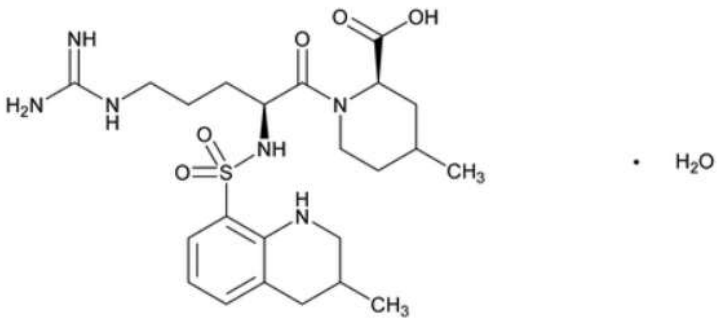

Figure 2: Structure of Argatroban.

Literature review for both the drugs reveals that no any analytical methods have been reported for the simultaneous estimation of Zonisamide and Argatroban drug combination. The present study was aimed to develop and validate Simple, Economical, Precise, Reproducible, Accurate UV & HPLC method for the simultaneous estimation of Zonisamide and Argatroban in their synthetic mixtures according to ICH guidelines.

I. MATERIAL AND METHOD

2.1 Materials

Zonisamide and Argatroban bulk drug was procured from the manufacturer for the analytical

work purpose. Analytical Grade of HPLC grade Distilled Water, Methanol and Acetonitrile was purchased from Merck Specialties Private Limited, India. All the chemicals were used of analytical grade.

2.2 Instrumentation

HPLC of Waters, 2646 was used for the study with the N power 3 software. An analytical balance used for weighing purpose was of Shimadzu Model 1800. Volumetric Flask and Pipettes were used of Borosilicate glasses. All the statistical calculations were done with the Microsoft excel.

2.3 Method

2.4.1 Preparation of stock solution

Drug was accurately weighed to make the standard stock solution 1000 & 1000 µg/mL for ZSM and ARB, respectively in 100 mL volumetric flask. Add 10 mL distilled water into it and stir well to make the drugs soluble properly. Makeup the volume up to the mark to give standard stock Solution.

2.4.2 Preparation of Working solution for Wavelength selection

Appropriate volume for ZSM and ARB were withdrawn from the Stock solution in separate 10 mL volumetric flask. The final volume was made up to the mark with DW in each volumetric flask this results in the final concentrations, 4 µg/mL for both the drugs. The solutions were scanned in the range of 200 – 400 nm and the overlain of zero order spectra.

2.4.3 Preparation of Standard stock solution of ZSM and ARB mixture

100 mg of Zonisamide and 100 mg of Argatroban was accurately weighed and transferred into 100 mL volumetric flask. Drugs were dissolved with 10 mL of Methanol with rigorous shaking and followed by sonicator for 5 min or till get completely dissolved and then diluted up to the mark with methanol which gives standard stock solution of ZSM and ARB mixture of 1000 µg/mL and 1000 µg/mL, respectively.

2.4.4 Selection of detection Wavelength

These drug solutions were scanned in the UV-region of 200-400 nm and the spectra were recorded to get maximum of analytes in mobile phase. ZSM and ARB were scanned in UV in which both the drugs show reasonably good response at 277 nm. So, for detection of ZSM and ARB 277 nm wavelength was selected.

