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Original Articles

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ORIGINAL ARTICLE

Development and Validation of High-Performance Thin Layer Chromatographic Method for the Simultaneous Estimation of Dapagliflozin and Vildagliptin in Fixed-Dose Combination

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ABSTRACT

Objectives: The objective of this study was to develop a simple, precise, and accurate high-performance thin-layer chromatographic (HPTLC) method for the simultaneous estimation of dapagliflozin (DAP) and vildagliptin (VIL) in a combined pharmaceutical formulation. Managing diabetes often involves using a combination of drugs to better control blood sugar levels. One such effective formulation is combination of DAP, an SGLT2 inhibitor, with VIL, a DPP-4 inhibitor, in a single formulation. To ensure the quality and consistency of these combination products, it is important to have a simple and reliable method for analyzing both drugs simultaneously.

Materials and Methods: An aluminium-backed pre-coated silica gel 60 F_{254} TLC plate was employed as the stationary phase. The mobile phase consisted of toluene, methanol, and ethyl acetate in a volumetric ratio of 5:3:2. Prior to plate development, the chamber was saturated with the mobile phase for 20 minutes. Detection was carried out at 210 nm, selected based on the isosbestic point of the analytes.

Results: The developed method successfully separated the analytes with retardation factor values of 0.57±0.02 for DAP and 0.26±0.02 for VIL. The method exhibited linearity in the concentration ranges of 0.6 to 1.4 µg per band for DAP, with a correlation coefficient (r^2) of 0.997 and 6 to 14 µg per band for VIL, with an r^2 of 0.998. The limit of detection was found to be 0.02 µg/band for DAP and 0.19 µg/band for VIL. Similarly, the limit of quantification was determined to be 0.07 µg/band for DAP and 0.58 µg/band for VIL.

Conclusion: The proposed HPTLC method allows for the simultaneous estimation of DAP and VIL with high accuracy, precision, and sensitivity. Owing to its satisfactory analytical performance, the method is suitable for routine quality control of combined dosage forms containing DAP and VIL.

Keywords: HPTLC, anti-diabetic, dapagliflozin, vildagliptin, method development

INTRODUCTION

Diabetes mellitus (DM) is a chronic, complex metabolic disorder associated with hyperglycemia. DM is primarily classified into type 1 and type 2 DM (T2DM). Type 1 DM is insulin-dependent, whereas type 2 is insulin-independent, which accounts for more than 85% of total affected patients worldwide.[1] Various therapeutic agents are available and are also being developed, targeting different pathophysiological aspects related to glycemic activity.[2] Metformin is one of the well-established oral anti-diabetic medications.[3] Early combination therapy provides more significant glycemic control than metformin monotherapy.[4]

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The fixed-dose combination (FDC) of dapagliflozin (DAP) and vildagliptin (VIL) is indicated for patients with T2DM uncontrolled by metformin monotherapy.[5]

DAP is chemically (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl)methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol;[6,7] it provides effective glycemic control with a low-risk of hypoglycemia, lowers body weight, and has adequate control over blood pressure. Its mechanism of action is insulin-independent.[8] It acts by inhibiting sodium glucose co-transporter 2, thereby blocking the glucose reabsorption from the kidney, which in turn increases the elimination of glucose in urine.[9,10] The structure of DAP is given in Figure 1.

VIL is chemically: (2S)-1-[2-[(3-hydroxy-1-adamantyl)amino] acetyl]pyrrolidine-2-carbonitrile.[11] It acts by enhancing the incretin levels by inhibiting dipeptidyl peptidase-4, which degrades the incretin, increasing insulin sensitivity, and decreasing glucagon secretion.[12-14] The structure of VIL is given in Figure 2.

According to the literature review, there are currently few published high-performance thin-layer chromatographic (HPTLC) methods, and only one HPTLC method is available for estimating DAP and VIL simultaneously. Still, the published HPTLC method has used benzene as a component of the mobile phase.[15] A comparison table for existing methods versus the current method is given in Table 1. We developed a safe, simple, precise, and accurate HPTLC method for the simultaneous estimation of DAP and VIL in tablet and bulk dosage forms. The method mentioned in the literature incorporated the use of benzene as a component of the mobile phase, which is a class 1 solvent and is considered carcinogenic, so we have proceeded with solvents such as toluene, ethyl acetate, and methanol, which are comparatively safer. The availability

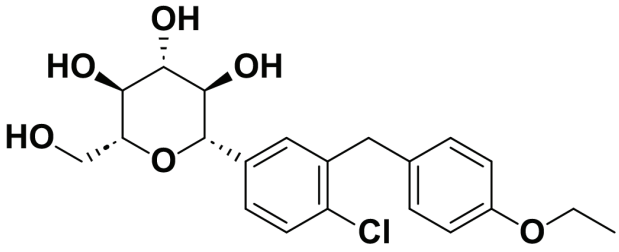

Figure 1. Structure of dapagliflozin

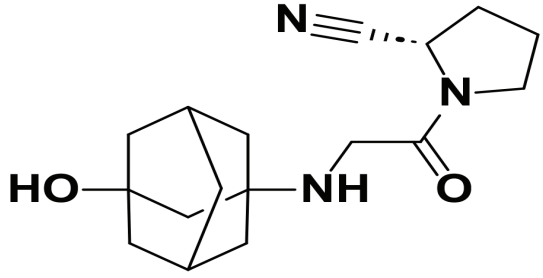

Figure 2. Structure of vildagliptin

Table 1. A comparison table for existing methods vs. the current method for simultaneous estimation of DAP and VIL

Comparison table		HPTLC		HPLC	
		Current method	Existing method	Existing method	Existing method
Stationary phase		Silica gel 60 F_{254}	Silica gel 60 F_{254}	C_{18} (250mm x 4.6mm, 5µm)	C_{18} (250mm x 4.6mm, 5µm)
Mobile phase		Toluene: ethyl acetate: methanol (5:2:3, *v/v/v*)	Acetonitrile: benzene: glacial acetic acid (9:1:2 *v/v/v*)	Methanol: 0.01% Trifluoroacetic acid (pH 2.78) (95:5% *v/v*) Isocratic mode	0.05 M KH2PO4: acetonitrile: methanol (35:10:55% *v/v/v*) Isocratic mode
Retardation factor for HPTLC	DAP	0.57	0.84	4.070 min	7.97 min
Retention time for HPLC	VIL	0.26	0.21	2.282 min	2.91 min
Linearity and range	DAP	0.6-1.4 µg/band (r^2=0.997)	0.2-2.5 µg/band (r^2=0.9931)	10-60 µg/mL (r^2=0.999)	1-5 µg/mL
	VIL	6.0-14 µg/band (r^2=0.998)	2.0-25 µg/band (r^2=0.9954)		10-50 µg/mL
LOD	DAP	0.02 µg/band	0.021 µg/band	0.3342 µg/mL	0.039 µg/mL
	VIL	0.19 µg/band	0.154 µg/band	0.9012 µg/mL	0.585 µg/mL
LOQ	DAP	0.07 µg/band	0.063 µg/band	1.0128 µg/mL	0.128 µg/mL
	VIL	0.58 µg/band	0.469 µg/band	2.7310 µg/mL	1.930 µg/mL

DAP: Dapagliflozin, HPLC: High-performance liquid chromatography, HPTLC: High-performance thin layer chromatography, LOD: Limit of detection, LOQ: Limit of quantification, VIL: Vildagliptin, min: Minimum

of robust, cost-effective, safe, and rapid analytical methods for the simultaneous estimation of these drugs in combined dosage forms remains limited. Therefore, the development and validation of an HPTLC method is essential to ensure quality control, regulatory compliance, and batch-to-batch consistency during pharmaceutical manufacturing. This study addresses this need by establishing a simple, precise, and reliable HPTLC method for the simultaneous quantification of DAP and VIL in FDCs. The developed chromatographic method was validated for multiple parameters, including linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and specificity, by ICH Q2(R1) guidelines.[16] The aim of this study is to develop a simultaneous quantitative HPTLC method for the estimation of DAP and VIL in FDCs.

MATERIALS AND METHODS

DAP and VIL pure substances were purchased from Yarrowchem (Mumbai, India) and Astitva Chemicals (Gujarat, India), respectively; the FDC tablet dosage form was procured locally; and toluene was procured from Sisco Laboratories Pvt. Ltd., Mumbai, India. Methanol (VetecTM) was obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Ethyl acetate (AvantorTM) was procured from Avantor Performance Materials, Maharashtra, India. All the reagents used for this analytical work were of Analytical Reagent grade.

Instrumentation and chromatographic conditions

Chromatography was performed by spotting the sample using a CAMAG Hamilton syringe with a capacity of 100 µL (Bonaduz, Switzerland) on a precoated silica gel 60 F254 aluminium-backed TLC plate [Merck, Darmstadt, Germany]. Using the CAMAG Linomat V applicator (Switzerland), the sample was spotted onto the plate with a bandwidth of 8 mm at a constant dosage speed of 150 nL s-1. For the chromatographic development, a 20x10 cm twin trough glass chamber (CAMAG, Switzerland) was used. We performed the chromatographic separation using the stationary phase of an aluminium-backed TLC plate pre-coated with silica gel 60 F254 (20x10 cm). The mobile phase for development was toluene, methanol, and ethyl acetate (5:3:2, *v/v/v*). For 20 minutes, the mobile phase was allowed to saturate the development chamber. At room temperature, the developed plate was air-dried for 10 minutes to evaporate the mobile phase. The CAMAG TLC scanner IV was used to scan the developed plate in the absorbance mode. The slit dimension of 6x0.45 mm and the scanning speed of 10 mm s-1 were selected for processing. For detection, a deuterium lamp was selected as the radiation source; we set the detection wavelength at 210 nm. Data analysis and interpretation were performed using CAMAG VisionCATS software (V 3.1).

Preparation of standard and sample solutions

Precisely 10 mg of DAP was weighed and transferred into a 10 mL volumetric flask. The standard DAP was then dissolved and made up to 10 mL using methanol to get the standard stock solution having a concentration of 1000 µg mL^{-1}. The working solution of the mixture of DAP and VIL was prepared by adding 1 mL of DAP stock solution into a 10 mL standard flask containing 10 mg of VIL. Using methanol, we adjusted the volume to 10 mL to get the working concentration of 100 µg mL^{-1} and 1000 µg mL^{-1} of DAP and VIL, respectively.

To prepare the sample solution, twenty tablets were accurately weighed and triturated. The powder weight equivalent to 10 mg of DAP was weighed and transferred into a 100 mL standard flask. Initially, the volume was made up to 50 mL using methanol, then sonicated for 30 minutes, and filtered. The final volume was adjusted to 100 mL by using methanol.

Validation of the chromatographic method

In accordance with ICH Q2(R1) guidelines, the developed HPTLC method was validated for specificity, accuracy, intra-day, inter-day precision, repeatability, linearity of sample application, and area under the curve measurement. Further, the LOD and LOQ for DAP and VIL were determined based on the standard deviation of the slope.

Linearity

For linearity of the method, an aliquot of 6-14 µL of working standard solution (DAP 100 µg mL^{-1} and VIL 1000 µg mL^{-1}) was used to obtain 0.6-1.4 µg/band for DAP and 6-14 µg/band for VIL. TLC plates were developed under optimized conditions and scanned using a densitometer. Peak areas were noted for the corresponding concentrations of DAP and VIL. Peak areas of DAP and VIL plotted against their corresponding concentrations were used to develop the standard calibration curve.

Accuracy

The developed HPTLC method was evaluated for accuracy at three levels in the drug product (50%, 100%, 150%) by adding a known amount of pure DAP and VIL to the product, and the recovery (%) was calculated. Triplicates were performed for each level. Results obtained from this study were then compared with those of the expected value.

Precision

To demonstrate the precision of the method, parameters such as intra-day precision, inter-day precision, and repeatability studies were employed.

Repeatability

Sample application repeatability

Sample application repeatability was evaluated by spotting DAP (0.8 µg/band) and VIL (8 µg/band) six times on a precoated TLC plate. The plate was then developed by using the optimized HPTLC method and scanned. The % relative standard deviation (RSD%) of peak areas for six spots of DAP and VIL was calculated.

Sample measurement repeatability

Sample measurement repeatability was evaluated by spotting DAP (0.8 µg/band) and VIL (8 µg/band) on a precoated TLC plate. The plate was developed. After development, the corresponding spots of DAP and VIL were scanned using a scanner six times, without any change in the position of the developed TLC plate, and the RSD% of peak areas obtained for six scans of each analyte was computed.

Intra-day precision and inter-day precision

Intra-day precision and inter-day precision were studied by analyzing the responses at three different concentration levels (0.6, 0.8, 1.0 µg/band for DAP and 6, 8, and 10 µg/band for VIL), and the RSD% values were calculated.

Detection limit and quantification limit

The LOD and LOQ for DAP and VIL of this method were calculated by using the formulae LOD = (3.3× standard deviation of intercept)/slope and LOQ = (10× standard deviation of intercept)/slope, where the slope is obtained from the line equation of the calibration graph of DAP and VIL individually.

Specificity

By comparing the band's peak start, peak apex, and peak end position spectra of standard drugs and samples, the specificity of the procedure was evaluated.

RESULTS

Optimization of chromatographic conditions and mobile phase composition

Initially, individual solvents were tried as the mobile phase based on the band shape, and further optimization of the retardation factor (R_f) values was conducted. Further, different mixtures of solvents (n-hexane: methanol: toluene, toluene: methanol: ethyl acetate) have been tried as a mobile phase for chromatographic development. In the mixture of n-hexane, methanol, and toluene, the band of VIL appeared at an R_f of 0.11, which is unacceptable. The combination of Toluene, methanol, and ethyl acetate resulted in better separation of both the standard drugs. Further, different compositions of the same mixture (Toluene: methanol: ethyl acetate, 4:4:2 *v/v/v*, 5:3:2 *v/v/v*) have been tried. Toluene: methanol: ethyl acetate, 5:3:2 *v/v/v,* resulted in proper separation and compact bands, which resulted in good band shape for both the analytes. When tried with methanol at lower levels in mobile phase composition, VIL resulted in R_f less than 0.2, which is usually an unacceptable R_f. However, higher methanol levels improved the R_f of VIL, but it resulted in the poor peak shape of DAP. Finally, the mobile phase composition was optimized to be toluene: methanol: ethyl acetate in the ratio of 5:3:2 *v/v/v*. Based on the isobestic point obtained from the overlay spectrum, 210 nm was selected as the detection wavelength. Optimization of saturation time was performed by trying different saturation periods of 10, 20, and 30 minutes. Among those, saturation times of 10 minutes, the solvent front was not linear as expected. However, with 20 and 30 minutes, a linear development was observed, and there was no significant difference between them. Therefore, 20 minutes was selected as the optimized saturation time for this method. A typical chromatogram of standard DAP and VIL separated using the proposed method is presented in Figure 3. Table 2 represents the optimal conditions for chromatography.

Linearity

The linearity of this method was established by spotting nine concentrations of the drug, which was prepared using methanol in the range of 0.6-1.4 µg/band for DAP and 6-14 µg/band for VIL.

Table 2. Fixed chromatographic conditions

Stationary phase	Silica gel 60 F_{254}
Mobile phase	Toluene: ethyl acetate: methanol (5:2:3, *v/v/v*)
Chamber saturation time	20 minutes
Bandwidth	8 mm
Slit dimension	6x0.45 mm
Detection wavelength	210 nm

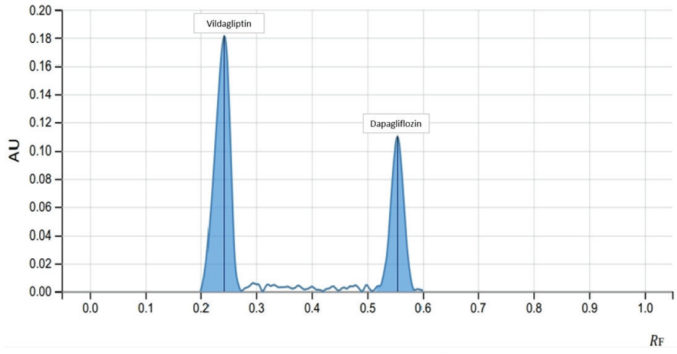

Figure 3. A typical densitogram of standard dapagliflozin and vildagliptin
AU: Absorbance unit

The calibration regression equation from the calibration plot of DAP was y = 0.0071x+0.0004, and the correlation coefficient (r^2) was 0.9972. The calibration regression equation from the calibration plot of VIL was y y = 0.0012x-0.0003, and the correlation coefficient (r^2) was 0.998. The standard calibration curves obtained for DAP and VIL are given in Figures 4 and 5.

Accuracy

The recovery (%) for DAP and VIL after spiking the known amount of standard into their pharmaceutical dosage forms fell between 95.50-100.04%, and their data are given in Table 3.

Precision

The precision of the developed chromatographic method was evaluated by calculating RSD% values for intra-day and inter-day precision, as well as the repeatability of the sample application and measurement of DAP and VIL. These values were found to be 2.0% or less, indicating that the developed method has achieved an acceptable level of precision. The data supporting the precision of the developed method are given in Tables 4 and 5.

Detection limit and quantification limit

The LOD and LOQ were calculated for the analytes DAP and VIL based on the standard deviation of the response and the slope method, as per ICH Q2(R1). The detection limit was found to be 0.02 µg/band for DAP and 0.19 µg/band for VIL, while the quantification limit was found to be 0.07 µg/band for DAP and 0.58 µg/band for VIL, respectively.

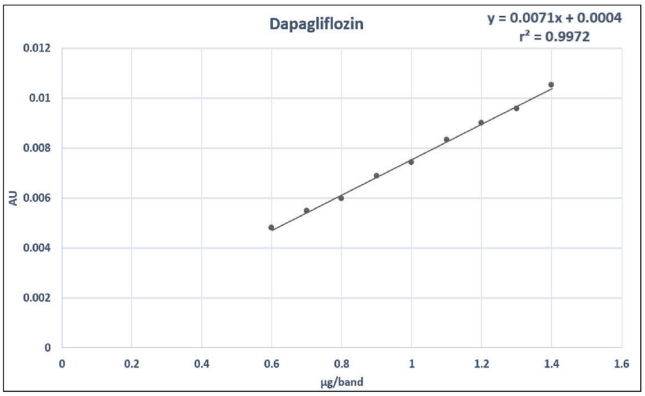

Figure 4. Calibration curve of dapagliflozin (0.6-1.4µg/band)
AU: Absorbance unit

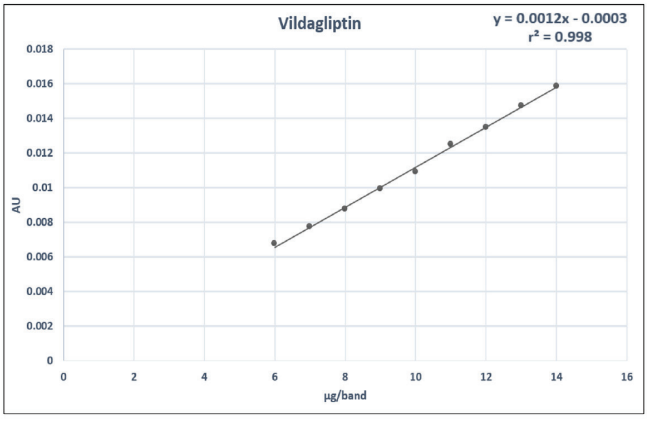

Figure 5. Calibration curve of vildagliptin (6-14µg/band)
AU: Absorbance unit

Specificity

The densitometric spectrum of the standard and the formulation was compared for both DAP and VIL at their corresponding R_f values, specifically at the peak start, apex, and end of the band. The absence of significant differences in the spectra of the standard and sample indicates that the developed method is specific.

Assay of the market available fixed-dose combination

The developed chromatographic method was used on the FDC of DAP and VIL to simultaneously estimate both analytes. The assay results obtained for the formulation are given in Table 6.

DISCUSSION

The HPTLC method is known for its ability to analyze several samples concurrently, which enables the analysis of more samples within a short time. Moreover, it is a cost-effective option.[17] In this study, an HPTLC method was developed and validated for the simultaneous estimation of DAP and VIL in an FDC.

Initial method development began with the use of a CAMAG TLC plate viewer, which operates at fixed wavelengths of 254 nm and 366 nm.[18] While DAP showed adequate ultraviolet (UV) absorption at these wavelengths, VIL did not exhibit a significant response, making it difficult to detect using this setup. This limitation required a shift in strategy. To overcome this, we proceeded with the CAMAG TLC Scanner IV, which allows scanning across a range of wavelengths. This enabled a more thorough spectral evaluation of both compounds. Through this approach, 210 nm was identified as a suitable detection wavelength, as both DAP and VIL showed adequate absorbance at this point. This wavelength provided clear, distinct bands for both analytes, making it ideal for simultaneous analysis.