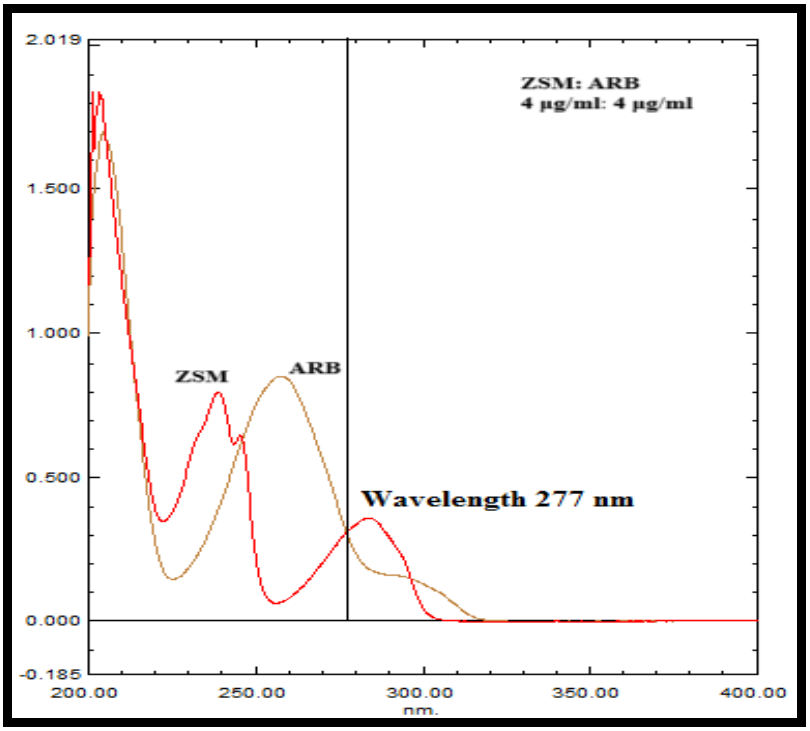

Figure 3: UV Zero order Spectra of ZSM & ARB (4 µg/mL).

2.4.5 Selection of Mobile Phase

Upon review of literature common solvents were taken as a mobile phase in a trial phase like Acetonitrile, Water, Methanol with different pH adjusted with the orthophosphoric acid with distinct compositions to get high resolution and sharp peaks of the drugs. Individual drugs and mixture of standard drugs were run in various ratio of Acetonitrile: Water, Methanol: Water and Acetonitrile: Methanol with adjusted pH. At pH 6.8 adjusted with orthophosphoric acid in Acetonitrile and Methanol + Water in the ratio of 60: 25 + 15 drugs were showing high resolution with sharp peaks.

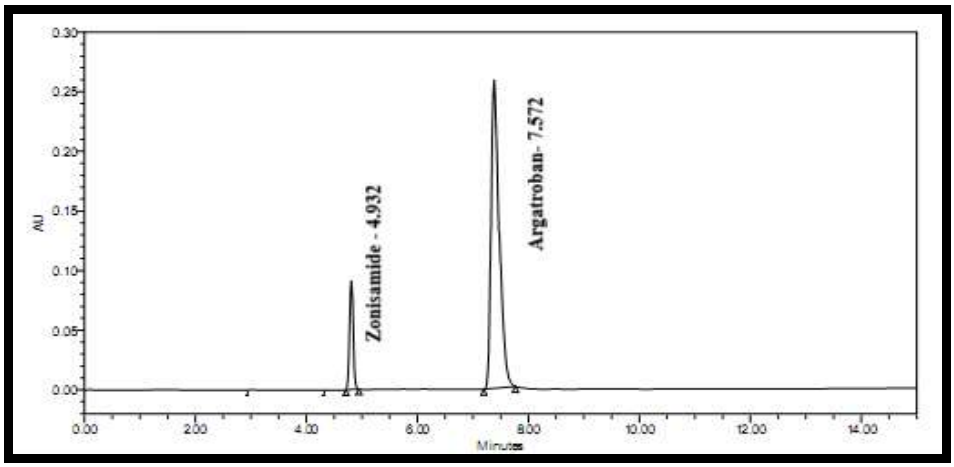

Figure 4: Chromatogram of ZSM and ARB (150 µg/mL) with optimized conditions.

Table 1: Optimized conditions for the ZSM & ARB Estimation.