Table 3. Recovery studies data for DAP and VIL (n=3)

Level	DAP		VIL	
	Recovery%	RSD*%	Recovery%	RSD*%
50%	97.92	1.08	98.17	0.81
100%	100.04	1.25	99.69	0.44
150%	95.50	0.92	96.86	1.71

DAP: Dapagliflozin, RSD: Relative standard deviation, VIL: Vildagliptin

Table 4. Intra-day and inter-day precision (n=3)

Precision studies	Analyte	µg/band	RSD%	Analyte	µg/band	RSD%
Intra-day precision	DAP	0.6	1.55	VIL	6	0.83
		0.8	1.07		8	1.46
		1.0	0.85		10	0.89
Inter-day precision	DAP	0.6	1.02	VIL	6	0.21
		0.8	0.96		8	0.93
		1.0	1.26		10	1.17

DAP: Dapagliflozin, RSD: Relative standard deviation, VIL: Vildagliptin

Table 5. Repeatability of the sample application and sample measurement

A) Repeatability of the sample application					
Concentration (µg/band)		**Peak area**		**RSD%**	
DAP	**VIL**	**DAP**	**VIL**	**DAP**	**VIL**
0.8	8	0.00592	0.00882	1.15	0.93
		0.00576	0.00872		
		0.00583	0.00868		
		0.00592	0.00882		
		0.00588	0.00891		
		0.00579	0.00877		
B) Repeatability of sample measurement					
Concentration (µg/band)		**Peak area**		**RSD%**	
DAP	VIL	DAP	VIL	DAP	VIL
0.8	8	0.00597	0.00876	0.89	0.63
		0.00590	0.00868		
		0.00592	0.00864		
		0.00584	0.00879		
		0.00598	0.00873		
		0.00589	0.00870		

DAP: Dapagliflozin, RSD: Relative standard deviation, VIL: Vildagliptin

Table 6. Result of formulation analysis (n=3)

Drug	Amount of drug (mg/tablet)		Label claim%	RSD%*
	Labelled	Estimated		
DAP	10	10.05	104.89	0.65
VIL	100	102.80	102.8	0.42

DAP: Dapagliflozin, RSD: Relative standard deviation, VIL: Vildagliptin

The issue encountered with VIL at fixed wavelengths underscores the importance of wavelength selection, especially in methods involving multiple compounds with different UV profiles. The flexibility offered by densitometric scanning allowed for effective method optimization, leading to a reliable and practical technique for routine quality control. This method demonstrates how careful wavelength selection and the right instrumentation can address detection challenges during method development. The outcome is a simple, reproducible, and sensitive procedure suitable for simultaneous estimation of DAP and VIL in combined pharmaceutical formulations.

CONCLUSION

An HPTLC method has been developed for the estimation of fixed-dose formulation of DAP and VIL. According to ICH Q2(R1) guidelines, the developed method was validated. The suggested approach was found to be accurate, precise, and specific in determining DAP and VIL in tablet formulation. As a result, the developed chromatographic method can be used for routine quality control analysis of DAP and VIL in an FDC to quantify both analytes simultaneously.

Ethics

Ethics Committee Approval: This research work does not require ethical committee approval.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: Y.V.K., S.A., Design: Y.V.K., S.A., Data Collection or Processing: Y.V.K., V.K., Analysis or Interpretation: Y.V.K., S.A., V.K., Literature Search: Y.V.K., Writing: Y.V.K., V.K.,

Conflict of Interest: The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Validation and Reliability of the Turkish Version of a Patient Satisfaction Survey for Comprehensive Medication Management

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ABSTRACT

Objectives: Comprehensive Medication Management (CMM) is pivotal in optimizing clinical outcomes through personalized medication review and patient engagement. Patient satisfaction surveys, such as the Medication Management Patient Satisfaction Survey (MMPSS), play a crucial role in assessing the quality of these services. However, there is currently no Turkish version of the MMPSS available. This study aimed to translate, culturally adapt, and validate the Turkish version of the MMPSS to assess patient satisfaction with CMM services provided by pharmacists in Türkiye.

Materials and Methods: Following established guidelines for cross-cultural instrument validation, the MMPSS was translated into Turkish and culturally adapted. The survey underwent forward translation, expert panel review, back-translation, and pilot testing. Data collection occurred in a tertiary care university hospital between September 9, 2022, and March 21, 2023. Psychometric analyses included reliability testing (Cronbach's alpha), factorial validity using confirmatory factor analysis, and test-retest reliability using the Intraclass Correlation Coefficient (ICC).

Results: A total of 124 participants (82.7%) completed the survey. Participants were mostly women (57.3%) and elderly, with a mean age of 70.43 years, three comorbidities, and six medications. The Turkish MMPSS demonstrated excellent internal consistency (Cronbach's α = 0.858) and test-retest reliability (ICC=0.937), confirming its reliability over time. Factor analysis supported a one-factor structure, consistent with the original MMPSS framework, and all items showed strong correlations.

Conclusion: The Turkish version of the MMPSS is a reliable and valid instrument for assessing patient satisfaction with CMM services in Türkiye. Its implementation can enhance the evaluation and improvement of clinical pharmacy services, ultimately promoting better patient care and outcomes.

Keywords: Patient satisfaction, pharmaceutical services, survey, questionnaire

INTRODUCTION

Medication therapy management is a service provided by pharmacists that involves reviewing and managing patients' medication regimens to optimize clinical outcomes and ensure that patients receive the most effective medication therapy to achieve their individual pharmacotherapeutic goals.[1,2] Comprehensive Medication Management (CMM) constitutes a systematic medication evaluation procedure for evaluating the appropriateness, effectiveness, safety, and practicality of medication usage, ensuring patient medication adherence. CMM represents the optimal standard of care; it involves the assessment of all medications, ranging from prescribed drugs

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to over-the-counter remedies and nutritional supplements.[3] The provision of CMM involves creating a personalized care plan aimed at achieving specific therapy goals, with active patient engagement.[4] It includes conducting thorough assessments of the patient's clinical condition regarding each medication and health concern, conducting follow-up evaluations to assess the patient's progress towards treatment goals, and collaborating with the healthcare team. In this way, patients' individual needs, medication-related problems and the outcomes of the care plan can be determined.[3] The personalized approach of the CMM service optimizes medication use and improves clinical outcomes.[4]

The relationship between CMM and patient satisfaction is significant, as CMM aims to optimize pharmacotherapeutic outcomes, which directly influences patients' perceptions of care quality. In the context of CMM, patient satisfaction is crucial for assessing the effectiveness of pharmacists' interventions and the overall quality of care.[5] Research shows that patients receiving CMM services report higher satisfaction due to improved communication, personalized medication planning, and greater involvement in their own care. Conversely, low satisfaction may reduce adherence and weaken the impact of CMM.[4,6]

Patient satisfaction is one of the most crucial components of quality assurance in healthcare services, measured by the patient's subjective experience.[7,8] By evaluating results of patient satisfaction surveys, healthcare providers can identify areas for improvement in the services offered to patients and optimize resource utilization.[8,9] Evaluation of satisfaction has led to a rise in projects focusing on understanding the concept of satisfaction, determining factors influencing patient satisfaction, and developing patient satisfaction questionnaires.[10,11] One study emphasized that patient satisfaction is associated with factors such as the quality of information provided, the level of attention received, and the time allocated, regardless of physical conditions.[9] A systematic review showed pharmacists' care services contribute to the management of medication-related problems, increase patient compliance, reduce health care costs, and increase patient satisfaction.[12] Previous studies have identified positive correlations between patient satisfaction and various factors such as patients' adherence to treatment, continuity of healthcare, collaboration with health professionals, and health outcomes.[13-15]

Therefore, evaluating patients' satisfaction with a CMM service is considered valuable in facilitating the dissemination and implementation of such a service.

While numerous instruments have been developed to assess patient satisfaction, none have been specifically designed to evaluate pharmacist-led CMM services, which were the focus of this study. A key strength of the Medication Management Patient Satisfaction Survey (MMPSS) is that it was specifically developed to assess patient satisfaction within the context of CMM services.

Additional strengths of the MMPSS include its focus on the process dimension of healthcare quality, as outlined by Donabedian's framework, in which emphasis is placed on the interactions and activities constituting care delivery, rather than structural components or solely outcomes.[16] This process-oriented focus aligns well with the personalized nature of CMM, involving patient engagement, medication education, and collaborative planning. Other strengths of the MMPSS include its brevity (10 items), clarity, and specificity to pharmacist-led interventions, making it suitable for routine use in clinical settings. However, despite these strengths, the scale has only been validated in limited cultural contexts.[4,6] No Turkish version of the MMPSS exists to date, and its adaptation may offer a valuable contribution to the assessment of CMM quality in Türkiye. Therefore, this study aims to translate, culturally adapt, and evaluate the psychometric properties of the MMPSS in Turkish, filling a notable gap in measuring patient satisfaction with CMM services in local healthcare settings.

MATERIALS AND METHODS

The MMPSS

Approval was obtained from the author of the original English version of the MMPSS for its use. As part of the present study, the Turkish version of the MMPSS was developed for the first time through translation, cultural adaptation, and psychometric validation.

The MMPSS was originally developed to assess patient satisfaction specifically with CMM services provided by pharmacists. The tool aims to measure whether pharmacists helped patients understand their medications, supported medication adherence, and encouraged active involvement in care decisions. It consists of 10 items covering three conceptual domains: (1) addressing medication-related needs, (2) patient activation through pharmacist-patient engagement, and (3) overall satisfaction with the service. The MMPSS consists of 10 items. The first 9 items are rated on a 4-point Likert scale (from "strongly agree" to "strongly disagree"), while the tenth assesses overall satisfaction using a 5-point scale (from "excellent" to "poor"). Additionally, a "not applicable" option is provided for 6 items. Furthermore, there is an open-ended free-text question concerning service improvement suggestions; however, it was excluded from the quantitative analysis. The first 9 items are combined into a total score, with lower scores reflecting greater satisfaction, while the 10th item is scored separately, with higher values indicating lower satisfaction.[6]

Study population and setting

This study was carried out in a tertiary care university hospital between September 9, 2022 and March 21, 2023. The study site is an academic teaching hospital with 216 beds, including four intensive care units (82 beds) and services (134 beds). Inclusion criteria were patients over the age of 18 who were hospitalized in internal medicine, pulmonary diseases, or infectious diseases departments for at least 48 hours and had at least one chronic disease. Patients not fluent in Turkish or diagnosed with dementia, Alzheimer's disease, or psychological disorders affecting compliance were excluded. A demographic data collection tool was used to collect data on patient

characteristics such as age, gender, education level, household size, comorbidities, and medications.

The study was carried out in alignment with the Helsinki Declaration, and the Lokman Hekim University Scientific Research Ethics Committee reviewed and approved the study protocol (approval number: 2022135, dated: 15.10.2022). All the participants provided informed consent.

Sample size

In research studies, determining an appropriate sample size is crucial for ensuring the validity and reliability of study findings. In scale-based research, it is often recommended to include a minimum of 10 respondents per scale item to ensure sufficient statistical power and reliability.[17,18] This guideline ensures adequate statistical power to identify significant relationships within the data.

In the present study, a 10-item scale was used to assess patient satisfaction. According to the above-mentioned approach, a minimum of 100 participants was initially targeted (10 items*10 respondents per item). Ultimately, the analysis was conducted using data collected from 124 participants.

This approach aligns with commonly applied practices in survey-based research and helps ensure that the sample size is sufficient to yield reliable results while remaining practical and manageable. By applying this method, the study aimed to contribute to the literature with statistically sound and generalizable findings.

Preparing the Turkish version of the survey

The survey was translated into Turkish by five independent pharmacists fluent in English and native Turkish speakers. English and Turkish versions of the survey were individually reviewed by an expert committee of four bilingual (Turkish/English) clinical pharmacists, one of whom is a professor of clinical pharmacy. The expert committee utilized a language consistency form[19] and a translation evaluation form[20] to identify and discuss any discrepancies in language and meaning. The agreed Turkish survey was subsequently back-translated into English by two pharmacists fluent in both languages, who were not previously involved in the translation process. The back-translated, survey was compared to the original English version by the expert committee. At this phase, additional changes were not required due to the close similarity between the back translations and the original text. The finalized Turkish survey underwent cultural adaptation with the participation of pharmacists for language and understandability assessment. Survey participants were asked to identify any incomprehensible items and offer suggestions as needed. Following the pilot study, the Likert scale rating was adjusted from strongly disagree to strongly agree, and the scoring of the last question was modified from 1 (very poor) to 5 (excellent). The Turkish version of MMPSS is provided in Supplementary 1.

Test-retest reliability assessment

To assess the test–retest reliability of the Turkish version of the MMPSS, the survey was administered twice to the same group of patients with a two-month interval between administrations. This interval was selected based on expert consultation involving clinicians, pharmacists, and statisticians, considering its suitability for potential future correlations with additional parameters such as medication adherence, patient knowledge, and others, which are commonly monitored over similar timeframes.[21] It was also assumed that patients with chronic conditions would maintain clinical stability during this period, and no major changes in health status were observed. The follow-up surveys were conducted via telephone, and both administrations were carried out by the same clinical pharmacist to ensure consistency in data collection. A total of 124 participants completed both the initial and follow-up surveys, administered two months apart, and their data were included in the test–retest reliability analysis.

Statistical analysis

Statistical analyses were conducted using R (version 4.3.1, https://cran.r-project.org), SPSS for Windows Version 23.0, and AMOS (23); conducted under the guidance of an academic biostatistician. The reliability (internal consistency, test-retest reliability) and validity (structural) of MMPSS were evaluated. Test-retest reliability was measured using the Intraclass Correlation Coefficient (ICC), with values between 0.60 and 0.80 indicating good reliability and values above 0.80 indicating excellent reliability. The Bland-Altman graphical approach, via the "BlandAltmanLeh" package, was used to evaluate agreement.[22] To assess test-retest reliability, the ICC was used, which is an appropriate method for evaluating the consistency of measurements for continuous data. In addition, the Bland-Altman analysis was performed to visually assess the agreement between two administrations of the scale and to examine potential systematic bias or limits of agreement. Together, these two methods provided a more comprehensive evaluation of the measurement stability. Internal consistency, indicating result homogeneity, was assessed with Cronbach's alpha, with values exceeding 0.80 considered to indicate high internal consistency.[23] The analysis was performed on the first nine items, excluding the 10th item due to its distinct scoring format. Confirmatory factor analysis verified the factor structure. To achieve the best-fit model, the following indices were targeted: a Tucker Lewis index ≥0.90; a Satorra-Bentler scaled chi-square/degrees of freedom ratio (CMIN/df) ≤3; a comparative fit index (CFI) ≥0.95; a normed fit index ≥0.90; a low root mean square error of approximation (RMSEA) ≤0.08; an incremental fit index (IFI) ≥0.90; and a goodness-of-fit index (GFI) ≥0.90.[24]

The "metan" package was used for Pearson correlation coefficients.[25] Discriminant validity was assessed using receiver operating characteristic (ROC) curve analysis and the corresponding area under the curve (AUC) values, to evaluate the ability of the scale to distinguish between patients receiving pharmaceutical care from different healthcare settings. Additionally, using the Kruskal–Wallis test, differences in total MMPSS scores were analyzed across the subgroups of Item 10, which measures overall satisfaction on a 5-point scale ranging from poor to excellent. Dunn's Bonferroni post-hoc

test was applied to determine which subgroup contributed to the observed differences. A two-tailed *p*-value of <0.05 was considered statistically significant.

RESULTS

Participants' characteristics

A total of 124 participants who completed both the initial and follow-up surveys, administered two months apart, were included in the analysis. Most of the participants were female (57.3%) and over 65 years of age (72.6%). Participants' mean age was 70.43±14.24 years. Among the participants, 88.7% reported living with someone and 62.1% had less than 8 years of education. The median number of comorbidities was 3 [interquartile range (IQR): 2–4], and the median number of medications was 6 (IQR: 4–10). A detailed summary of the demographic characteristics is presented in Table 1.

Construct validity

Factor analysis

Factor analysis was conducted during the questionnaire validation process to define constructs (factors) and their associated items. A conceptual one-factor structure was applied to the model using data from 124 participants. Fit measures were assessed and detailed in Table 2. Fit indices were calculated, including CMIN/df=1.549, CFI=0.981, IFI=0.981, GFI=0.946, RMSEA=0.067. Based on the modification indices provided in Table 2, it was concluded that the values are in an acceptable range for the measurement model's fit. As a result, a scale structure consisting of 9 items and one dimension was validated. Figure 1 displays the results of the confirmatory factor analysis. As seen in the diagram, all items loaded significantly on a single latent factor, with standardized factor loadings ranging from 0.85 (Item 4) to 1.00 (Item 3). These high factor loadings suggest that all items are strongly related to the underlying construct measured by the scale, supporting its unidimensionality.

A positive correlation was found between all items in the scale. The strongest correlation (0.85) was seen between Item 4 "My clinical pharmacist helped me find easier ways to take my medicines" and Item 5, "My clinical pharmacist helped me understand the best ways to take my medicines". This strong relationship suggests that the two items conceptually overlap and measure similar components of patient satisfaction. The weakest correlation (0.19) was observed between Item 1 and Item 4, as well as between Item 2 and Item 8. These low correlations indicate that the items represent different aspects of satisfaction and contribute to the overall diversity of the scale. Inter-item correlations are visualized in the matrix presented in Figure 2.

There was a statistically significant difference in the total MMPSS scores (Items 1–9) across the response categories of Item 10, which ranges from "poor" to "excellent" ($p < 0.001$). Participants who have a score of 4 on Item 10 have a higher MMPSS score (median 24). This finding supports the criterion validity of the scale, suggesting that patients who are generally

Table 1. Participant's demographic characteristics

		Values, n (%)
Age	Mean ± standard deviation	70.43±14.24
	≥65 age	90 (72.6)
Gender	Female	71 (57.3)
	Male	53 (42.7)
Body mass index	Mean ± standard deviation	29.19±6.97
Living alone	Yes	14 (11.3)
	No	110 (88.7)
Literacy	Literate	106 (85.5)
	Illiterate	18 (14.5)
Educational qualification	<8 years	77 (62.1)
	8-12 years	25 (20.2)
	>12 years	22 (17.7)
Smoking status	Smoker	16 (12.9)
	Former smoker	34 (27.4)
	Non-smoker	74 (59.7)
Number of comorbidites	Median (IQR 25–75%)	3 (2–4)
Number of medications	Median (IQR 25–75%)	6 (4–10)

IQR: Interquartile range

Table 2. Confirmatory factor analysis results

Parameter	Abbreviation	Acceptable range	Initial model	Final model
Chi-square fit test	*CMIN/df*	2≤ CMIN/df ≤3	9.176	1.549
Comparative fit index	*CFI*	0.95≤ CFI ≤0.97	0.635	0.981
Goodness of fit index	*GFI*	0.85≤ GFI ≤0.90	0.707	0.946
Normal fit index	*NFI*	0.90≤ NFI ≤0.95	0.613	0.949
Tucker-Lewis index	*TLI*	*TLI ≥0.95*	0.513	0.967
Incremental fit index	*IFI*	0.90≤ IFI ≤0.95	0.640	0.981
Root square mean error of approximation	*RMSEA*	0.05≤ RMSE ≤0.08	0.258	0.067

CMIN/df: Chi-square minimum/degrees of freedom ratio, CFI: Comparative fit index,GFI: Goodness-of-fit index, IFI: Incremental fit index, RMSEA: Root mean square error of approximation, NFI: Normed fit index, TLI: Tucker–Lewis index

satisfied with their medication management services tend to report higher satisfaction across specific service aspects assessed by the first 9 items. Moreover, in practice, Item 10 may function as a quick screening item for identifying patients with potential dissatisfaction. For instance, a low score on Item 10 could trigger an alert in an electronic system, prompting the healthcare provider to review detailed responses to Items 1–9 to identify which aspects of the CMM service may require improvement. This enhances the utility of the MMPSS not only as a research tool but also as a practical instrument for ongoing quality improvement in pharmacist-led care.

ROC analysis

In this study, the MMPSS scale, which was analysed for validity and reliability, was applied to patients with at least one chronic disease who had been hospitalized for a minimum of 48 hours. These patients were divided into two groups: those who received pharmaceutical care from the clinical pharmacy unit of a hospital and those who received care from a community pharmacy. To evaluate the discriminant validity of the scale, a ROC analysis was conducted, using the type of pharmaceutical care setting as the criterion variable. This analysis aimed to determine the ability of the total MMPSS score to distinguish between groups. The rationale for using ROC analysis in this context is to assess how well the scale differentiates patients based on the type of pharmaceutical care setting. The AUC for 9 items of the MMPSS scale was 0.909 (p-value<0.001), indicating excellent discriminative ability. As shown in Figure 3, the scale demonstrated strong performance in distinguishing between patient groups.

Reliability

Internal consistency

Cronbach's alpha was employed to evaluate the internal consistency reliability of the scale. Cronbach's alpha coefficient (α=0.858) was calculated for the first nine items of the MMPSS, as the 10th item uses a different response format and was excluded from internal consistency analysis. This result indicates excellent internal consistency. These results are shown in Table 3.

Test-retest reliability

A total of 124 participants completed the scale again two months later for test-retest analysis. The baseline and follow-up mean scores were 21.16 and 21.31, respectively. Test-retest reliability was found to be excellent, with an ICC value of 0.937 (95% confidence interval: 0.912–0.956). The data points in the Bland-

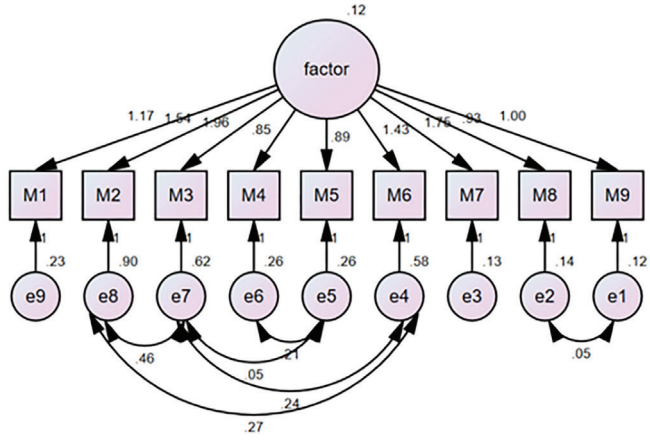

Figure 1. Diagram of confirmatory factor analysis (adjusted model)

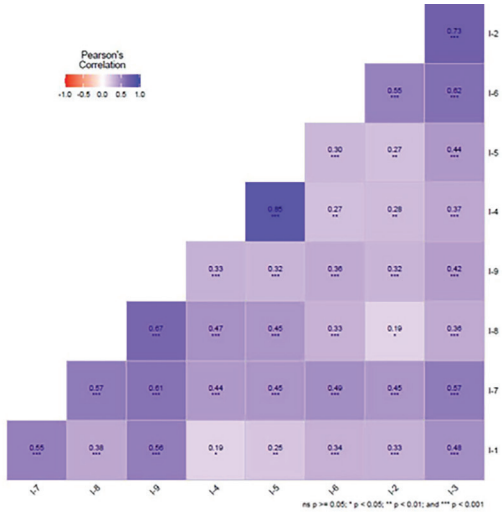

Figure 2. Correlation matrix plot for items (1-9)

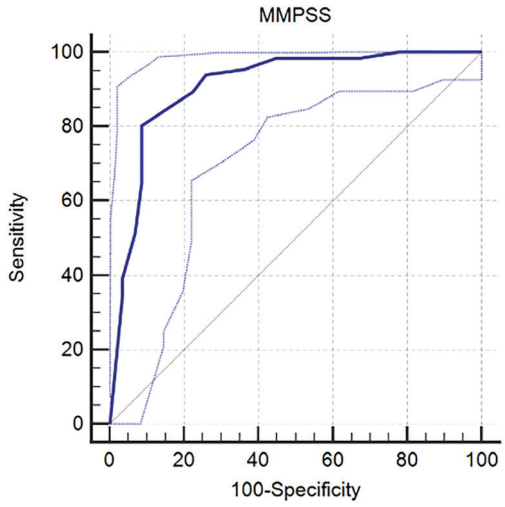

Figure 3. ROC curves plot

MMPSS: Medication Management Patient Satisfaction Survey, ROC: Receiver operating characteristic

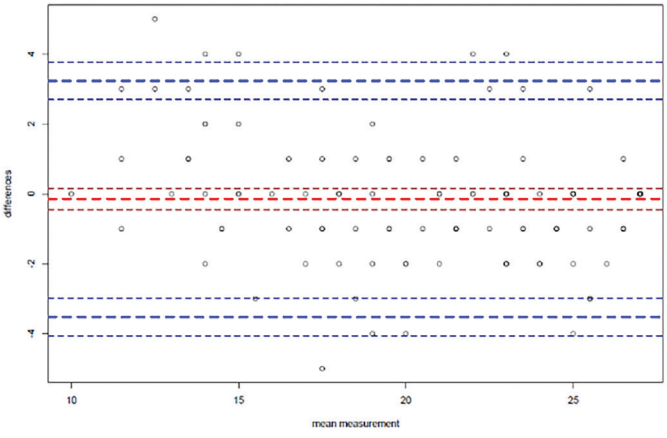

Figure 4. Test-retest results with Bland-Altman plot of MMPSS

MMPSS: Medication Management Patient Satisfaction Survey

Altman graphs are very close to the zero line, indicating that the agreement between the test-retest results is at a reliable level. Figure 4 illustrates the Bland-Altman plot for test-retest reliability. The majority of data points lie within the 95% limits of agreement, and no systematic bias was observed, indicating good agreement between the two administrations of the scale.

DISCUSSION

Patient satisfaction is a critical component of quality assurance in healthcare services. Evaluating satisfaction helps identify areas for improvement, optimize resource use, and improve overall patient care. Previous studies have shown that high levels of patient satisfaction correlate with better adherence to treatment, continuity of care, and improved health outcomes.[11-13] In this context, there is an increasing necessity for user-friendly and comprehensible assessment tools to evaluate patients' perceptions of the CMM services provided by pharmacists, the quality of these services, and patients' satisfaction with them. To the best of the authors' knowledge, this is the first study validating and exploring the psychometric properties of the Turkish version of the MMPSS. The study aimed to translate, culturally adapt, and assess the psychometric properties of the MMPSS for use in Türkiye. In addition to the original English version, there is only a Lebanese version of the MMPSS. The results demonstrated that the Turkish version of the MMPSS is a reliable and valid instrument for evaluating patient satisfaction with CMM services provided by pharmacists.

Reliability and validity of the Turkish MMPSS

The psychometric analysis of the Turkish MMPSS demonstrated excellent reliability and validity. Internal consistency, assessed by Cronbach's alpha (α=0.858), had high reliability, similar to the Lebanese version (α=0.90) and the original version (α=0.95).[4,6] Unlike the Lebanese version, test-retest reliability was analysed in the Turkish version and the ICC value was excellent (0.937),[4] suggesting that the survey consistently measures patient satisfaction over time. The Turkish version showed a lower RMSEA value (0.067) compared to the Lebanese version (0.10),

Table 3. Item-level analysis and Cronbach's alpha coefficients for the MMPSS

Scale	Subjects (n)	Mean (SD)	Range (min.-max.)	Corrected item-total correlation	Cronbach's alpha if item deleted
Item-1	124	2.69 (0.629)	0-3	0.527	0.848
Item-2	124	1.56 (1.091)	0-3	0.599	0.848
Item-3	124	1.94 (1.046)	0-3	0.763	0.823
Item-4	124	2.60 (0.596)	1-3	0.525	0.849
Item-5	124	2.63 (0.604)	1-3	0.555	0.846
Item-6	124	1.81 (0.914)	0-3	0.608	0.841
Item-7	124	2.39 (0.707)	1-3	0.722	0.830
Item-8	124	2.76 (0.500)	1-3	0.548	0.849
Item-9	124	2.77 (0.491)	1-3	0.596	0.846

Cronbach's alpha=0.858. SD: Standard deviation, min.-max: Minimum-maximum, MMPSS: Medication Management Patient Satisfaction Survey

indicating a better fit between the model and the observed data. The confirmatory factor analysis supported a one-factor structure, with fit indices within acceptable ranges, further affirming the survey's validity.

These findings suggest that the Turkish version of the MMPSS is not only psychometrically sound but also practical for use in longitudinal studies and routine clinical settings. The strong internal consistency and test-retest reliability support its applicability in monitoring patient satisfaction over time in various healthcare environments, including hospitals and community pharmacies. Furthermore, the validated scale can serve as a valuable tool for national quality improvement initiatives aiming to enhance the delivery of pharmacist-led CMM services in all healthcare settings across Türkiye.

When comparing the Turkish version with the existing Lebanese adaptation, some methodological and structural differences can be observed. For instance, in the Lebanese version, the word "clinical pharmacist" was removed and replaced with "pharmacist", and exploratory factor analysis was performed. Explanatory factor analysis was not performed in the present study, as no modifications were made to the original survey structure. Similar to what was observed in the Lebanese version, positive correlations were observed among all items in the current study. The strongest correlations were identified between similar items (Items 4 to 9) in both the Turkish and Lebanese versions.

Cultural adaptation and its challenges

The process of translating and culturally adapting the MMPSS involved multiple steps to ensure the survey was both linguistically and contextually appropriate for Turkish patients. The translation by five independent pharmacists and subsequent review by a bilingual expert committee ensured that the survey retained its original meaning and relevance. The back-translation process confirmed the accuracy of the Turkish version.

A pilot study was conducted to assess the clarity and comprehensibility of the Turkish version. Participants were asked to identify any unclear expressions or questions; no major linguistic challenges were reported. While the 4-point Likert scale structure of the original tool was retained and easily understood by respondents, a minor modification was made to the final question's scoring direction—from "excellent to poor" to "poor to excellent"—to align with the general scoring format used in Türkiye.

In addition, although there are cultural differences between this study and other versions, the demographic characteristics of the participants are similar. In the Turkish version of the study, most of the participants were female and over the age of 65, similar to the other studies. The number of comorbidities and medications reported by participants was similar to those reported in the Lebanese version of the study.

Implications for practice and policy

Building upon these findings, considering how the Turkish version of the MMPSS may contribute to improving pharmaceutical care services and inform healthcare strategies at clinical and policy levels. Importantly, the ultimate goal of pharmaceutical care is to improve patients' quality of life through the responsible provision of drug therapy to achieve defined outcomes.[26] When the goal is to enhance humanistic outcomes such as patient satisfaction, these outcomes must be measurable. However, there is currently no widely adopted tool specifically designed to assess this domain. The MMPSS fills this gap by offering a standardized and validated instrument that helps identify deficiencies in pharmacist-led care processes, thereby supporting systematic improvement in pharmaceutical care quality.

Given the strong association between patient satisfaction and quality of life, regular assessment using a tool like the MMPSS becomes critical. The high ICC and Cronbach's alpha values reported in this study indicate that the Turkish version is reliable for longitudinal evaluations and can be used in routine clinical settings such as hospitals and community pharmacies. Moreover, it holds value for integration into national quality improvement programs and health policy initiatives. Embedding the MMPSS into electronic health records would allow the standardized collection of patient satisfaction data, producing actionable insights to guide the enhancement of pharmacist-led care services across Türkiye.

Study limitations

While this study provides a robust tool for assessing patient satisfaction with CMM services in Türkiye, there are limitations to consider. Although all patients were recruited from a single tertiary healthcare institution, the pharmaceutical care services they received were delivered across different healthcare settings. Therefore, while the recruitment site was singular, the care contexts were diverse. Moreover, the patient population represents a relatively homogeneous group from a specific geographical region, which allows for consistent evaluation but may limit broader applicability. To enhance generalizability, future research should validate the Turkish version of the MMPSS in more heterogeneous populations across multiple regions and healthcare institutions.

Another limitation is the two-month interval between the test and retest administrations. This duration was selected based on its suitability for potential future correlations with parameters such as medication adherence and knowledge level, which are commonly evaluated over similar periods in chronic care settings. A multidisciplinary team also agreed that this timeframe would not likely result in major changes in the health status of patients with stable chronic conditions, making it appropriate for test–retest analysis. Nonetheless, the extended interval may have introduced some variability and should be considered when interpreting the results.

Future research should aim to validate the Turkish MMPSS in diverse healthcare environments and with larger, more varied patient populations. Additionally, increasing the sample size could enhance the modification indices and further reduce the root mean square error. This study is the first to validate the psychometric properties of the Turkish version of MMPSS.

Exploring the impact of CMM services on clinical outcomes and healthcare costs in the Turkish context would provide a more comprehensive understanding of its benefits.

CONCLUSION

The primary objective of the present study was the validation and reliability assessment of a patient satisfaction survey for CMM. Evidence indicates a strong correlation between patient satisfaction, treatment adherence, and positive health outcomes; this underscores the importance of using satisfaction assessment tools. The findings indicate that the Turkish version of the MMPSS is a reliable and valid instrument for evaluating patient satisfaction with CMM services. The scale demonstrated high internal consistency (α=0.858), excellent test-retest reliability (ICC=0.937), and satisfactory model fit indices, confirming its robustness for repeated applications. Given its strong psychometric performance, the Turkish MMPSS can be used not only in research settings but also as a practical tool for routine use in hospitals, community pharmacies, and national quality improvement efforts. Future studies are recommended to explore its applicability in various healthcare settings and to assess its utility in intervention studies targeting pharmacist-led care. The Turkish MMPSS will serve as a valuable instrument for systematically evaluating and improving patient-centered pharmacy services across Türkiye.

Ethics

Ethics Committee Approval: The study was carried out in alignment with the Helsinki Declaration, and the Lokman Hekim University Scientific Research Ethics Committee reviewed and approved the study protocol (approval number: 2022135, dated: 15.10.2022).

Informed Consent: All the participants provided informed consent.

Footnotes

Authorship Contributions

Concept: C.A., A.Ç., Ş.A., Design: C.A., A.Ç., Ş.A., Data Collection or Processing: C.A., P.B., Analysis or Interpretation: C.A., P.B., H.A., A.Ç., Ş.A., Literature Search: C.A., P.B., A.Ç., Ş.A., Writing: C.A., P.B., H.A.

Conflict of Interest: The authors declare no conflicts of interest.

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Supplementary 1. Turkish patient satisfaction survey for comprehensive medication management

#	Sorular	Kesinlikle katılmıyorum	Katılmıyorum	Katılıyorum	Kesinlikle katılıyorum	
1	Klinik eczacım ilaçlarımın her birini neden kullandığımı anlamama yardımcı oldu.					
2	Klinik eczacım ilaçlarımın işe yarayıp yaramadığını nasıl anlayacağım konusunda yardımcı oldu.					
3	Klinik eczacım (ilaçlarımın olası yan etkilerini bilerek ve ilaç etkileşimlerini önleyerek) ilaçlarımın güvenli olduğundan emin oldu.					
4	Klinik eczacım ilaçlarımı kullanmanın daha kolay yollarını bulmama yardımcı oldu.					
5	Klinik eczacım ilaçlarımı kullanmanın en iyi yollarını anlamama yardımcı oldu.					
6	Klinik eczacım, benimle ilgilenen diğer sağlık çalışanları ile birlikte takımın bir üyesi olarak çalışıyor.					
7	Klinik eczacımla konuştuktan sonra, ilaçlarımı yönetme konusunda kendime daha çok güveniyorum.					
8	Klinik eczacım ilaçlarım hakkındaki endişelerimi dinledi.					
9	Klinik eczacımı aileme veya arkadaşlarıma tavsiye ederim.					
#	Sorular	1 (Çok kötü)	2	3	4	5 (Mükemmel)
10	Genel olarak, klinik eczacıdan aldığınız bakım ve hizmetlerin kalitesini nasıl değerlendirirsiniz?					

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ORIGINAL ARTICLE

The Administration of Melatonin Improved Depressive Behavior in Both Maximal Electroshock Seizure-Prone and Non-Seizure Mice After Undergoing Levetiracetam Treatment

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ABSTRACT

Objectives: Comorbid psychiatric disorders, especially depression, pose challenges in epilepsy. Antiepileptic drugs, including levetiracetam, can also have psychiatric adverse effects, necessitating strategies to address mood regulation. The study aims to assess the impact of melatonin administration on depressive behavior in epileptic and non-epileptic mice.

Materials and Methods: Male albino mice were assigned to different treatment groups. Levetiracetam (20 mg/kg ip) was injected for 14 days; melatonin (25 mg/kg ip) was injected for 7 days. Additional groups were included for epileptic mice. Maximal electroshock was used to induce seizures: locomotor activity, immobility time in the forced swimming test (FST), latency, and food consumption were measured in the novelty-suppressed feeding test (NSFT).

Results: There were insignificant differences in locomotor activity between groups. In the FST, levetiracetam administration significantly increased the immobility duration compared to the control group in epileptic and non-epileptic mice ($p<0.05$). The immobility duration in the levetiracetam-melatonin groups of both epileptic and non-epileptic mice significantly decreased compared to the levetiracetam alone group ($p<0.01$). In NSFT, the levetiracetam group exhibited a significantly longer latency ($p<0.01$) and less food intake ($p<0.05$) compared to the control group; these changes were reversed when levetiracetam-melatonin was administered. In epileptic groups, the difference in latency was insignificant, while food consumption increased significantly ($p<0.05$) in the levetiracetam-melatonin group compared to the levetiracetam-alone group. The results observed with melatonin were similar to those of imipramine.

Conclusion: Melatonin was found to reduce depressive behavior in both non-epileptic and epileptic groups. These results suggest that melatonin could be a potential therapeutic agent for countering the depressive effects of levetiracetam.

Keywords: Depression, melatonin, levetiracetam, epileptic seizure, animal behavior

INTRODUCTION

Depressive symptoms in individuals with epilepsy can have a profound impact on their quality of life, cognitive function, and treatment outcomes, necessitating a comprehensive understanding of the underlying mechanisms and potential interventions.[1,2] Antiepileptic drug (AED) therapy, while essential for seizure control, has been associated with an increased risk of psychiatric adverse effects, including depression.[3] Levetiracetam, a widely prescribed AED, has been specifically implicated in the development or exacerbation of depressive symptoms in patients.[4] Consequently, exploring

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strategies to mitigate the negative impact of AED treatment on mood regulation becomes crucial in optimizing the overall well-being of individuals with epilepsy.[5]

Melatonin, a hormone released by the pineal gland, has garnered considerable attention for its potential therapeutic effects beyond its primary role in sleep regulation.[6] Beyond its established circadian rhythm regulatory functions, melatonin has demonstrated neuroprotective, anti-inflammatory, and antioxidant properties, suggesting a broader role in neurological and psychiatric disorders.[7] In the context of epilepsy, studies have indicated melatonin's anticonvulsant properties and its potential to enhance the efficacy of AEDs, including levetiracetam.[8]

Preclinical studies have confirmed that melatonin administration can alleviate depressive-like behaviors in animal models, including those with epilepsy-related comorbidities.[9] Notably, melatonin has been shown to regulate the levels of neurotransmitters such as serotonin and dopamine, which play crucial roles in mood regulation. By targeting these pathways, melatonin may help restore the imbalance associated with depressive symptoms in individuals with epilepsy.[10,11] Furthermore, an investigation demonstrated that sub-chronic melatonin treatment extended the time it took for pilocarpine-induced convulsions to occur in rats.[12] In a separate study, the persistent use of a melatonin receptor agonist was linked to decreased immobility time in Flinders Sensitive Line rats during the forced swimming test (FST) and an antidepressant influence.[13]

This study aims to evaluate the impact of melatonin administration on depressive behavior in mice treated with levetiracetam, considering the presence or absence of seizures. Behavioral paradigms targeting depressive-like symptoms and locomotor activity will be utilized.

MATERIALS AND METHODS

Animals

Male albino mice weighing 27±2 g (6-8 weeks old) were housed under standard conditions, with access to standard mice chow and tap water, and maintained in a 12-hour light-dark cycle. The mice were acclimated to the behavioral laboratory for 48 hours prior to the experiments. All experimental procedures were conducted in accordance with the guidelines set by the National Ethical Committee of Iran (approval number: IR.MUI.RESEARCH.REC.1401.042, dated: 26.04.2022), to minimize animal distress and the number of animals used in the study. To minimize potential interventions on the animals' behavior, the tests were conducted between 8 AM and 2 PM.

Chemicals

Levetiracetam (Amin Daru Isfahan, Iran), melatonin (Nutralab, Canada), and imipramine (Sigma-Aldrich, Germany).

Study design

The animals were divided randomly into 13 groups, each consisting of 6. A group of mice received levetiracetam (20 mg/kg).[14] The control group received normal saline for 2 weeks. A group received melatonin (25 mg/kg);[15] the control group received the melatonin vehicle (2% ethanol, dissolved in normal saline) for 7 days. A group received levetiracetam for 14 days and melatonin from days 7 to 14. A group received levetiracetam for 14 days and imipramine (10 mg/kg) from days 7 to 14.

There were additional groups for the epileptic mice. The normal group did not receive an electric shock. The study included groups that received levetiracetam (20 mg/kg) or normal saline for 14 days, followed by an electric shock on the 14th day. Groups that received melatonin (25 mg/kg) or imipramine (10 mg/kg) for 7 days were given an electric shock on the 7th day. Two combination therapy groups, the levetiracetam-melatonin and levetiracetam-imipramine groups, were included, which received levetiracetam for 14 days and either melatonin or imipramine from day 7th to 14th, and an electric shock on day 14. All the drugs were injected intraperitoneally; the concentration for all injections was 1mL/100g.

Maximal electroshock (MES)

For the induction of seizures using the MES method, mice were subjected to an electrical stimulus produced by means of an alternating current provided by a Hugo Sachs generator (Rodent Shocker, type 221, Freiburg, Germany). The electrical stimulus (35 mA with a 0.6 second duration) was delivered via ear-clip electrodes connected to the stimulator. The intensity of the electrical current was adjusted to elicit generalized tonic-clonic seizures, characterized by bilateral limb extension and loss of postural control.[16]

Locomotor activity test

This method involved using an open box measuring 40×40×40 cm to evaluate rodents. The animals were placed inside the chamber and allowed to explore for 3 minutes while facing the wall. Total activity was measured by manually recording the vertical activity, while the horizontal movements were recorded by a device that utilized infrared beams to detect the animals' positions.[14]

Forced swimming test (FST)

To conduct this assessment, the mice were placed inside a 2-liter glass container filled with water maintained at 23–25 degrees Celsius and measuring 15 cm in depth. The entire test lasted 6 minutes, with the initial 2 minutes serving as an adaptation period. The subsequent 4 minutes were dedicated to recording the duration of immobility, swimming, and climbing behaviors exhibited by the mice. To prevent hypothermia, the animals were dried in a warm room following the test.[17-19]

Novelty-suppressed feeding test (NSFT)

After a 24-hour period of food deprivation, the animals were placed in a new environment containing food in the center. The test consisted of a measurement: the latency to initiate eating was used as an indicator of feeding motivation and potential depressive-like behaviors. Following the delay period, the amount of food consumed by each animal within the designated 20-minute time frame was measured. This measurement provided insight into the animal's feeding behavior and appetite in response to the novel environment.[20]

Statistical analysis

The mean ± standard error of the mean was used to express the results of all groups. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison tests as a post hoc analysis. Results with *p*-values less than 0.05 were considered statistically significant. Data analysis was performed using Excel and GraphPad Prism 9 software.