Chromatographic Conditions	Results
Elution	Binary gradient
Column	C_{18} (5µ, 250 x 4.5 mm)
Mobile phase Composition (% v/v)	ACN: MeOH + Water (60: 25 + 15 v/v)
pH	6.8 Adjusted with OPA
Flow rate	1.0 mL/min
Detection wavelength	277 nm
Injection volume	20 µl
Run time	10 min
Retention Time (min)	ZSM – 4.932, ARB – 7.572

2.4.6 Validation of Method[12-13]

I. Linearity

Appropriate aliquots from ZSM and ARB stock solutions were withdrawn in separate 10 mL volumetric flask and final volume was made up to the mark with Distilled water to get the final concentration 50, 100, 150, 200 and 250 µg/mL of both the drugs.

II. Precision

a. Repeatability

Repeatability of the method was performed by six different solutions of same concentration prepared from single stock solution and were analyzed containing 150 & 150 µg/mL of ZSM and ARB, respectively.

b. Precision

Intraday & Interday precision of the proposed method was performed by analysing 3 concentration (in µg/mL) levels i.e. low, medium and high* in triplicates on the same day for Intraday study (with the interval of 2 hour) and on 3 different days for Interday study.

III. Accuracy (Recovery study)

Accuracy was determined by performing recovery studies by spiking different concentration of drug to pre-analysed sample solution of 50 µg/mL for ZSM & ARB. With different levels i.e., 80%, 100% and 120% the experiment was performed in triplicate. The result was evaluated in terms of %Recovery.

IV. Assay

500 mg of Synthetic mixture of ZSM and & ARB was prepared as per conventional drug combination ingredients with 100 mg of each drug for single drug formulation. For Synthetic

mixture formulation, whole 500 mg was taken into the mortar pestle to mix it completely. The fine mixed powder was taken in to the 100 mL volumetric flask and dissolved it with 10 mL of distilled water. Sonication was done for 10 min for complete dissolution of mixture into solvent and final volume was made up to the mark. Appropriate aliquots of solution were taken into 10 mL volumetric flask to make 150 µg/mL concentration of each drug as a working solution.

V. Robustness

Robustness of proposed method was evaluated by deliberately changing parameters like Wavelength, Mobile phase composition and pH.

2.4.7 RESULT AND DISCUSSION

A. Linearity

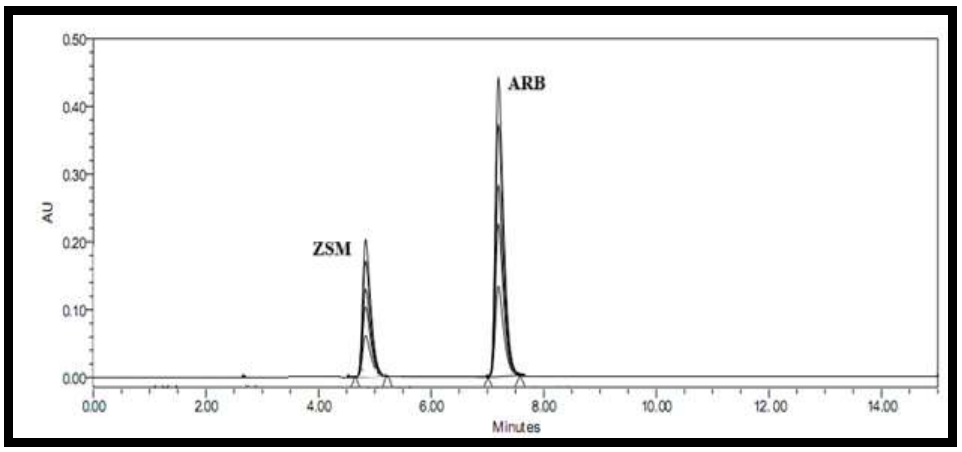

Figure 5: Overlain Chromatogram of ZSM & ARB (50 – 250 µg/mL).

Table 2: Linearity data of ZSM & ARB.