RESULTS

Effect of MES

The epileptic group was subjected to an electrical stimulus of a predetermined value of 35 mA with a 0.6-second duration, and the tonic bilateral or hind limb extension was taken as the endpoint. Six groups were challenged with electroshocks to ensure that each group yielded at least six mice with seizures. Limb stretching was observed in animals with seizures, and the hind limbs of these animals were outstretched 180° to the plane of the body axis. Animals were housed in a controlled environment for 24 hours, after which the next tests were conducted.

Effect of treatments on FST

In non-epileptic mice, in terms of immobility duration, the group receiving levetiracetam showed a significant increase compared to the control group (NaCl) ($p<0.05$). However, in the groups receiving levetiracetam-melatonin and levetiracetam-imipramine, immobility time significantly decreased compared to the levetiracetam alone group ($p<0.01$) (Table 1, Figure 1A). Regarding swimming duration, the swimming duration in the levetiracetam group was significantly lower than the control group (NaCl) (Table 1, Figure 1B) ($p<0.05$). In the levetiracetam-melatonin and levetiracetam-imipramine groups, swimming durations significantly increased compared to the levetiracetam alone group ($p<0.001$). Results of climbing duration did not yield any significant differences among the experimental groups (Table 1, Figure 1C).

In epileptic mice, the groups receiving levetiracetam or NaCl (control) exhibited a significantly increased average immobility duration compared to the normal group ($p<0.05$), indicating MES-induced depression. The group receiving melatonin showed a significant decrease in immobility duration compared to the control group, suggesting the antidepressant effects of melatonin ($p<0.05$). Similarly, the group receiving imipramine also demonstrated a significant decrease in immobility duration compared to the control group ($p<0.01$). The group receiving levetiracetam-melatonin, similar to the group receiving levetiracetam-imipramine, exhibited significant reductions in immobility duration compared to the levetiracetam alone group ($p<0.01$) and the control group ($p<0.01$) (Table 1, Figure 1E). The group receiving melatonin displayed a significant increase in swimming duration compared to the control group ($p<0.01$), indicating its antidepressant effects, similar to the imipramine group. The group receiving levetiracetam-melatonin and the levetiracetam-imipramine group also exhibited significant increases in swimming duration (Table 1, Figure 1F). No significant differences were observed among the tested groups in terms of climbing duration (Table 1, Figure 1G). There was no significant difference in the locomotor activity when compared to the control group (Table 1).

Effect of treatments on NSFT

In terms of the delay in eating, non-epileptic mice in the levetiracetam group had a significantly longer duration compared to the control group ($p<0.05$). However, the levetiracetam-melatonin group, similar to the levetiracetam-imipramine group, demonstrated a significant decrease in latency compared to the levetiracetam monotherapy group ($p<0.05$) (Table 2, Figure 2A). The amount of food consumed by the levetiracetam group was reduced compared to the control group ($p<0.01$), while melatonin increased food consumption significantly compared to the vehicle EtOH ($p<0.05$) (Table 2, Figure 2B). The levetiracetam-melatonin group, similar to the levetiracetam-imipramine group, demonstrated a significant

Table 1. The results of the total activity count in the locomotor activity test, and the forced swimming test in epileptic and non-epileptic mice

Non-epileptic groups (n=6)		**Control (NaCl)**	**Lev**	**EtOH**	**Mel**	**Lev-Mel**	**Lev-Imi**
Locomotor activity (count)	-	163.3±48.4	214.3±32.9	139.4±48.7	143.6±34.8	153±72.3	127.9±57.3
Immobility time (s)	-	137.4±15.4	181.5±10.1*	123.1±16.2	92.0±16.8	95.9±18.5$^{\#\#}$	86.5±16.8$^{\#\#}$
Swimming time (s)	-	75.8±10.6	39.7±7.6*	68.8±8.6	78.5±7.0	102.1±10.3$^{\#\#\#}$	101.7±6.4$^{\#\#\#}$
Climbing time (s)	-	26.6±14.8	18.7±8.0	48.0±10.5	60.4±16.4	42.0±11.4	50.2±19.2
Epileptic groups (n=6)	**Normal**	**Control (NaCl)**	**Lev**	**Imi**	**Mel**	**Lev-Mel**	**Lev-Imi**
Locomotor activity (count)	155.5±25.6	156.1±21.97	179.0±29.5	119.0±47.57	151±58.3	167±49.2	122.9±54.6
Immobility time (s)	140.8±13.1	179.1±13.3^	178.7±8.3^	105.2±14.2**	122.7±8.3*	110.5±15.8**,$^{\#\#}$	95.8±11.7***,$^{\#\#\#}$
Swimming time (s)	71.3±4.7	47.5±5.1	49.0±5.3	118.7±11.3***	101.0±7.0**	91.2±7.9*,$^{\#}$	114.0±6.9***,$^{\#\#\#}$
Climbing time (s)	17.8±9.0	5.1±2.6	12.3±4.6	16.2±7.7	16.3±3.0	24.8±5.3	30.1±9.4

The data of all groups are expressed as mean ± standard error of the mean. The results were analyzed using one-way analysis of variance with Tukey's supplementary test. The control group received NaCl. The melatonin control group received the vehicle (2% ethanol solution, EtOH). The normal group did not receive maximal electroshock. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control (NaCl) group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. levetiracetam-only group; ^$p<0.05$ vs. normal group. Mel: Melatoni, Lev: Levetiracetam, Imi: Imipramine

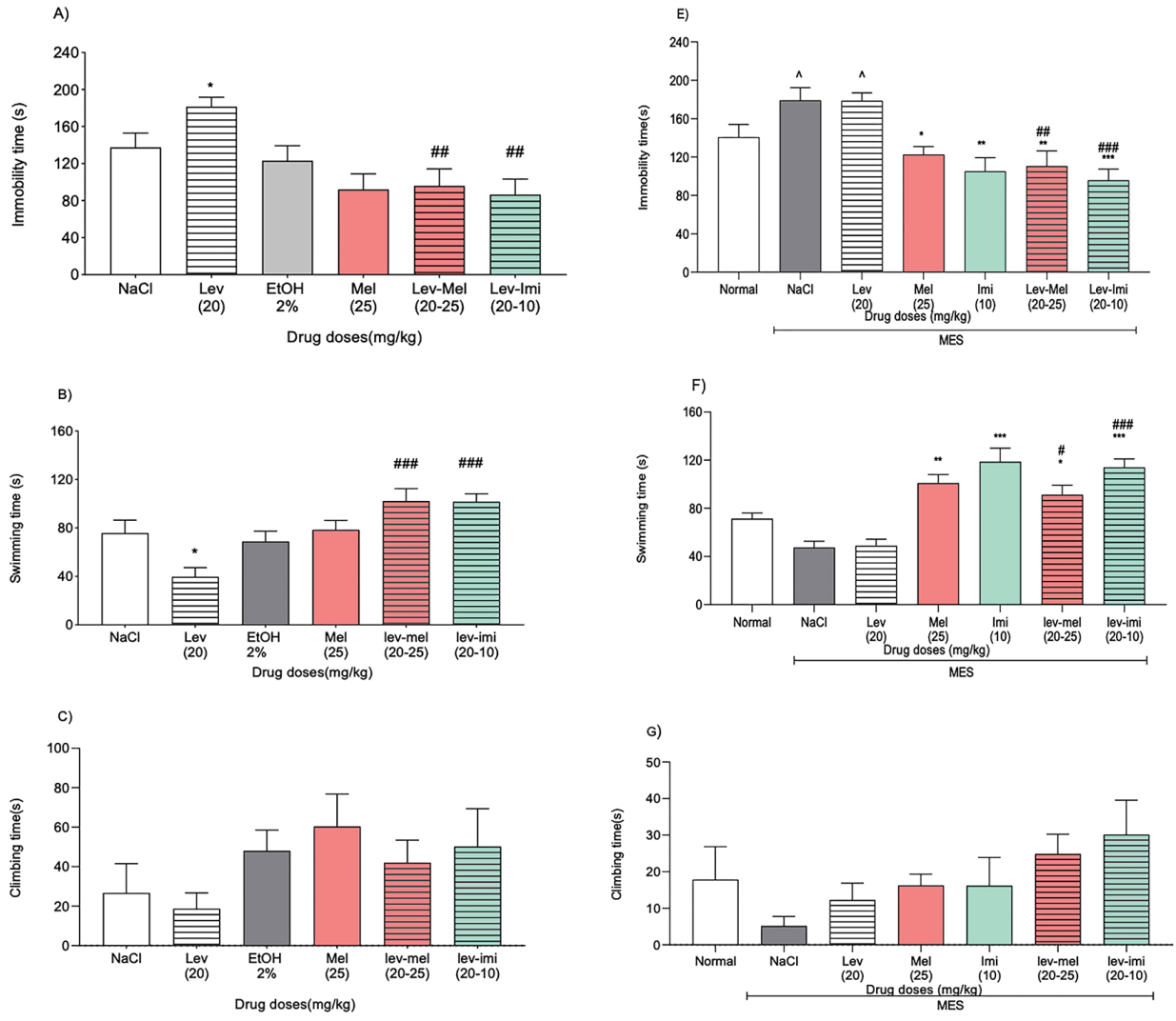

Figure 1. The results of the forced swimming test in epileptic and non-epileptic mice. (A) Immobility duration in non-epileptic mice. (B) Swimming duration in non-epileptic mice. (C) Climb duration in non-epileptic mice. (D) Immobility duration in epileptic mice. (E) Swimming duration in epileptic mice. (F) Climb duration in epileptic mice. Data are expressed as mean ± standard error of the mean. Results were analyzed using one-way analysis of variance with Tukey's post hoc test. The control group received NaCl. The melatonin control group received the vehicle (2% ethanol solution). *p<0.05, **p<0.01, ***p<0.001 vs. control (NaCl) group; #p<0.05, ##p<0.01, ###p<0.001 vs. levetiracetam-only group; ^p<0.05 vs. normal group. Mel: Melatoni, Lev: Levetiracetam, Imi: Imipramine, MES: Maximal electroshock

increase in food consumption compared to the levetiracetam monotherapy group (p<0.05). Based on the findings in epileptic mice, the difference in average duration of eating delay among the experimental groups was insignificant (Table 2, Figure 2C).

Turning to Figure 2D, the average amount of food consumed varied across the different groups. In comparison, the group receiving melatonin exhibited a significant increase (p<0.05) in food consumption compared to the control group, with the average amount indicated in Table 2. The group receiving levetiracetam-melatonin consumed significantly more food (p<0.05 vs. control and levetiracetam alone group), while the difference was insignificant in the levetiracetam-imipramine group. This value in the melatonin and levetiracetam-melatonin groups was significantly higher than the normal group (p<0.05) (Table 2, Figure 2D).

DISCUSSION

According to the results, melatonin co-administration with levetiracetam improved despair behavior during FST and food intake behavior during NSFT in both epileptic and non-epileptic mice. The treatments, including levetiracetam, melatonin, imipramine, and their combinations, did not have a significant impact on the locomotor activity of the mice in this study, regardless of their epileptic or non-epileptic status. Further investigations utilizing additional behavioral tests and depression-related outcome measures would provide an understanding of the effects of these treatments in both epileptic and non-epileptic models.

In this study, it was observed that administration of levetiracetam in non-epileptic mice significantly increased the duration of immobility in the FST, compared to the

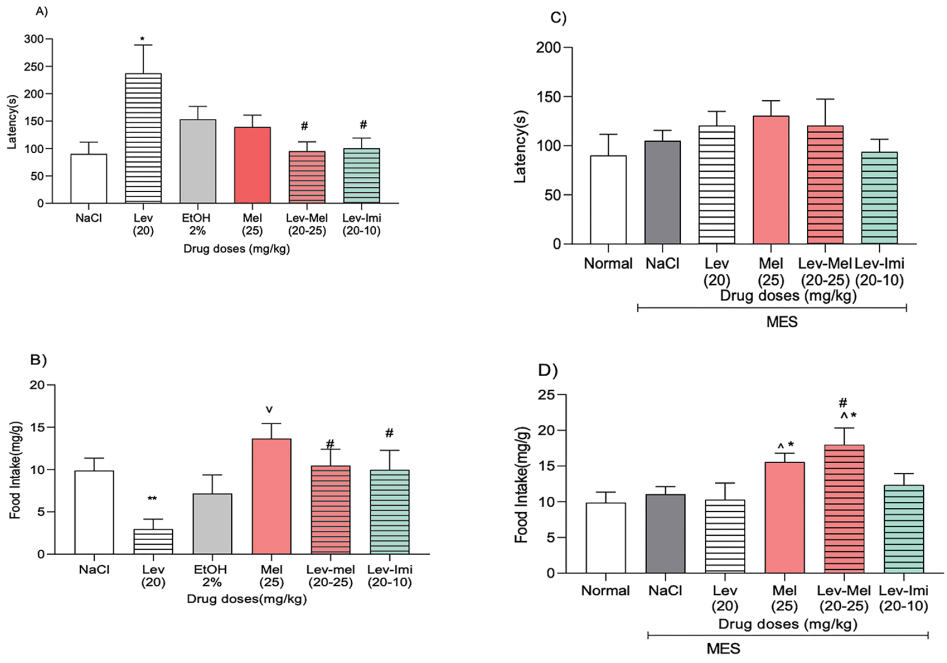

Figure 2. Results of the novelty-suppressed feeding test in epileptic and non-epileptic mice. (A) Latency duration in non-epileptic mice. (B) Food intake in non-epileptic mice. (C) Latency duration in epileptic mice. (D) Food intake in epileptic mice. Data are expressed as mean ± standard error of the mean. Results were analyzed using one-way analysis of variance with Tukey's post hoc test. Control group received NaCl. The melatonin control group received the vehicle (2% ethanol solution). *p<0.05, **p<0.01, ***p<0.001 vs. control (NaCl) group; #p<0.05, ##p<0.01, ###p<0.001 vs. levetiracetam-only group; v sign p<0.05 compared with EtOH group; ^p<0.05 vs. normal group. Mel: Melatoni, Lev: Levetiracetam, Imi: Imipramine, MES: Maximal electroshock

Table 2. The results of the Novelty-suppressed feeding test in epileptic and non-epileptic mice

Non-epileptic groups (n=6)	**Control (NaCl)**	**Lev**	**EtOH**	**Mel**	**Lev-Mel**	**Lev-Imi**
Latency (s)	90.1±21.4	237.3±51.4*	153.3±23.6	139.5±21.2	95.3±16.8###	100.5±18.7###
Food intake (mg/g)	9.8±1.4	2.9±1.1**	7.1±2.2	13.6±1.7v	10.4±1.9#	9.9±2.3#
Epileptic groups (n=6)	**Normal**	**Control (NaCl)**	**Lev**	**Mel**	**Lev-Mel**	**Lev-Imi**
Latency (s)	90.1±21.4	105.0±10.6	120.4±14.5	120.5±27.0	120.5±31.5	93.7±27.0
Food intake (mg/g)	9.8±1.4	11.1±1.0	10.3±2.3	15.5±1.2^'*	18.0±2.3#'^'*	12.4±1.6

The data of all groups are expressed as mean ± standard error of the mean. The results were analyzed using one-way analysis of variance with Tukey's supplementary test. The control group received NaCl. The melatonin control group received the vehicle (2% ethanol solution, EtOH. *p<0.05, **p<0.01, ***p<0.001 vs. control (NaCl) group; #p<0.05, ##p<0.01, ###p<0.001 vs. levetiracetam-only group; ^p<0.05 vs. normal group; v sign p<0.05 compared with EtOH group; ^p<0.05 vs. normal group. Mel: Melatoni, Lev: Levetiracetam, Imi: Imipramine

control group. Additionally, co-administration of melatonin or imipramine with levetiracetam significantly reduced the duration of immobility. These findings suggest that melatonin has antidepressant effects against levetiracetam-induced depression. Previously, it has been demonstrated that the introduction of depression, either by using only levetiracetam or after inducing pentylenetetrazole-triggered seizures in mice by FSTs, led to heightened immobility periods.[21,22]

Previously, melatonin treatment prevented lipopolysaccharide-induced depressive-like behavior in the FST and tail suspension tests, without affecting locomotor activity assessed in the open field test. Melatonin also attenuated the lipopolysaccharide-induced increase in tumor necrosis factor-α levels and the decrease in BDNF levels in the hippocampus. Moreover, melatonin treatment prevented the increase in lipid peroxidation and the decrease in hippocampal glutathione levels.[23] In the current study, a similar pattern was observed in epileptic mice. The group receiving levetiracetam alone exhibited a significant increase in sedentary duration compared to the normal group in FST, but as a similar pattern was observed in the control group, this could be the direct effect of MES. Furthermore, in a separate study investigating the antiepileptic effects of ethosuximide and levetiracetam in WAG/Rij rats with induced epilepsy, ethosuximide was found to improve depression-like behavior; in contrast, levetiracetam exacerbated this symptom.[24]

On the other hand, in the melatonin and imipramine group that received MES, immobility time was significantly lower than the control group. Studies on the effect of melatonin on seizures show that melatonin can reduce seizures.[12,25]

Scientists have noted the anticonvulsant effects of melatonin on penicillin-induced epileptiform behavior in rats. When administered intracerebroventricularly, melatonin extended the delay in epileptiform behavior onset, assessed through electrocorticogram analysis.[26] Experts propose that this observed effect of melatonin might stem from its beneficial influence on GABA-ergic transmission.[12] However, melatonin, like imipramine, also improved despair behavior during the FST in levetiracetam-treated MES epileptic mice, suggesting their antidepressant effect. Therefore, in agreement with both preclinical and clinical research, indicating the advantageous impacts of the melatonin system on anxiety, depression, and epilepsy, melatonin-related substances could offer effectiveness in addressing associated behavioral complexities in epilepsy. This extends beyond simply regulating disrupted sleep-wake patterns.[27] Melatonin and serotonin are primarily involved in regulating sleep and mood. Their synthesis is closely linked, as melatonin is derived from serotonin.[28] On the other hand, daily treatment with melatonin increased serotonin levels, which could be related to its effect on depression, in several brain regions such as the amygdala and midbrain.[29]

The classification of active behaviors during FST into swimming and climbing time would facilitate the differentiation between serotonergic and noradrenergic classes of antidepressant drugs.[30] In epileptic and non-epileptic mice, co-administration of melatonin or imipramine with levetiracetam significantly increased swimming duration, while there was no significant difference in the climbing time. Climbing behavior may not be sensitive enough to detect subtle differences caused by the treatments.[31]

These findings indicate that melatonin can effectively counteract the depressive effects induced by levetiracetam in epileptic mice. Results suggest that both melatonin and imipramine antidepressant effects are possibly mediated through altering the serotonergic system. A related study focused on investigating the antidepressant effects of N-methyl-D-aspartate receptor (NMDAR) blockers following levetiracetam administration in the modified FST showed that levetiracetam significantly decreased swimming time, while the NMDAR blockers increased swimming duration compared to the control group.[14] Therefore, it was supposed that reduced serotonin levels are related to levetiracetam-induced depression. Consistent with these results, a separate study investigating the effects of melatonin and agomelatine on anxiety and depressive-like behaviors induced by doxorubicin in rats observed that pretreatment with melatonin and agomelatine significantly reduced immobility time and increased swimming time in the FST compared to the doxorubicin alone group.[32]

The NSFT is a commonly used experimental approach to assess alterations in eating behavior. By subjecting animals to a novel environment, researchers can examine the impact of unfamiliar surroundings on their food consumption, thereby offering valuable insights into the intricate interaction between environment, stress, and feeding behavior.[33] Notably, despite FST, which is an acute model that evaluates despair behavior, the NSFT detects depressive-like behaviors in mice subjected to chronic treatment, surpassing other assessment methods in this regard.[14] In non-epileptic mice, the latency to eating differed. In the levetiracetam-melatonin group, there was a significant reduction in the eating delay time compared to the levetiracetam group. A similar eating delay was seen with imipramine co-administration. Consistent with these findings, a separate study investigating eating suppression in a novel environment reported that chronic administration of exogenous corticosterone increased the delay time before feeding. This anxiety-related behavior was reversed by chronic administration of melatonin.[34] Significant differences in food consumption were observed in the combined treatment groups of levetiracetam-melatonin and levetiracetam-imipramine compared to the levetiracetam-only group. In epileptic mice, there was no difference in latency time compared with the control group, which implied the dominant effect of MES on the results. The study indicates that melatonin could not reduce the latency time while co-administered with levetiracetam when epilepsy was induced by MES. On the other hand, in epileptic mice, significant differences in food consumption were found among the treatment groups. However, the levetiracetam-melatonin group significantly increased food consumption compared to the groups receiving levetiracetam alone, the control group, and the normal group.

CONCLUSION

Melatonin exhibited beneficial efficacy in alleviating depressive behavior in both epileptic and non-epileptic mice groups. Thus, melatonin holds potential as a therapeutic agent to counteract the depressive effects associated with levetiracetam treatment. Further investigation is warranted to elucidate the underlying mechanisms responsible for melatonin's antidepressant properties. Melatonin, recognized as a naturally produced hormone with potential antiepileptic and antidepressant effects, might hold significant promise in mitigating depression during epilepsy treatment. However, additional investigation through clinical studies is required to further explore this potential.

Ethics

Ethics Committee Approval: All experimental procedures were conducted in accordance with the guidelines set by the National Ethical Committee of Iran (approval number: IR.MUI.RESEARCH.REC.1401.042, dated: 26.04.2022), to minimize animal distress and the number of animals used in the study.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: A.M., Design: A.M., M.R., Data Collection or Processing: A.M., A.Mo., Analysis or Interpretation: A.M., M.R., A,M., Literature Search: A.M., A,Mo., Writing: A.M., M.R., A,Mo.

Conflict of Interest: The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Pharmacoeconomics Education in Pharmacy Faculties: Status in Türkiye and Other Countries

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ABSTRACT

Objectives: Pharmacoeconomics is an important branch of science that should be taken into account by countries' social security institutions in order to rationally manage drug expenditures within healthcare budgets for the aging population. Pharmacists trained in pharmacoeconomics make a great contribution to this field. This study aims to draw attention to the inclusion of pharmacoeconomics education as a compulsory course in the curricula of pharmacy faculties in Türkiye.

Materials and Methods: Fifty-one pharmacy faculties in Türkiye were analysed. The pharmacoeconomics courses and their contents in the curriculum of these faculties were evaluated. The course contents, European Credit Transfer System and credits, weekly and meeting hours of the faculties offering pharmacoeconomics courses were analyzed.

Results: There are 51 pharmacy faculties in Türkiye. Of these pharmacy faculties, 33 are operating under state universities and 18 under foundation universities. There is no pharmacoeconomics course in the curriculum of 82.35% of the pharmacy faculties (n=42). In the other 17.65% (n=9) of the faculties, there is a pharmacoeconomics course in the curriculum. The course contents of the faculties are similar, and basic pharmacoeconomics information is generally given. There are no faculty members who have completed their PhDs in this field.

Conclusion: This study, the first to systematically evaluate the situation in all pharmacy faculties in Türkiye, has revealed that pharmacoeconomics education is limited. Making pharmacoeconomics courses mandatory in the curriculum of pharmacy faculties is necessary to comply with international standards and enable pharmacists to contribute more effectively to rational drug use and the sustainability of healthcare systems.

Keywords: Pharmacoeconomics, pharmacy education, pharmacy curriculum, health expenditures, Türkiye

INTRODUCTION

In our age of increasing life expectancy, the potential increase in pharmaceutical expenditures due to an ageing population and changing treatment options makes the forecasting of health expenditures even more important.[1] Developing technology and medical innovations have led to major advances in the treatment of chronic diseases and new drug discoveries. However, this situation challenges countries in the provision of health services, social security, and welfare.[2]

Non-communicable diseases have become a major health problem worldwide,[3] accounting for 63% of all deaths. The high costs of newly discovered drugs prevent the widespread use of these treatments, leading to an increase in unmet medical needs.[4] In particular, developments in gene therapies offer important options for rare diseases and intensify scientific studies in this field.[5]

As the average life expectancy increases, health expenditures also increase. Consequently, the high treatment costs of the elderly population cause major debates in social security systems.[6] Pharmaceutical expenditures constitute a significant component of health expenditures. In 2022, approximately 1.48 trillion in pharmaceutical expenditure was spent globally. This figure is estimated to reach 1.9 trillion dollars by 2027.[7]

Increasing health expenditures force countries to make more rational and systematic plans for financial sustainability. Pharmaceutical expenditures, which constitute a large portion

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of health expenditures, are increasing, especially with the introduction of next-generation drugs. In Organisation for Economic Co-operation and Development (OECD) countries, pharmaceutical expenditures reached 20 percent of total health expenditures in 2013.[8] This situation has intensified even more with the introduction of new drug treatments to the market. The Coronavirus Disease 2019 (COVID-19) pandemic has also caused these expenditures to rise.[9]

It has led to the systematic development of pharmacoeconomic evaluations in health economics to answer the question of how financial resources should be allocated to different drugs.[10,11] Pharmacoeconomic studies propose the most appropriate alternative by addressing the healthcare expenditures. By making detailed analyses, it ensures rational drug use. Pharmacoeconomics is necessary for industry, national regulatory authorities, and health financiers to make rational decisions.[12] Additionally, it establishes a framework for making economic decisions by evaluating all stages from the drug's pharmacological effects to its pricing. Cost analyses allow pharmacists and reimbursement institutions to make better and more informed decisions.[11]

Pharmacists can contribute to the rational management of drug expenditures with knowledge of pharmacoeconomics. For this, they should receive pharmacoeconomics education in pharmacy faculties. However, pharmacoeconomics education is not provided in most pharmacy faculties in Türkiye.[13] Pharmacoeconomics education improves students' decision-making and skills in the critical analysis of evidence by enabling them to make more informed decisions.[14]

This study aims to evaluate the current status of pharmacoeconomics education in pharmacy faculties in Türkiye and to emphasise the importance of including this education in the curriculum.

MATERIALS AND METHODS

This study was designed as a descriptive, cross-sectional analysis. All 51 pharmacy faculties in Türkiye that were actively providing education during the 2023–2024 academic year were included. Data were obtained from the official websites of the faculties and the Yükseköğretim Kurulu Atlas (Higher Education Council Atlas) database.[15,16]

The curricula of each faculty were examined for the presence of a pharmacoeconomics course. When available, the following parameters were recorded: course title, whether the course was compulsory or elective, the semester in which it was offered, European Credit Transfer System credits, and weekly course hours. In addition, the detailed content of the pharmacoeconomics courses and the reference books used was reviewed.

Statistical analysis

Categorical variables such as the presence (yes/no) and status (mandatory/elective), of pharmacoeconomics courses in public versus foundation faculties were compared using chi-square or Fisher's exact test, with a significance level set at $p<0.05$. Results were presented as numbers (n) and percentages (%).

Data organization and tabulation were performed in Microsoft Excel for Microsoft 365, and statistical analyses were carried out using Social Sciences for Windows, (version 22.0; IBM Corp., Armonk, IBM Corp., USA).

The study is based on publicly available open-source data from university websites. No live material or human participant data were used. Therefore, ethical approval and patient consent were not required.

RESULTS

There are 208 universities in Türkiye, 133 of which are state universities and 75 are foundation universities. Among these universities, 55 pharmacy faculties (37 state and 18 foundation) have been established under the relevant rectorates. Among these faculties, 51 pharmacy faculties have started education. Thirty-three of these pharmacy faculties operate within state universities, and eighteen operate within foundation universities (Figure 1).

There is no pharmacoeconomics course in the curriculum at 82.35% (n=42) of the pharmacy faculties. In the other 17.65% (n=9) of the faculties, there is a pharmacoeconomics course in the curriculum.

Among 33 state faculties, 7 (21.2%) offered a pharmacoeconomics course, while 2 of 18 foundation faculties (11.1%) included such a course. The difference was not statistically significant ($\chi^2=0.27$, $p=0.603$) (Table 1).

Among the nine faculties offering pharmacoeconomics courses, three state and two foundation faculties provided the course as mandatory, while four state faculties offered it as an elective. No significant difference was observed (Fisher's exact test, $p=0.444$) (Table 2).

When the course contents of the faculty offering pharmacoeconomics courses were examined, it was observed that the scope and depth of the courses were heterogeneous across institutions. The most frequently included topics were basic economic concepts, pharmacoeconomic evaluation, and cost-effectiveness analysis, while advanced methods such as Markov modelling were not included (Table 3).

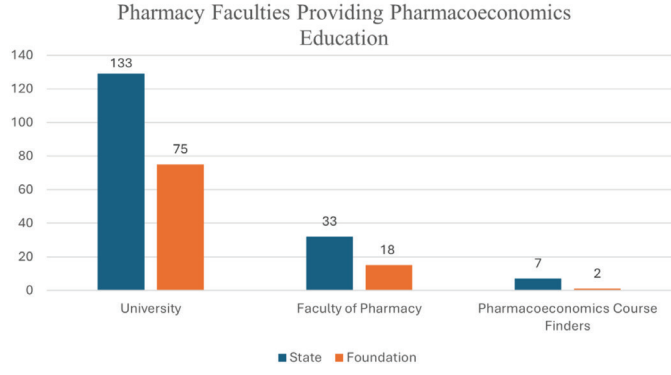

Figure 1. Universities, pharmacy faculties and pharmacoeconomics courses in Türkiye[15,16]

Table 1. Availability of pharmacoeconomics courses in pharmacy faculties in Türkiye

Faculty type	Pharmacoeconomics course available	No course	Total
State (n=33)	7 (21.2%)	26	33
Foundation (n=18)	2 (11.1%)	16	18
Total (n=51)	9 (17.6%)	42	51

χ^2=0.27, *p*=0.603

Table 2. Status of pharmacoeconomics courses as mandatory or elective

Faculty type	Mandatory	Elective	Total
State (n=33)	3	4	7
Foundation (n=18)	2	0	2
Total (n=51)	5	4	9

Fisher's exact test, *p*=0.444

Table 3. Content of pharmacoeconomics courses across faculties

Content area	Faculties covering topic (n, %)
Basic economic concepts & health economics	9 (100%)
Pharmacoeconomics & economic evaluation	8 (88.9%)
Cost-effectiveness analysis	8 (88.9%)
Cost-utility analysis (QALY)	7 (77.8%)
Cost-benefit/cost of illness analysis	6 (66.7%)
Case studies	6 (66.7%)
Decision analysis	4 (44.4%)
Economic modelling	3 (33.3%)
Retrospective databases	1 (11.1%)
Markov modelling	0 (0%)

QALY: Quality-adjusted life year

Findings indicate that there is not enough awareness in Türkiye about pharmacoeconomics education. Stakeholders managing health economics should pay more attention to this issue.

DISCUSSION

Health expenditures are increasing annually worldwide. According to the Fiscal Sustainability of Health Systems report published by the OECD, the share of health expenditures in the Gross Domestic Product (GDP) of OECD member countries has been increasing since the COVID-19 pandemic. This share will reach 11.2% of GDP in 2040. In Türkiye, the ratio of health expenditures to GDP was 5.8% in 2009 and 4% in 2022.[17]

Considering the increasing pharmaceutical expenditures, pharmacists trained in pharmacoeconomics will significantly contribute to managing public spending in this area. However, while there is a need for experts in the field of pharmacoeconomics, which is critical for rational drug expenditures, it is paradoxical that pharmacists are not trained in pharmacoeconomics. The fact that only eight pharmacy faculties in Türkiye provide basic pharmacoeconomics education may delay access to medicines in the future, due to financial reasons. In Türkiye, the Social Security Institution, which finances health expenditures, has legislated that pharmacoeconomic evaluation recommended by companies will be taken into consideration in drug reimbursement, but it does not specify how this will be done.[18]

The rationalization of health expenditures is possible through smarter management of health care economics. To manage pharmaceutical expenditures, there is a great need for specialized pharmacists who know pharmacoeconomics. Current knowledge suggests that pharmacists, who have a critical role in drug counseling, clinical pharmacy, and drug supply, will not only ensure rational drug use but also reduce drug expenditures for social security systems and save money with the pharmacoeconomics training they will receive.[19] Therefore, it is important to include a pharmacoeconomics course in the curriculum of pharmacy faculties. With the savings to be achieved, these institutions will be able to better address unmet medical needs for patients. It is therefore important to include a pharmacoeconomics course in the curriculum of pharmacy faculties.

However, pharmacoeconomics should not be limited to cost containment or drug expenditure reduction. It also encompasses broader domains such as cost-effectiveness, cost-utility analyses including quality-adjusted life years, budget impact evaluations, and health technology assessment (HTA).[20] Training pharmacists in these areas would enable them not only to contribute to rational drug spending but also to improve clinical outcomes and strengthen evidence-based decision making.

Previous studies on pharmacoeconomics education in Türkiye were limited to a small number of faculties. Our study provides an original contribution by systematically evaluating the curricula of all pharmacy faculties in Türkiye for the first time.

Pharmacoeconomics influences not only the decisions of drug reimbursement agencies but also the cost savings for hospitals in procuring medicines. In the study conducted by Javor et al.,[21] the study stated that the inclusion of pharmacists in pharmaceutical procurement in hospitals will provide significant savings for health expenditures.

Pharmacoeconomics education is widely included in the curricula of pharmacy faculties in many countries, especially in the United States of America (USA).[13] Makhinova and Rascati[22] conducted a study on pharmacy faculties in the USA and reported that in 2011, 87 pharmacy faculties included pharmacoeconomics courses in their curricula and 90% of these faculties required pharmacoeconomics courses. According to a study conducted by Adunlin et al.[23] in accredited pharmacy faculties in the USA, 111 of 141 pharmacy faculties had pharmacoeconomics courses. The study emphasized the importance of pharmacoeconomics education for improving

clinical outcomes and reducing health expenditures. In our study, it was determined that pharmacoeconomics courses are offered in only one-fifth of pharmacy faculties in Türkiye.

The status of pharmacoeconomics education in pharmacy faculties in 22 countries in the Middle East and North Africa region was analysed in a study. According to a study by Farid and Baines,[24] 80 of 176 pharmacy faculties in 14 countries included pharmacoeconomics courses in their curricula. However, in this training, the basics of pharmacoeconomics draw attention. Similarly, when the course contents are analysed in our study, we understand that basic pharmacoeconomics information is given.

In a study on pharmacoeconomics education in pharmacy faculties in Bosnia and Herzegovina, it was reported that only one faculty offers a pharmacoeconomics course in the curriculum.[25] According to another study conducted by Freitas and Balbinotto,[26] there is a pharmacoeconomics course in 4 out of 55 pharmacy faculties in Brazil. It was emphasized that the pharmacoeconomics course is important for the Brazilian Health System. In a study conducted on the pharmacy faculties in Egypt, it was emphasised that pharmacoeconomics courses are given in 7 out of 20 pharmacy faculties, highlighting a need for faculty members in this field. Additionally, education in this area is still at the initial level.[27] In Türkiye, there is a similar situation in terms of faculty members, since doctoral programmes in pharmacoeconomics have yet to be established in pharmacy faculties. According to a study, there are no specialised pharmacoeconomics pharmacists in Türkiye.[28] This deficiency stands as a significant challenge to the provision of pharmacoeconomics education in pharmacy faculties in Türkiye.

According to the standards of organizations such as the International Society for Pharmacoeconomics and Outcomes Research and Accreditation Council for Pharmacy Education, pharmacoeconomics education should go beyond basic concepts and include topics such as cost minimization, cost-effectiveness, cost-benefit analyses, economic modeling (e.g., Markov models); HTA, and budget impact analysis.[29-31] Furthermore, teaching methods should include case-based learning and practical projects to ensure that graduates are competent not only in theory but also in practice.

In a study conducted by Şencan et al.[28] among students in five pharmacy faculties in Türkiye, it was stated that the science of pharmacoeconomics is not well understood by students and that a course should be added to the curriculum on this subject. In this study conducted with students, it was concluded that they were aware of the necessity of pharmacoeconomics education and that the course should be given in the fourth year. In our study, it was observed that education occurred mostly in the fourth and fifth grades.

Pharmacy faculties in the USA are ahead of other countries in pharmacoeconomics education.[23] The situation in the countries mentioned in the literature is similar to Türkiye.[25,26]

The findings of this study may accelerate the decisions of policymakers concerning social security expenditures to employ more pharmacists, who have knowledge in pharmacoeconomics, in the public sector. In this study, it evaluated whether the pharmacoeconomics course was included in the education curriculum of pharmacy faculties in Türkiye, and a comparison was made with the situation in some countries, especially in the USA, where data are available in the literature.[22,23,32,33] More academic research is needed to reinforce the importance of education in this field.

Study limitations

The main limitation of this study is that it relies solely on course content information available on pharmacy faculty websites. The knowledge and competency levels of students taking the pharmacoeconomics course could not be assessed. Furthermore, no data could be obtained regarding the educational outcomes of the faculties or the effectiveness of the courses. Future studies should examine the impact of pharmacoeconomics education on post-graduation contributions and employment opportunities for students.

CONCLUSION

This study is one of the first comprehensive investigations examining the inclusion of pharmacoeconomics courses in the curricula of all pharmacy faculties in Türkiye. The findings indicate that the course is only offered in a limited number of faculties. Making pharmacoeconomics education mandatory in all faculties will enable pharmacists to play a more effective role in health policies and drug expenditures. This will strengthen both rational drug use and the economic contribution to the health system.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Footnotes

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ORIGINAL ARTICLE

Bioavailability Enhancement and Polymorphic Stabilization of One BCS Class IV Metastable Drug Through Novel Formulation Approach

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ABSTRACT

Objectives: This study aims to enhance the bioavailability and polymorphic stability of Ticagrelor, a metastable, low-soluble and low-permeable Biopharmaceutics Classification System Class IV drug, by exploring different formulation approaches.

Materials and Methods: Ticagrelor was taken as a model drug for the enhancement of bioavailability and polymorphic stability. Initially, various techniques, such as micronization, amorphous solid dispersion (ASD), and Self-Microemulsifying Drug Delivery System, were evaluated for dissolution enhancement. Based on the improvement in dissolution rate, polymorphic stability, and process viability, an ASD technique was selected for dissolution enhancement of Ticagrelor. Co-povidone VA 64 and vitamin E TPGS were used as carriers for the preparation of Ticagrelor solid dispersion (SD) by the solvent evaporation technique. The formulation was optimized and further evaluated for dissolution performance in biorelevant media fasted state simulated gastric fluid and fasted state simulated intestinal fluid. The bioavailability of the Ticagrelor SD tablet formulation was compared with a conventional immediate release tablet formulation prepared by wet granulation process in line with reference product Brilinta® (AstraZeneca LP). *In vivo* pharmacokinetic (PK) studies were carried out in Wistar rats with due approval from ethics committees such as CPCSEA and IAEC (CPCSEA/DIPS/02/23/61). Patients are not involved in this study, hence informed consent not applicable.

Results: The relative bioavailability and peak plasma concentration (C_{max}) of Ticagrelor SD formulation compared to conventional immediate release tablet formulation in line with Brilinta® (AstraZeneca LP) were found to be 141.61±2.29% and 137.0±0.59%, respectively. Further, based on a dose-adjusted PKs study of Ticagrelor SD, a 70 mg Ticagrelor tablet formulated with the SD technique was found to be equivalent to a 90 mg dose of Ticagrelor conventional immediate release tablet formulation with a comparable C_{max}, area under the curve $(AUC)_{0-24}$, and $AUC_{0-\infty}$. Visual observation of the dissected gastric organ through a stereomicroscope revealed no redness or bleeding post-administration of Ticagrelor SD formulations.

Conclusion: The SD technique with carrier co-povidone VA 64 and vitamin E TPGS prepared by the solvent evaporation process could yield a Ticagrelor formulation with improved bioavailability and polymorphic stability.

Keywords: Bioavailability, pharmacokinetics, polymorphic stability, gastrointestinal bleeding study, Ticagrelor, amorphous solid dispersion

INTRODUCTION

Drug dissolution and permeability play a critical role in achieving the desired bioavailability and pharmacological response, which in turn affects the clinical safety and efficacy of a drug significantly. In addition to drug dissolution and permeability, polymorphic transformation of metastable drugs during manufacturing, storage, and gastrointestinal (GI) transit adds to the challenges in achieving the intended clinical safety and efficacy.