Sr no.	ZSM			ARB		
	Conc. (µg/mL)	Mean of Area ± SD (n=6)	% RSD	Conc. (µg/mL)	Mean of Area ± SD (n=6)	% RSD
1	50	4277148 ± 53896	1.260	50	4851966 ± 63147	1.301
2	100	8605300 ± 46821	0.544	100	9852469 ± 66242	0.672
3	150	12762736 ± 37068	0.290	150	14621719 ± 58642	0.401
4	200	17241088 ± 99808	0.579	200	19704939 ± 132483	0.672
5	250	21291442 ± 106332	0.499	250	24545005 ± 39624	0.161

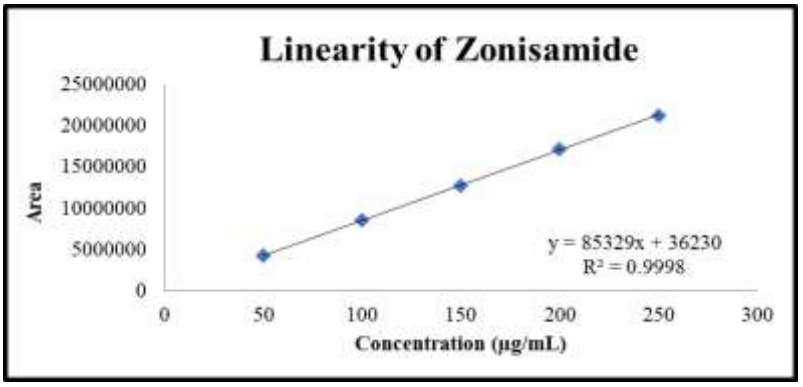

Figure 6: Calibration Curve of ZSM.

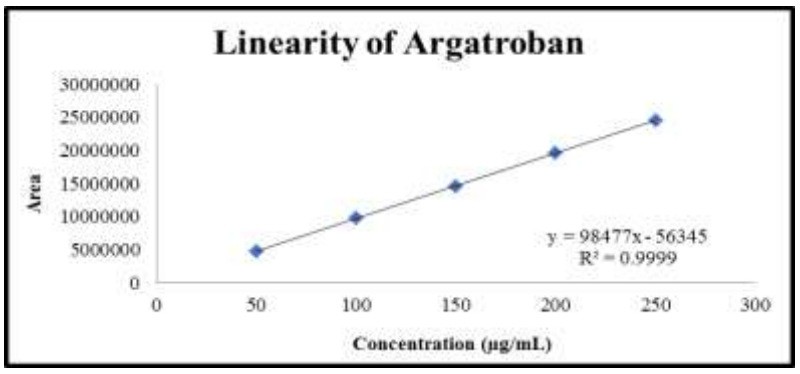

Figure 7: Calibration Curve of ARB.

Discussion: Linear correlation was obtained between absorbance vs. concentration of ZSM and ARB in concentration range of **50 – 250 µg/mL,** respectively. The linearity of calibration curve was validated by high value of correlation coefficient of regression which was 0.999 for both the drugs.

B. Precision

Table 3: Precision data of ZSM and ARB.

Repeatability (n = 6)							
Drug	**Concentration (in µg/mL)**			**Mean ± SD**			**%RSD**
ZSM	150			4412628.84 ± 25386.28			**0.58**
ARB	150			4728851.34 ± 64798.05			**1.37**
Drug	**Concentration (in µg/mL)**	**Intraday**			**Interday**		
		Area	**SD**	**% RSD**	**Area**	**SD**	**% RSD**
ZSM	**50**	4427671	2546	**0.057**	4424283	1985	**0.045**
	100	8679987	62742	**0.723**	8667177	27600	**0.318**
	150	12661611	91175	**0.720**	12692540	110662	**0.872**
ARB	**50**	4842748	12572	**0.260**	4966634	22516	**0.453**
	100	9841639	12676	**0.129**	9822615	84119	**0.856**
	150	14688135	86059	**0.586**	14710154	39921	**0.271**

Discussion: The lower RSD values of Precision study for ZSM and ARB respectively, reveals that the proposed method is precise.

C. Accuracy

Table 4: Pre-analysed conc. of ZSM & ARB.