One such Biopharmaceutics Classification System (BCS) Class IV, cardiovascular medication is Ticagrelor (not ionized in the physiological pH range), which has a moderate intrinsic permeability and very poor solubility (less than 10 µg/mL). Brilinta®, AstraZeneca LP (Ticagrelor), has a median t_{max} of 1.50

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hours and an absolute bioavailability of roughly 36%. According to reports, the active metabolite of Ticagrelor has a median half-life of 2.5 hours. Ticagrelor demonstrates polymorphism. There have been reports of Forms I, II, III, and IV (non-solvated) and numerous solvated (metastable) forms, suggesting that formulation and processing parameters can have a substantial impact on Ticagrelor's dissolution and clinical performance. Thus, it is imperative to improve Ticagrelor's bioavailability.[1,2]

Currently, many techniques, such as micronization, solid dispersion (SD), polymeric amorphization, complexation, micro- and nano-emulsification [i.e., Self-Microemulsifying Drug Delivery System (SMEDDS) and Self-Nanoemulsifying Drug Delivery System], the co-crystal approach, the use of surfactants, and liposomal drug delivery, are being explored for enhancing the solubility and bioavailability of low-solubility drugs. Nonetheless, all these techniques have their own sets of advantages and disadvantages regarding drug loading, stability, and *in vivo* permeation. P-glycoprotein, an efflux transporter, plays an important role in drug transport. Surfactants like Vitamin TPGS and polysorbate are reported as permeation enhancers and are considered to play an important role in increasing intestinal permeability through the inhibition of the P-gp pump. Conventional mechanical micronization helps in enhancing intrinsic dissolution, but is limited to many drugs due to thermal and chemical degradation during the process. Novel techniques such as SD, micro-emulsifying drug delivery, co-crystal, and liposomal drug delivery are considered to improve both solubility and stability of drug formulations, significantly and hence could be the optimal alternatives to the conventional approaches of dissolution enhancement.[3-7]

Little research has been published to improve Ticagrelor's oral bioavailability; however, numerous crucial factors, including formulation stability, polymorphic transformation, and process technology viability, have not received enough attention. Amorphous SD (ASD) is one prominent technique explored to improve both the solubility and the permeability of BCS class IV drugs.[8] The use of vitamin E TPGS as a carrier of solid dispersion has been reported to enhance drug dissolution.[8-12] Also, it is reported that Ticagrelor absorption is facilitated through the inhibition of P-glycoprotein. However, its sticky nature and low thermal stability lead to crystallization of drugs and may cause stability issues with the formulations. [8-12]

The current study intends to design an appropriate formulation technology that would not only enhance Ticagrelor's bioavailability but also improve polymorphic stability, ensuring consistent therapeutic antiplatelet effects, especially during the critical initial hours of treating an acute coronary event. The selection of an appropriate formulation technology was based on a preliminary screening study on three different formulation technologies, including SMEDDS, ASD, and micronization.

For the preparation of ASD of meta-stable Ticagrelor, various polymers Co-povidone VA 64, Soluplus®, and HPMCAS were explored initially based on their crystallization driving force (CDF), glass transition temperature (Tg) and hydrophobicity. The medium chain triglyceride, i.e., Labrafac lipophile WL 1349, co-surfactant (Transcutol HP), surfactant (Polysorbate 80), and solubilizer diethylene glycol monoethyl ether were selected for the preparation of a self-microemulsifying drug delivery system for Ticagrelor.[13-18]

An appropriate analytical toolbox was explored to monitor the polymorphic transformation of Ticagrelor formulations during the preparation and storage period. A discriminatory dissolution method was developed for evaluating the *in vitro* dissolution performance of Ticagrelor formulations, considering pH 6.8 without surfactant as the dissolution medium with phosphate buffer. A biorelevant dissolution study was performed in FaSSGF and FeSSIF to simulate the GI environment.[19,20]

One *in vivo* pharmacokinetic (PK) study was carried out in Wistar rats (CPCSEA/DIPS/02/23/61) and aimed to compare the bioavailability of the ASD of Ticagrelor with a conventional immediate-release tablet formulation in line with the reference product Brilinta® (AstraZeneca LP). In a dose-adjusted PKs study in Wistar rats, a 70 mg dose of Ticagrelor ASD was compared with a 90 mg dose of a conventional immediate-release tablet formulation of Ticagrelor. GI bleeding studies were carried out to evaluate any episode of bleeding in the stomachs of Wistar rats.

MATERIALS AND METHODS

Materials

Ticagrelor was acquired as a gift sample from Mankind Research Center (a division of Mankind Pharma Ltd., India). Kolidone VA 64 and Soluplus were provided as a gift sample from BASF (Ludwigshafen, Germany). HPMC Acetate Succinate was collected as a gift sample from Shin Etsu Co. Pearlitol SD 200 (Roquette) was supplied by the Signet Chemical Corporation (Mumbai, India). Anhydrous calcium hydrogen phosphate was supplied by Sudeep Pharma (India), sodium starch glycollate was supplied by Amit Hydrocolloid (India), and Polyplasdone XL was supplied by Ashaland (New Milford CT, USA). Labrafac Lipophile WL 1349 and Transcutol HP were provided by Gattefosse (Saint Priest, Cedex, France). A gift sample of vitamin E TPGS was taken from Seqens GmbH (Germany), Opadry YS-1-7040 white from Colorcon Asia Pvt Ltd. (India), and Polysorbate 80 purchased from Croda Singapore PTE Ltd. Acetonitrile and methanol (HPLC grade) were received from Merck Chemicals (Germany). Ammonium acetate, ammonium hydroxide and potassium dihydrogen orthophosphate (KH2PO4) were collected from Qualigen (Thermo Fisher Scientific, Mumbai, India). The analytical column, Chromosil, 250 × 4.6 mm, 5.0 µm, was purchased from Chrom Separations Inc (USA). FaSSIF, FeSSIF and FaSSGF 3F Powder were received from Biorelevant (UK).

HPLC analysis

Shimadzu LC-10 AT VP HPLC with PDA detector (Shimadzu Corporation, Japan) was used for quantification of Ticagrelor. Reverse-phase chromatography was adopted as described by Bueno et al.[21] with some modifications. A C8 reverse-phase analytical column (Chromosil, 250 × 4.6 mm, 5.0 µm) and

a mobile phase of 50 mM acetonitrile: ammonium acetate (57:43 *v/v* with pH adjusted to 8.2) were used in the analysis. The column temperature was set to 25 ˚C, with an injection volume of 20 µL, and a flow rate of 0.7 mL/min was maintained. Ticagrelor absorbance was taken at 270 nm.

Ultraviolet (UV) analysis

A UV-Visible spectrophotometer (Shimadzu 1900 series with Lab Solution software) was used for the dissolution of Ticagrelor formulations. For standard preparation, phosphate buffer pH 6.8 and methanol were prepared in a 70:30 ratio. For the standard calibration curve, different concentrations of Ticagrelor standard solution were prepared from 2 µg/mL to 20 µg/mL, and absorbance was measured at 222 nm. Similarly, dissolution samples of Ticagrelor withdrawn at 5, 10, 15, 30, 45, 60, and 75 minutes were diluted with the dissolution medium, and their absorbance was taken at 222 nm.

Screening and optimization of formulation technology for Ticagrelor

In a preliminary screening study, different formulations of Ticagrelor were prepared using the techniques of conventional wet granulation, SD, and micro-emulsification (SMEDDS). Conventional wet granulation was adopted to prepare a film-coated tablet dosage form with a similar Qualitative (Q1) and Quantitative (Q2) composition to that of the reference product Brilinta® (AstraZeneca LP). Ticagrelor of differing particle size (D90) was used in the formulation of an immediate-release tablet using the wet granulation process. Solvent evaporation techniques were performed to prepare Ticagrelor SD. Co-povidone (Kolidone VA 64), Soluplus, HPMC acetate succinate, and permeation enhancer vitamin E TPGS were explored for the preparation of SD. The SD thus obtained was further blended with other excipients for tablet compression, followed by film coating. Ticagrelor (SMEDDS) was prepared with medium-chain triglyceride (Labrafac lipofile WL 1349, Gattefosse), co-surfactant Transcutol HP (Gattefosse), surfactant Polysorbate 80, and solvent ethanol. The SMEDDS thus obtained were filled into size X hard gelatin capsules. The above dosage forms of Ticagrelor prepared with different formulation technologies were evaluated to select a stabilized Ticagrelor dosage form with a superior dissolution profile.

Preparation of conventional immediate release tablets (TICA-IR)

A conventional immediate-release Ticagrelor film-coated tablet formulation was prepared by an aqueous wet granulation process. Two different particle size ranges of Ticagrelor, (A) D90: 26.96 microns, and (B) D90: 12.88 microns, were considered. The qualitative (Q1) and quantitative (Q2) composition, as well as the manufacturing process, were similar to the reference product Brilinta® (AstraZeneca LP). Ticagrelor and intragranular excipients were sifted and blended. The blended dry mix was then granulated with purified water and dried to achieve a loss on drying of 1.0-1.50% *w/w*. The dried granules are sifted and milled to obtain granules sized at mesh 30. The sifted granules were then blended with extra granular ingredients and compressed into tablets, followed by film coating.

Preparation and optimization of immediate-release Ticagrelor tablets with SD technology (TICA-SD)

Polymers co-povidone VA 64, Soluplus, and HPMC Acetate succinate were screened for their drug loading efficiency and their capacity to form an ASD. Ticagrelor and polymer were dispersed in ethanol under continuous stirring using a magnetic stirrer at 300 rpm for 1 h. The drug-polymer dispersion in ethanol was then processed in a rotary evaporator at 50 ˚C to obtain an SD of Ticagrelor. The SDs thus obtained were extra-dried and sifted through a sieve of mesh # 30 and characterized by XRPD. The sized SD powder was blended with Pearlitol SD 200, calcium hydrogen phosphate dihydrate, sodium starch glycolate, and hydroxypropyl-cellulose in a blender for 10 minutes at 12 RPM. Magnesium stearate was added and blended further for 3 minutes at 12 RPM. The blended mass is then compressed into a tablet in a rotary compression machine. The compressed tablets were coated with Opadry YS-1-7040, with an inlet temperature of 65±5 ˚C, at 6 RPM. Placebo tablets without Ticagrelor were also prepared by the same method as that used for preparing TICA-SD tablets.

The formulations of Ticagrelor SD were optimized with different ratios of Ticagrelor (TICA), vitamin E TPGS, and carriers, i.e., co-povidone VA 64 (CP VA64), Soluplus, and hypromellose acetate succinate (HPMCAS). The solvent evaporation technique was explored for the preparation of ASD. For the preparation of Ticagrelor SD (TICA-SD) with carrier co-povidone VA 64, different combinations such as TICA: TPGS: CP VA 64-1: 0.4: 1, TICA: TPGS: CP VA 64-1: 0.4: 2, and TICA: TPGS: CP VA 64-1: 0.4: 4 were used. The same combinations were followed for the preparation of Ticagrelor SD (TICA-SD) with Soluplus and HPMCAS also.

Preparation of immediate-release Ticagrelor capsules with SMEDDS technology (TICA-SMEDDS)

TICA-SMEDDS were prepared and optimized with a composition of Ticagrelor (10% *w/w*), Labrafac Lipofile WL 1349 (45% *w/w*), Transcutol HP (35% *w/w*), Polysorbate 80 (10% *w/w*) and ethanol (5% *w/w*). The solvent ethanol is lost during the homogenization process. The microemulsion pre-concentrate thus obtained was filled in a size 00 hard gelatin capsule and evaluated for dissolution.

Characterization of Ticagrelor and its formulations

Polarized microscopy

The crystal properties and morphology of pure crystalline Ticagrelor and Ticagrelor SD were evaluated by polarized microscopy (Olympus BX 53) with IPV P Class software (Image Pro-Vision). The samples were evaluated by polarized microscopy with a magnification of 20X, 40X, and 100X, with and without a polarizer, to identify small traces of crystalline Ticagrelor in samples of ASD. Further, the particle size, shape, and the presence of any agglomerates were also evaluated with the help of IPV-P Class software.

PXRD

PXRD analysis was performed on the SD and its tablet formulations (initial and 6-month stability) to determine the Ticagrelor crystallinity. PXRD patterns were recorded using the Rigaku Miniflex 600 XRD System (Tokyo, Japan) with Ni-filtered Cu-Kα radiation at 1.54 Å, power of 40 kV and 15 mA. The sample scanning was carried out from 2θ of 5˚ to 120˚, at 0.02˚ steps and with a 5˚/minute increment.

Differential scanning calorimetry (DSC)

The DSC curve for the SD sample was obtained using a DSC 60 plus thermal analyzer (Shimadzu Asia Pacific Pte Ltd.). The required quantity of samples [TICA active pharmaceutical ingredient and (API) TICA-SD] was taken in the DSC pan, and the thermogram was recorded at a heating rate of 10 ˚C/min from 0 to 350 ˚C.

Dynamic vapor sorption (DVS)

To understand the moisture-induced glass transition event of Ticagrelor and its SD, water vapor sorption experiments were carried out on a DVS automated moisture sorption instrument (DVS Adventure/Resolution instruments, Surface Measurement Systems UK) at 25 ˚C. The sample of Ticagrelor API and SD (11-50mg) was dried for about 300 minutes under a continuous air flow to achieve the dry mass before being subjected to relative humidity (RH) of 0% to 95% at a ramping rate of 2% RH per hour. The partial pressure was then decreased in a similar manner. The camera accessory was used to take sample images during the experiment.

Assay by HPLC

To prepare a standard stock solution, the 10 mg Ticagrelor reference standard was transferred to a 10 mL volumetric flask. The sample was added to 5 mL of methanol and sonicated to dissolve. We made up the final volume to 10 mL with methanol, and mixed well. The standard solution was prepared by diluting 2 mL of a standard stock solution to 20 mL with the mobile phase. The test solution for Ticagrelor SD was prepared by dissolving a SD sample equivalent to 90 mg of Ticagrelor in 25 mL of methanol. The solution was sonicated to get a clear solution and filtered through a 0.45 micron syringe filter. The first few mL of filtered solution was discarded, and the Ticagrelor was quantified using HPLC at 270 nm. A test solution for Ticagrelor film-coated tablets was prepared by dissolving samples from 20 crushed tablets equivalent to 90 mg of Ticagrelor, and the further processing was conducted as per the Ticagrelor SD method. A test solution for the Ticagrelor capsule was prepared by dissolving the capsule content (from 20 capsules) equivalent to 90 mg of Ticagrelor, and the further process was followed as per the Ticagrelor SD.

In vitro dissolution study

Dissolution testing of Ticagrelor formulations was conducted using a USP II paddle apparatus with the aid of a dissolution tester (Electrolab TDT-08 L instrument). The dissolution method includes a phosphate buffer with pH 6.8, 900 mL, a paddle at 75 rpm, with a sampling time of 10, 20, 30, 45, and 75 minutes. A comparative dissolution profile of the dosage forms prepared with conventional wet granulation, SD, and micro-emulsification (SMEDDS) techniques was carried out to select the best formulation technique suitable for the dissolution enhancement of Ticagrelor.

Researchers carried out Bio-relevant dissolution for the finalized formulation of SD with co-povidone VA 64 in FaSSIF and FaSSGF to assess the *in vivo* absorption of the Ticagrelor dosage form. The above media were prepared by taking a specified quantity of FaSSIF powder and making up the volume to 1 L with monobasic sodium phosphate monohydrate (NaH_2PO_4. H_2O) buffer of pH 6.5. The absorbance of the Ticagrelor standard solution and sample solution was measured using a UV-Visible spectrophotometer (Shimadzu 1900 series with LabSolutions software) at 222 nm.

Dissolution samples were withdrawn at the 5, 15, 30, 60, and 75 min time points and filtered through a 0.45 µm syringe filter. The dilution of the filtrate was done with a mixture of methanol-phosphate buffer 6.8 (70:30), and absorbance was measured in a UV-Visible spectrophotometer (Shimadzu 1900 series with Lab solution software) at 222 nm to calculate the dissolution of Ticagrelor.

Stability study

Ticagrelor tablets prepared by SD were packed in an HDPE container with induction sealing and were subjected to the stability study at accelerated (40 ˚C/75% RH) and long-term storage (30 ˚C/75% RH) conditions as per ICH. Stability of the Ticagrelor tablet with SD technology was evaluated for assay, related substance, dissolution, and PXRD at both initial and 6-month time intervals.

In vivo experiments

PK study

An *in vivo* study was carried out in Wistar rats to evaluate the relative bioavailability between conventional Ticagrelor tablets 90 mg and Ticagrelor tablets 90 mg prepared with an SD approach. Further, a dose-adjusted comparative bioavailability study was carried out between the Ticagrelor conventional TABLET 90 mg and the Ticagrelor SD tablet 70 mg for the assessment of *in vivo* bioequivalence.

Wistar rats weighing 350-400 g were housed in individually ventilated cages and provided with an autoclaved pellet diet, bedding, and water ad libitum. The Wistar rats were maintained in a light and noise-controlled environment. The rats were put into four groups at random with n=2 per group, namely TICA-IR TABLET-90, TICA-IR TABLET-90M, TICA-SD TABLET-90, and TICA-SD TABLET-70. A test sample equivalent to 90 mg Ticagrelor was administered orally to the animal using normal saline as a vehicle. Blood was withdrawn at 1, 3, 5, 8, 12, and 24 hours by orbital puncture, and plasma was separated by centrifugation. Plasma samples were precipitated with methanol. Methanol samples were centrifuged to remove suspended debris. The supernatant methanol samples were analyzed by RP-HPLC on a BDS Hypersil C 8 Column, mobile phase methanol: Water (20:80, *v/v*), a flow velocity of 1.0 mL/min, and a UV detector with a wavelength of 254 nm were used.

The primary PK parameters, such as C_{max}, T_{max}, AUC_0-t, $AUC_{0-\infty}$, and $t_{1/2}$ were calculated using PKSolver® software (Version 2.0).

Construction of standard calibration curve: Blood samples were collected from Wistar rats and serum was separated through centrifugation. Different dilutions, i.e., 100 ng/mL, 500 ng/mL, and 2000 ng/mL, were prepared in the serum. The serum was precipitated using methanol and centrifuged with a Remi benchtop clinical centrifuge at 1000 RPM for 10 minutes. The supernatant methanol samples were analyzed by RP-HPLC on a BDS Hypersil C 8 Column with a mobile phase of methanol: water (20:80, *v/v*), a flow rate of 1.0 mL/min, and a UV detector at 254 nm. The standard calibration curve was constructed with the data obtained for 100 ng/mL, 500 ng/mL, and 2000 ng/mL.

Monitoring of GI bleeding

Test samples equivalent to 90 mg Ticagrelor were administered orally to Wistar rats with normal saline as a vehicle. Animals were observed for 12 hours for side symptoms and then sacrificed. The gastric organs were dissected and observed for bleeding and other related symptoms through a stereo microscope with 10X magnification.

RESULTS

Screening and optimization of formulation technology for Ticagrelor

An ASD formulation with co-povidone, based on the screening dissolution study of Ticagrelor dosage forms, yielded a superior dissolution rate compared to Ticagrelor SMEDDS capsules and conventional immediate release tablets with micronized API. Hence, the SD formulation technology was selected for further investigations. The physical parameters for the dosage forms prepared with different formulation technologies are shown in Table 1. The images of the dosage forms prepared with different formulation technologies are depicted in Figure 1.

Characterization of Ticagrelor and its formulations

Ticagrelor formulations prepared with different dissolution enhancement techniques, such as micronization, SD, and micro-emulsifying drug delivery (SMEDDS) were evaluated for their dissolution profiles in a discriminatory dissolution method. Ticagrelor tablets prepared with SD technology were evaluated for dissolution profile, PXRD, and DSC. The dissolution profiles for all the formulations prepared with the above technology were compared with the conventional immediate-release tablet formulation that has a similar Q1/Q2 (composition) and process technology to the reference product Brilinta® (AstraZeneca LP).

Particle morphology of Ticagrelor by polarized microscopy

The particle morphology and crystal properties of two different lots of Ticagrelor were evaluated with a polarized microscope (Olympus-BX 53). Ticagrelor API, taken for this study, showed positive birefringence with a polarized microscope as shown in Figure 2, indicating the crystalline nature of the API.

Particle size distribution (PSD) of Ticagrelor

Ticagrelor with two different particle size ranges was considered in the formulation process. Ticagrelor API was analyzed with a polarized microscope (Olympus BX 53) equipped with image provision technology-polarizing (IPV-P) class software for PSD, shape and agglomerates. Using the IPV-P class software,

Table 1. Physical evaluation parameters for the Ticagrelor dosage form prepared with different formulation technology

Parameters	TICA-IR tablets	TICA-SD tablets	TICA-SMEDDS capsules (hard gelatin)
Size/shape/dimensions	9.5 mm, Round, Bi convex	19x8 mm, Oblong, Biconvex	Size 00, lock length -23 mm
Weight	309 mg±2% *w/w*	1340 mg±2% *w/w*	900 mg±2% *w/w*
DT	4-5 minutes	4-5 minutes	3-4 minutes

DT: Disintegration time, IR: Immediate release, SD: Solid dispersion, SMEDDS: Self-Microemulsifying Drug Delivery System, TICA: Ticagrelor

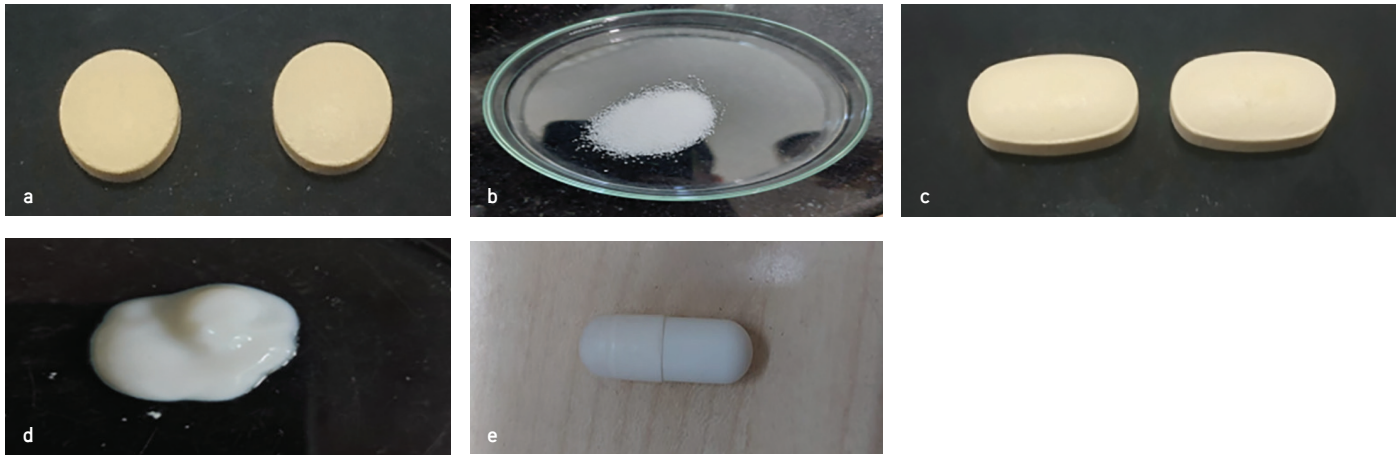


Figure 1. Ticagrelor formulations prepared with different dissolution enhancement techniques. (a) TICA-IR Tablets (In line with Brilinta® AstraZeneca LP); (b) TICA-SD; (c) TICA-SD tablets; (d) TICA-SMEDDS (e) TICA-SMEDDS Capsules (Hard gelatin)

TICA: Ticagrelor, IR: Immediate release, SD: Solid dispersion, SMEDDS: Self-Microemulsifying Drug Delivery System

PSD with D10, D50, and D90 were obtained and presented in Table 2. The optical microscopy images of the two different lots of API presented in Figure 2.

Optical microscopy for Ticagrelor SD

The Ticagrelor ASD sample was analyzed using an optical microscope (Olympus-BX 53) with and without the polarizer, at magnifications of 20X and 100X. Birefringence, an optical property of the crystalline solid, can be clearly observed with the help of a polarized optical microscope. Small traces of undesirable crystalline Ticagrelor in the sample of ASD could be identified when analyzed using the optical microscope equipped with a polarizer. No birefringence could be observed in the sample when analyzed by the optical microscope without a polarizer, as shown in Figure 2.

The impact of the traces of crystalline solids in the ASDs of Ticagrelor was further evaluated during the accelerated stability study to rule out any crystal growth and subsequent crystallization that could lead to a polymorphic transformation.

The particle size, shape, and presence of agglomerates were also evaluated with the help of IPV-P Class software. The results are presented in Table 3 and Figure 3.

Powder X-ray diffraction (PXRD)of Ticagrelor

The Ticagrelor API used in the current study was analyzed with the Rigaku Miniflex 600 XRD System. The PXRD pattern of Ticagrelor is shown in Figure 4. Sharp diffraction peaks of 2θ at 10.50^0, 13.36^0, 14.74^0, 18.18^0, 19.06^0, 21.12^0, 22.52^0, and 24.12^0 were observed against the reported 2 theta (2θ) values, Table 4, indicating that the Ticagrelor API used in the current study is a mixture of polymorphic forms II, III, and IV of Ticagrelor. Hence, it can be considered a metastable form of Ticagrelor.

Ticagrelor exhibits polymorphism. Four non-solvated polymorphs (Polymorph I, II, III and IV) and many solvated crystalline polymorphs are reported, which can be clearly distinguished by X-ray powder diffraction. Apart from the

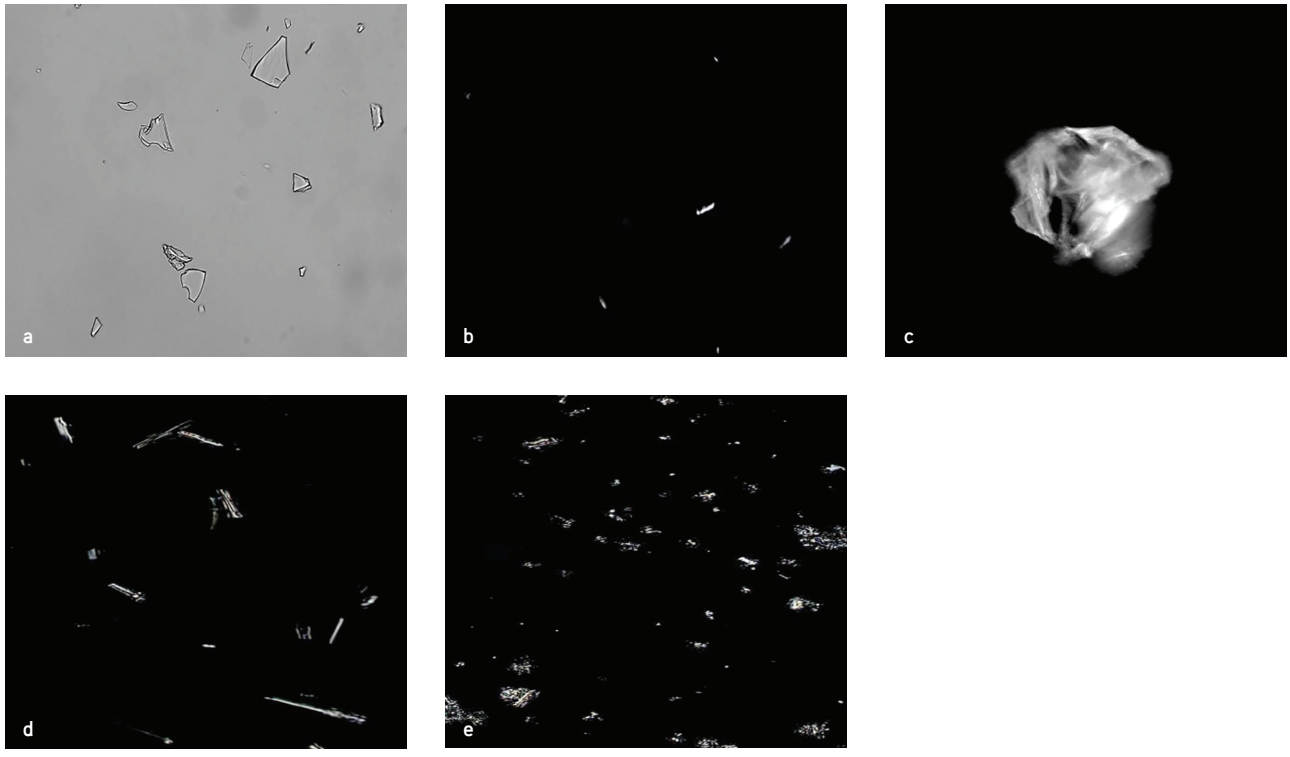


Figure 2. Optical microscopic images of Ticagrelor SD. (a) TICA-SD co-povidone VA 64, 20X without polarizer; (b) TICA-SD co-povidone VA 64, 20X with polarizer; (c) TICA-SD co-povidone VA 64, 100X with polarizer; (d) TICA-API (D90: 26.969 µm), 20X with polarizer; (e) TICA-API (D90: 12.8805 µm), 20X with polarizer obtained from polarized microscope (Olympus -BX 53) with IPV-P class software

API: Active pharmaceutical ingredient, IPV-P: Image provision technology–polarizing, SD: Solid dispersion, TICA: Ticagrelor

Table 2. Particle size and shape for two different lots of Ticagrelor analyzed through a polarized microscope (at 20X) with IPV-P class software (image provision technology)

PSD (µm)	Sample A			Sample B		
	Particle	Agglomerate	Total	Particle	Agglomerate	Total
D_{10}	3.946	3.946	3.946	3.355	9.3666	3.3550
D_{50}	10.011	10.011	10.011	6.344	14.9524	6.5353
D_{90}	26.969	26.969	26.969	11.839	26.5635	12.8805

IPV-P: Image provision technology-polarizing, PSD: Particle size distribution

above four polymorphic forms, Form α is also reported as an anhydrous form, which has no sharp peak in the PXRD. It is reported that form II is a metastable form, whereas form I is a thermodynamically stable form. The interconversion of form II to form I and vice versa takes place reversibly through a temperature-induced phase transition upon heating/cooling.[22,23]

Different crystal habits of Ticagrelor (TICA) form II were studied and reported by Ren et al.[24] The impact of the anisotropic surface chemistry of the crystal on the physicochemical properties of the crystal, such as solubility and tableting, was reported.

Table 3. PSD of SD of Ticagrelor prepared with co-povidone VA 64

PSD for TICA-SD			
Parameters	**Particle**	**Agglomerate**	**Total**
D10	2.290	0.000	2.2896
D50	3.946	0.000	3.9463
D90	12.952	0.000	12.9522

PSD: Particle size distribution, SD: Solid dispersion, TICA: Ticagrelor

PXRD for Ticagrelor SD

The PXRD pattern of crystalline Ticagrelor and Ticagrelor SD prepared with co-povidone VA 64, Soluplus, and HPMCAS is shown in Figure 5. Sharp crystalline peaks with 2θ at 10.50^0, 13.36^0, 14.74^0, 18.18^0, 19.06^0, 21.12^0, 22.52^0 and 24.12^0 were observed for the API Ticagrelor, whereas all the crystalline peaks disappeared in the SD samples.

Crystalline Ticagrelor was completely converted to amorphous form for three formulations prepared with co-povidone VA 64, Soluplus and HPMCAS. However, the stability of the ASD depends on the hygroscopicity and CDF. Faster dissolution and satisfactory stability of the ASD prepared with carrier co-povidone VA 64 could be attributed to its low CDF.[16]

The PXRD pattern of Ticagrelor API, SD with co-povidone VA 64, tablets prepared with SD, and placebo is shown in Figure 6.

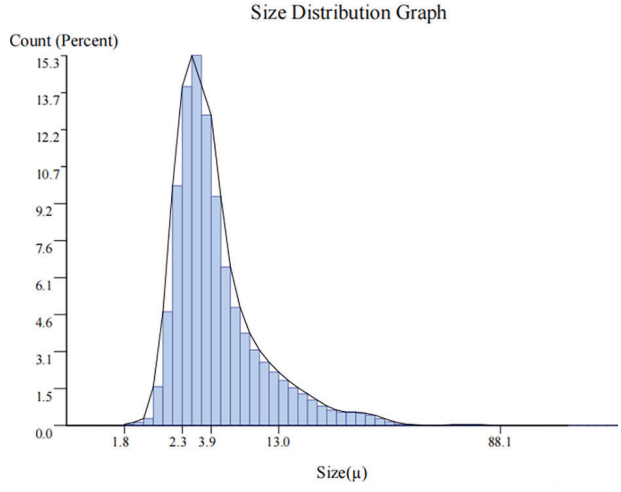


Figure 3. PSD of SD of Ticagrelor prepared with co-povidone VA 64

PSD: Particle size distribution, SD: Solid dispersion

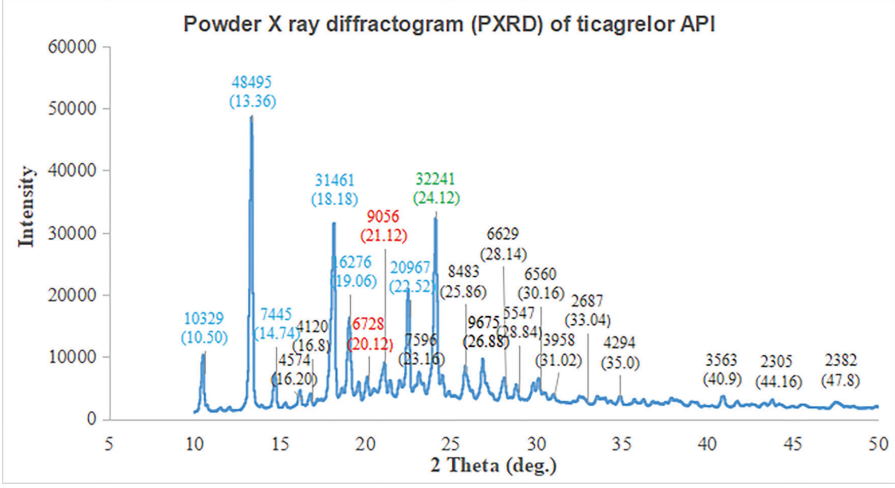


Figure 4. X-ray diffractogram of the Ticagrelor API used in the present study

API: Active pharmaceutical ingredient

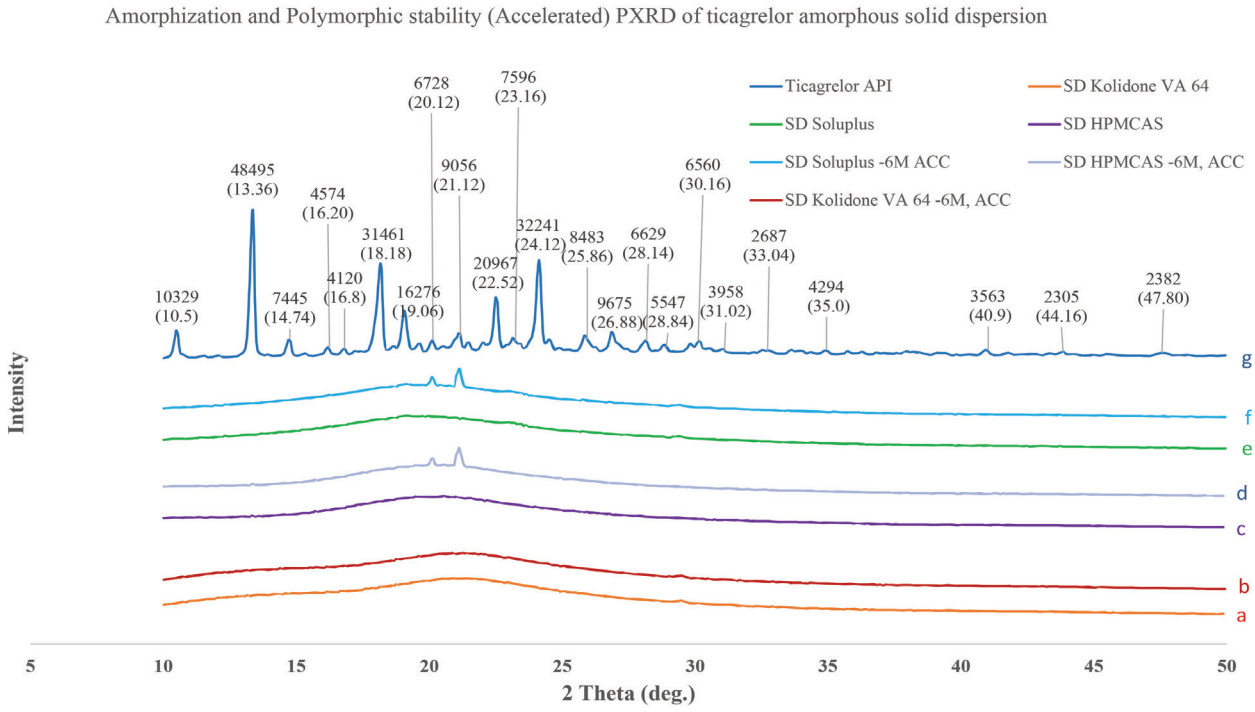


Figure 5. Amorphization and comparative polymorphic stability of Ticagrelor SD at accelerated storage condition. (a) PXRD of Ticagrelor SD to Kolidone VA 64; (b) PXRD of Ticagrelor SD with Kolidone VA 64 at 6M Accelerated condition; (c) PXRD of Ticagrelor SD HPMCAS; (d) PXRD of Ticagrelor SD HPMCAS at 6M Accelerated condition; (e) PXRD of Ticagrelor SD with Soluplus; (f) PXRD of Ticagrelor SD with Soluplus at 6M Accelerated condition; (g) PXRD of Ticagrelor API

API: Active pharmaceutical ingredient, D90: Diameter at which 90% of the particles are smaller (particle size distribution parameter), IPV-P: Image provision technology-polarizing (microscope imaging software, as stated), SD: Solid dispersion, TICA: Ticagrelor

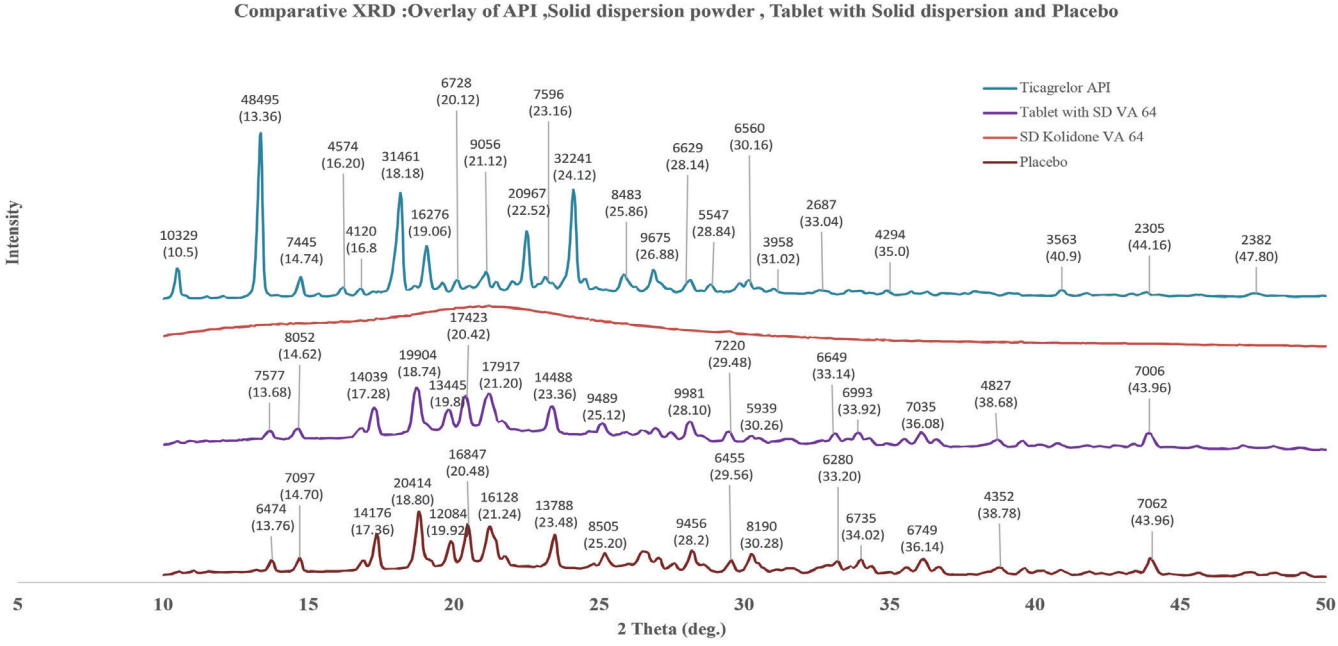


Figure 6. PXRD pattern of Ticagrelor API, placebo, optimized SD and its tablet formulation

API: Active pharmaceutical ingredient, PXRD: Powder X-ray diffraction, SD: Solid dispersion

The sharp characteristic peaks of the crystalline Ticagrelor API with 2θ at 10.50°, 13.36°, 14.74°, 18.18°, 19.06°, 21.12°, 22.52° and 24.12° were found to have disappeared in the SD formulation. However, in the PXRD of the SD tablets, only the placebo peaks appeared. Hence, it is concluded that no polymorphic change is observed in the Ticagrelor ASD during or after compression of the tablet.

Stability study of Ticagrelor SD

No significant change was observed in the assay, dissolution, and related substances for the optimized Ticagrelor tablets prepared with SD [TICA-SD Tablet with co-povidone (1:0.4:4)]. Further, in the PXRD of the stability sample, no crystalline peaks were observed up to 6 months.

In the PXRD of stability samples of Ticagrelor SD prepared with carrier Soluplus and HPMCAS, two crystalline-characteristic peaks 2θ at 20.12° and 21.12° corresponded to Ticagrelor polymorphic form I. However, no crystalline peaks were observed for the stability sample of Ticagrelor SD, prepared with carrier Co-povidone VA 64. The comparative polymorphic stability of Ticagrelor ASD with different carriers is depicted in Figure 5.

Differential scanning calorimetry (DSC)

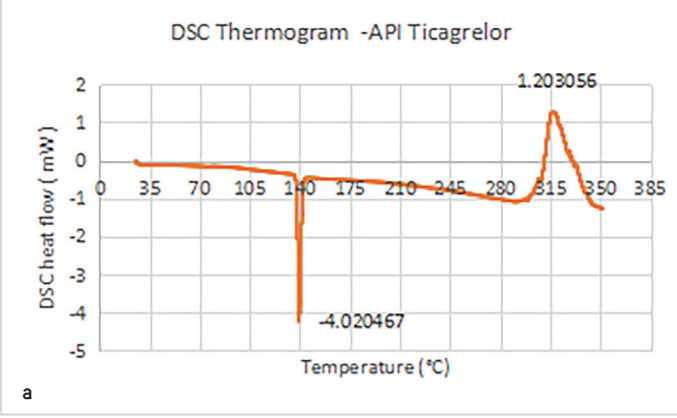
The DSC thermogram of Ticagrelor used in the present study was generated at a 10 °C/min heating rate over a range of 0 to 350 °C to understand transitions as a function of temperature and time. Also, specific information, i.e., glass Tg, melting point (Tm), and crystallization temperature (Tc) obtained from the endothermic and exothermic processes was studied. The endothermic peak at 140 °C and exothermic peak at 315 °C in Figure 7 indicate the melting and crystallization temperatures, respectively. The reported onset melting temperature for different polymorphs of Ticagrelor is given in Table 4. It can be noted that the precise value of the melting point is influenced by the purity of the compound, the sample weight, the heating rate, and the particle size. Hence, the experimental values may slightly differ from the reported values.