Drug	Conc. (µg/mL)	Mean area	Pre-analysed conc. (µg/mL) ± SD	%RSD	Mean Conc. obtained (%) ± SD
ZSM	50	4346089	50.562 ± 0.25	0.50	101.24 ± 0.5103
ARB	50	4879587	50.123 ± 0.23	0.47	100.24 ± 0.4764

Table 5: Recovery study of ZSM & ARB (n=3)

Level (%)	ZSM					ARB				
	Std conc. spiked	Total conc. taken	Mean % Recovery at diff. levels ± SD	Overall Mean % Recovery (%± SD)	% RSD	Std conc. spiked	Total conc. taken	Mean % Recovery at diff. levels ± SD	Overall Mean % Recovery (%± SD)	% RSD
80	40	90	100.45 ± 0.2184	100.41 ± 0.35	**0.35**	40	90	100.20 ± 0.2073	100.12 ± 0.21	**0.20**
100	50	100	100.74 ± 0.3177			50	100	100.29 ± 0.0531		
120	60	110	100.04 ± 0.2875			60	110	99.89 ± 0.1973		

Discussion: Obtained results reveals that % recovery of ZSM and ARB are within acceptance criteria given in ICH i.e., 98-102%.

D. Limit of Detection and limit of quantification

Table 6: LOD and LOQ for ZSM and ARB.

Parameters	Zonisamide	Argatroban
LOD (µg/mL)	4.4863	1.5240
LOQ (µg/mL)	13.5948	4.6181

Discussion: LOD was found to be **4.4863 µg/mL** and **1.5240 µg/mL** respectively and LOQ was found to be **13.5948 µg/mL** and **4.6181 µg/mL** for ZSM and ARB respectively. The proposed method can detect and quantify small amount of drugs precisely. So, it was concluded that the proposed method is very sensitive in nature.

E. Analysis of ZSM and ARB in Synthetic Mixture: (Assay)

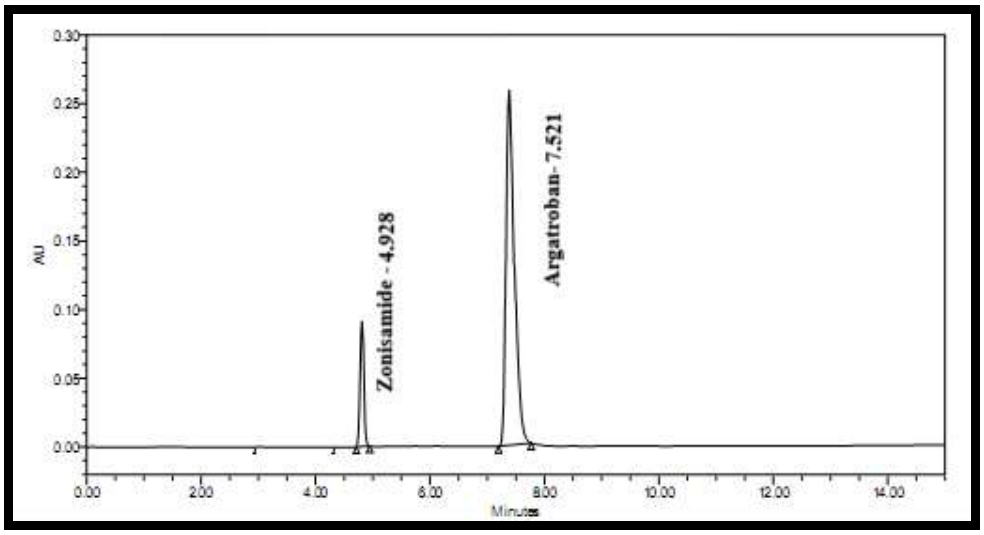

Figure 8: Chromatogram of Synthetic Mixture of ZSM and ARB (150 µg/mL).

Table 7: Analysis of ZSM and ARB (n=6).

Drug	Formulation (Synthetic mixture)		% Assay	SD	%RSD
	In mg	In µg/mL			
ZSM	100	150	101.00	0.0155	0.0154
ARB	100	150	100.76	0.0979	0.0972

Discussion: Formulation claims to contain 100 mg both ZSM and ARB. % Assay of ZSM and ARB was found in an acceptance limit so, this method could be used for analysis of this combination.

F. Robustness

Table 8: Robustness Study of ZSM and ARB.

Sr. No	Factor	Level	Peak Area* ± SD	% RSD	Rt ± SD	%RSD
ARG (100 µg/ml)						
1	Change in Flow rate ± 0.2 ml/min	0.8	9837298 ± 719	0.0073	7.522 ± 0.0015	0.0203
		1.2	9837661 ± 1062	0.0108	7.528 ± 0.0015	0.0203
2	Change in Wavelength (nm) ± 2 nm	277	9837992 ± 1083	0.0110	7.526 ± 0.0025	0.0334
		281	9837201 ± 608	0.0061	7.525 ± 0.0026	0.0352
3	Mobile Phase pH ± 0.2 pH	6.8	986905 ± 526	0.0053	7.526 ± 0.0021	0.0277
		7.2	9837075 ± 705	0.0070	7.528± 0.0015	0.0200
ZON (100 µg/ml)						
1	Change in Flow rate	0.8	8644238 ±	0.0064	4.953 ±	0.0420

	± 0.2 ml/min		555		0.0021	
		1.2	8646459 ± 1203	0.0139	4.954 ± 0.0015	0.0308
2	Change in Wavelength (nm) ± 2 nm	275	8644367 ± 332	0.0038	4.952 ± 0.0020	0.0308
		279	8644784 ± 443	0.0051	4.952 ± 0.0006	0.0117
3	Mobile Phase pH ± 0.2	6.6	8644738 ± 905	0.0104	4.951 ± 0.0015	0.0309
		7.0	8646043 ± 462	0.0050	4.955 ± 0.0015	0.0310

Discussion: The study suggested that all the parameters have no significant influence on the determination. Results indicates that the selected factors remained unaffected by small variations and % RSD was less than 2 %, which demonstrates that the proposed method was robust.

Table 9: Result summary of HPLC method for ZSM and ARB Combination

Parameters		**ZSM**	**ARB**
System suitability parameters	Retention time (min) ± SD	4.926 ± 0.08	7.542 ± 0.83
	Area ± SD	12693746 ± 36924	14621324 ± 58253
	Theoretical plates ± SD	4688.20 ± 23.35	3252.20 ± 26.19
	Tailing factor ± SD	1.05 ± 0.01	0.97 ± 0.01
	Resolution	NA	2.410 ± 0.03
Concentration range (µg/mL)		50 – 250	50 – 250
Slope		85329	98477
Intercept		36230	-56345
Correlation coefficient (R^2)		0.9998	0.9999
Precision (% RSD)		< 2.0	
Accuracy (% Recovery) ± SD		100.41 ± 0.35	100.12 ± 0.21
LOD(µg/mL)		4.4863	1.5240
LOQ(µg/mL)		13.5948	4.6181
%Assay ± SD		101.00 ± 0.01	100.76 ± 0.09
Robustness (% RSD)		< 2.0	

I. CONCLUSION

A simple, specific, and precise Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed for the simultaneous quantification of Zonisamide and Argatroban. The method utilizes cost-effective solvents that are commonly available in most quality laboratories. The results followed Beer's law, demonstrating good correlation. Minimal variation was observed in repeatability and reproducibility, with consistent and accurate outcomes from multiple measurements. The RP-HPLC method showed strong agreement with the labelled claims and was validated for the rapid determination of

Zonisamide and Argatroban in routine analyses. Validation was carried out in accordance with ICH guidelines, ensuring the method is linear, accurate, precise, specific, and robust. Given these advantages, the method is well-suited for use in quality control laboratories.

II. ACKNOWLEDGEMENTS

The authors would like to thank Laxminarayan College of Pharmacy and Dr. Hasumati Raj to give us support and encourages at every stage.

III. CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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