DSC for Ticagrelor and its SD

The DSC thermogram of pure Ticagrelor and Ticagrelor SD is presented in Figure 7. For the Ticagrelor API, the endothermic and exothermic peaks at 140 °C and 315 °C indicate the melting and crystallization temperature, respectively, whereas the endothermic peak of Ticagrelor SD at 52.72 °C indicates the glass transition temperature (Tg).

Stabilized amorphous Ticagrelor reported by Davis et al.[25] EP 2 813 216 A1. Amorphous Ticagrelor has a relatively low glass transition temperature (Tg), about 46 °C. It is, therefore, essential to prevent re-crystallization by increasing the glass transition temperature (Tg) to stabilize the ASD of Ticagrelor.

From the DSC thermogram, it is evident that the glass transition temperature (Tg) of Ticagrelor SD is increased to 52.72 °C due to the presence of Co-povidone VA 64 as a carrier. Co-povidone VA 64 is an excellent crystallization inhibitor and a matrix-forming agent with a glass transition temperature (Tg) of 101 °C. The absence of any exothermic peak in the DSC thermogram indicates the absence of crystallization in the sample of SD prepared with co-povidone VA 64.



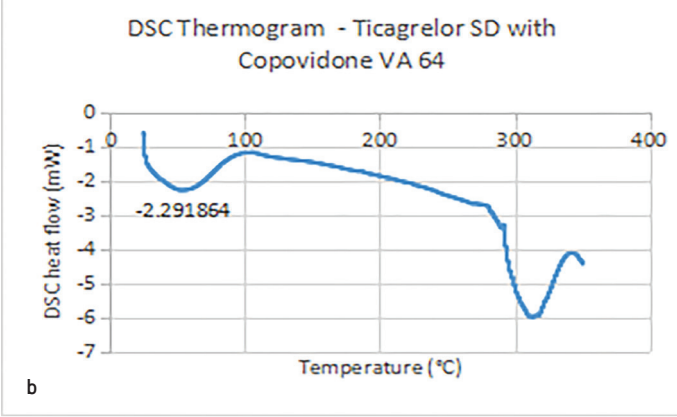


Figure 7. DSC thermogram of (a) pure Ticagrelor API and (b) Ticagrelor SD

API: Active pharmaceutical ingredient, DSC: Differential scanning calorimetry, SD: Solid dispersion

Table 4. Reported 2 Theta (2θ) value and onset melting temperature for different polymers of Ticagrelor[22]

Polymorphic form	2 theta value (XRD)	Characteristic high intensity peaks	On set of melting (DSC)
Form I	5.3,8.0, 9.6, 13.9, 15.3. 20.1,20.7,21.0,21.3, 26.2, 27.5	5.3,20.1,20.7,21.0,21.3	151 °C (146 -152 °C)
Form II	5.5, 6.8, 10.6,13.5, 14.9, 18.3, 19.2,22.7,24.3, 27.1	5.5,13.5,18.3,22.7,24.3	137.5 °C (136-139 °C)
Form III	5.6, 12.5, 14.0,17.4,18.4, 21.4, 22.2, 22.9,24.1, 24.5	14.0,17.4,18.4,21.4, 24.1	132 °C (127-132 °C)
Form IV	4.9, 6.0,9.2,11.6, 12.8, 15.6,16.4, 17.2, 18.1	4.9,9.2,11.6,15.6,16.4	139 °C

DSC: Differential scanning calorimetry, XRD: X-ray diffraction

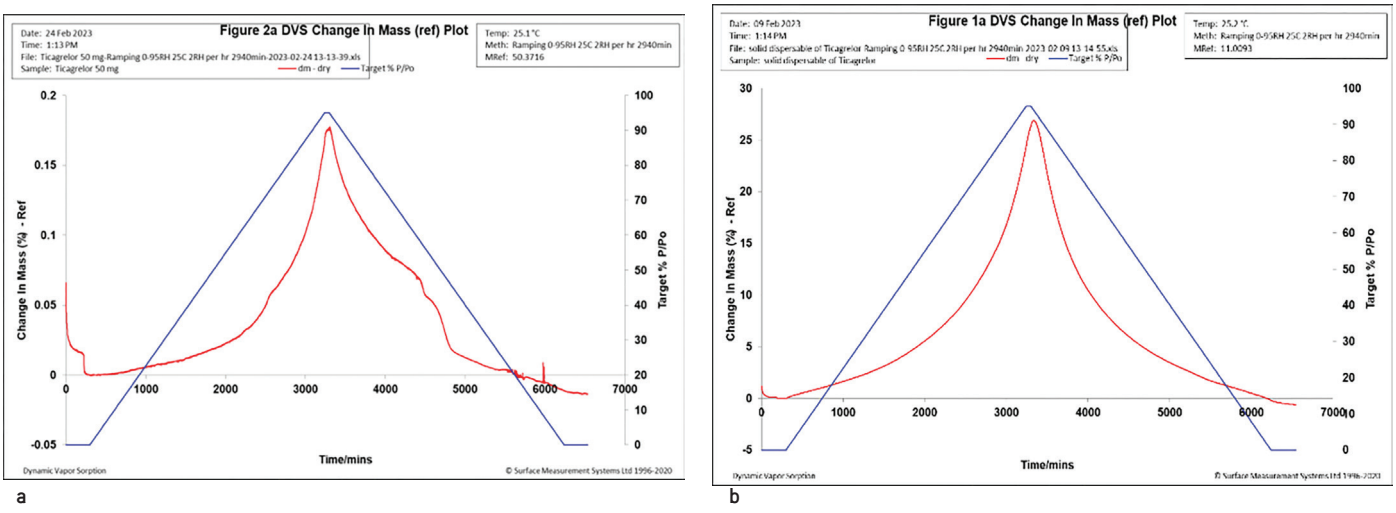
DVS

For the ramping experiment, the % change in mass versus time plots for the sample of Ticagrelor API and SD of Ticagrelor at 25 °C are shown in Figure 8, respectively.

In Figure 8, the red line indicates the net percent change in mass with respect to time, while the blue line represents the sample RH. The profile of the Ticagrelor SD sample shows a relatively higher mass uptake than the Ticagrelor API. This suggests that there was a bulk sorption mechanism occurring in the sample of SD of Ticagrelor, whereas a surface sorption process occurred in the Ticagrelor API sample.

The video camera accessory captured images of the SD sample of Ticagrelor and its API. Figure 9 shows snapshots of the SD of Ticagrelor during the RH ramping experiment at 25.0 °C to visualize RH-induced transformation. For a sample of Ticagrelor SD, there were distinct changes in the visual appearance. However, this is not observed in the case of the Ticagrelor API samples.

The RH where transformation occurs from surface adsorption to bulk absorption represents the glass transition relative humidity (RHg). The glass transition RH values for Ticagrelor API and Ticagrelor SD were found to be 59.9% and 64.4%, respectively.



a b

Figure 8. RH ramping experiment for (a) Ticagrelor API; (b) SD of Ticagrelor representing glass transition RH at 25.0 °C

API: Active pharmaceutical ingredient, RH: Relative humidity, SD: Solid dispersion

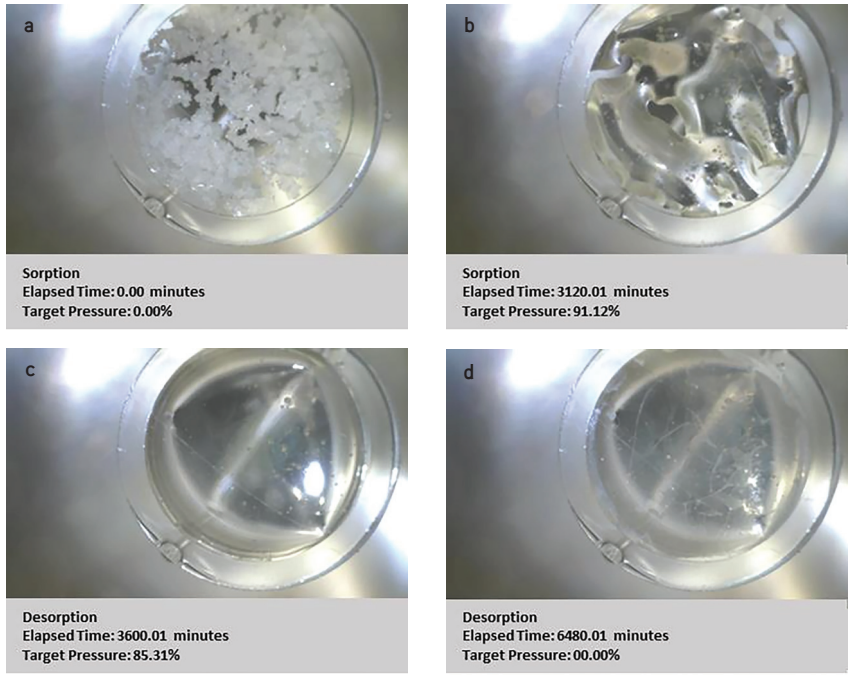


Figure 9. Real-time images of the Ticagrelor SD sample during the RH ramping experiment at 25.0 °C represent the RH-induced transformations and glass transitions. (a) sorption at 0 % pressure; (b) sorption at 91.12 % pressure; (c) desorption at 85.31 % pressure; (d) desorption at 0 % pressure

RH: Relative humidity, SD: Solid dispersion

In vitro dissolution study

To evaluate the dissolution performance of the Ticagrelor dosage form, a dissolution study was performed with the USP II paddle apparatus at 75 RPM and 900 mL of phosphate buffer pH 6.8; both with and without a surfactant. With its good discriminatory power, phosphate buffer pH 6.8 without surfactant was selected for the formulation trials. The sample analysis was performed with a UV-Visible spectrophotometer (Shimadzu 1900 series with LabSolutions software) at 222 nm.

The dissolution profiles for all the formulations of Ticagrelor were generated in phosphate buffer pH 6.8, as presented in Figure 10. As per the result, the dissolution after 75 minutes was found to be 10% (% RSD-1.1), 15% (% RSD-3.0), 24% (% RSD-0.5), 107% (% RSD-0.6), 30% (% RSD-2.8) and 27% (% RSD-0.3) for TICA-IR TABLET 90 (D90:26.96 µ), TICA-IR TABLET 90 (D90:12.88 µ), TICA-SD TABLET 90 with co-povidone VA 64 (1:0.4:1), TICA-SD TABLET 90 with co-povidone (1:0.4:4), TICA-SD TABLET 90 with soluplus (1:0.4:4), TICA-SD TABLET 90 with HPMCAS (1:0.4:4) and TICA-SMEDDS CAPSULE 90 respectively. The dissolution profile obtained for the conventional immediate release tablet formulation [TICA-IR TABLET 90 (D90:26.96 µ)] was found to be similar to the reported dissolution profile of reference product BRILINTA® (AstraZeneca LP).[18]

Bio-relevant dissolution

Based on the comparative dissolution profile of formulations prepared with different technologies, the SD technique with co-povidone VA 64 was superior and yielded faster dissolution compared to other techniques such as micronization and microemulsifying drug delivery. Hence, Ticagrelor tablets with SD technology, i.e., TICA-SD tablets with co-povidone (1:0.4:4), were further evaluated with biorelevant media (FaSSGF & FeSSIF) to simulate the *in vivo* realistic environment.

Based on the results of the TICA-SD Tablet with co-povidone (1:0.4:4), the dissolution rates at 75 minutes were found to be 107% (% RSD-0.6), 100% (% RSD-0.5) and 100% (% RSD-0.6) in phosphate buffer pH 6.8, FaSSGF, and FaSSIF, respectively (Figure 10).

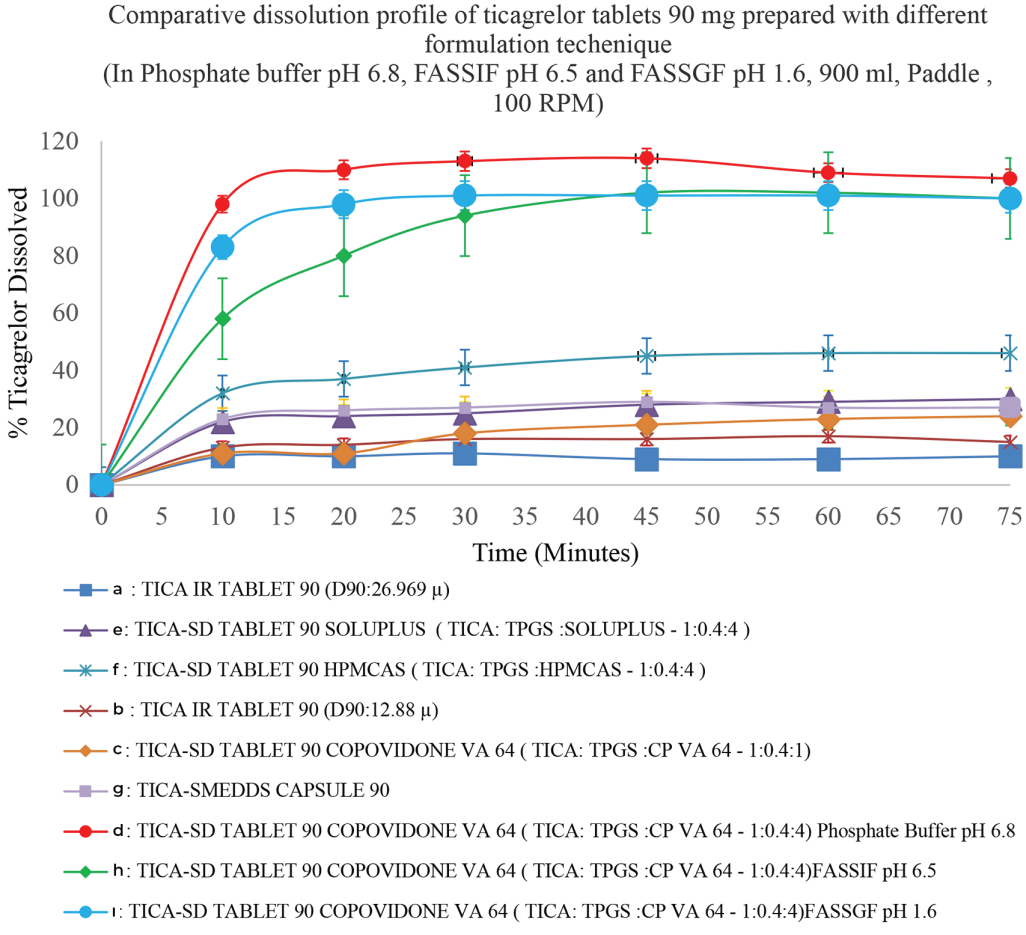


Figure 10. Comparative dissolution profile of Ticagrelor formulations, (a) conventional IR tablet (D90:26.96 µ); (b) conventional IR tablet (D90:12.88 µ); (c) TICA-SD tablet with copovidone (1:0.4:1); (d) TICA-SD TABLET 90 with copovidone VA 64 (1:0.4:4); (e) TICA-SD tablet with Soluplus (1:0.4:4); (f) TICA-SD tablets 90 with HPMCAS (1:0.4:4); (g) TICA-SMEDDS capsule 90; (h) TICA-SD TABLET 90 with copovidone VA 64 (1:0.4:4); in FASSIF pH 6.5; (i) TICA-SD TABLET 90 with copovidone VA 64(1:0.4:4) in FASSGF pH 1.6

IR: Immediate release, SD: Solid dispersion, SMEDDS: Self-microemulsifying drug delivery system, HPMCAS: Hydroxypropyl methylcellulose acetate succinate, FaSSIF: Fasted state simulated intestinal fluid, FaSSGF: Fasted state simulated gastric fluid

Dissolution data are presented as mean ± SD, where n is the number of observations (n=6).

In vivo results for Ticagrelor formulations

PK study

To evaluate comparative bioavailability, the Wistar rats were randomly divided into 4 groups (n=6), namely TICA-IR TABLET 90, TICA-IR TABLET 90M, TICA-SD TABLET 90, and TICA-SD TABLET70, for dosing as outlined in Table 5 below. The samples equivalent to the required dose were administered orally to the Wistar rats using normal saline as a vehicle. The animal dose for the Wistar rat was calculated considering the human dose using the equation below.[26]

HED (mg/kg) = Animal dose (mg/kg) x (Animal km)/ (Human km)

Where HED is the human equivalent dose

Km is the correction factor estimated by dividing the average body weight (kg) of a species by its body surface area (m^2).

The relative bioavailability and peak plasma concentrations (C_{max}) of Ticagrelor formulations are summarized in Table 6. The plasma concentration-time graph for the optimized Ticagrelor SD formulation is presented in Figure 11.

Monitoring of GI bleeding

Visual observation of the dissected gastric organ through a radical stereo microscope with 10X magnification (Figure 12) revealed no redness or bleeding post administration of the formulations of Ticagrelor.

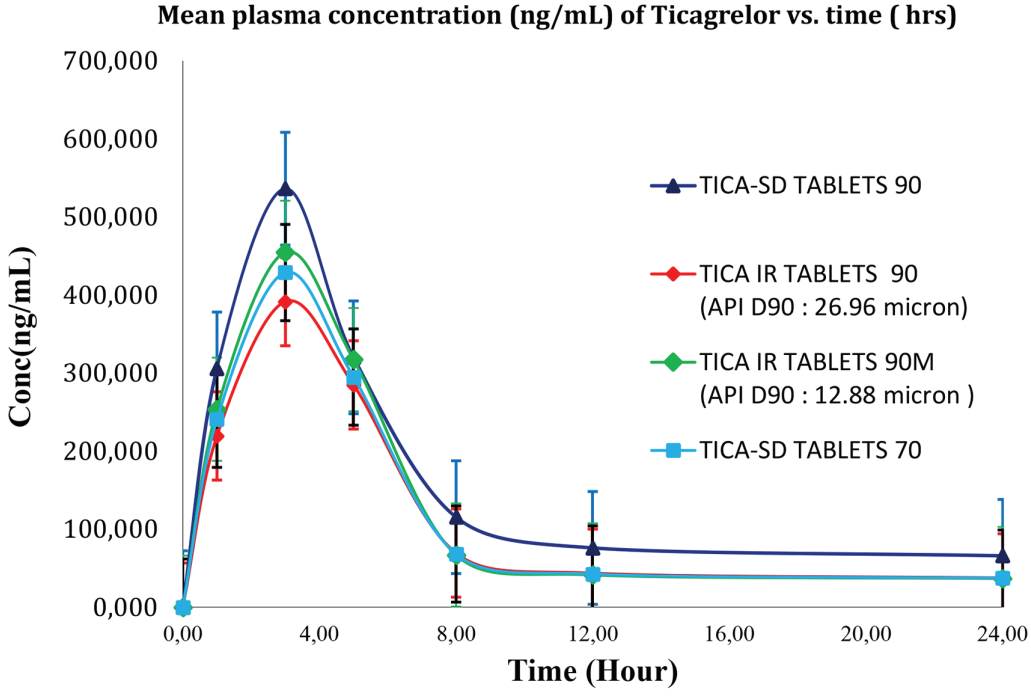


Figure 11. Comparative plasma concentration-time profiles of Ticagrelor in Wistar rats following oral administration of Ticagrelor conventional immediate release tablet formulations (TICA-IR TABLETS 90, TICA-IR TABLETS 90M, n=6) and Ticagrelor SD tablet formulations (TICA-SD TABLETS 90, TICA-SD TABLETS 70, n=6)

IR: Immediate release, n: Number of animals (sample size), SD: Solid dispersion, TICA: Ticagrelor

Table 5. Details of the formulation dosing group, along with the formulation code for PK study.

Group (n=6)	Formulation code	Formulation technology	Ticagrelor PSD (D_{90})	Dose
I	TICA-SD TABLET 90	SD	D_{90}: 26.96 µm	9 mg/kg
II	TICA-IR TABLET 90M	Conventional wet granulation (with micronized Ticagrelor)	D_{90}: 12.88 µm	9 mg/kg
III	TICA-SD TABLET 70	SD	D_{90}: 26.96 µm	7 mg/kg
IV	TICA- IR TABLET 90	(Conventional immediate release tablet formulation in line with Brilinta® AstraZeneca LP)	D_{90}: 26.96 µm	9 mg/kg

IR: Immediate release, PSD: Particle size distribution, SD: Solid dispersion, TICA: Ticagrelor

Table 6. Details of PK parameters for different Ticagrelor tablet formulations

PK parameters	TICA-SD TABLET 90	TICA-IR TABLET 90M	TICA-SD TABLET 70	TICA-IR TABLET 90
C_{max} (ng/mL)	536.25±7.8	454.75±11.2	428.77±5.4	391.45±7.4
AUC_{0-t} (h*ng/mL)	3736.90±71.00	2867.75±87.1	2757.28±59.9	2639.68±92.70
$AUC_{0-\infty}$ (h*ng/mL)	4462.23±154.27	3191.73±150.30	3096.55±135.40	2992.89±160.25
T_{max} (h)	3.0±0.00	3.0±0.00	3.0±0.00	3.0±0.00
$T_{1/2}$ (h)	7.63±0.30	6.094±0.40	6.46±0.20	6.50±0.40
Relative bioavailability (%)	141.61±2.29	108.65±0.52	104.48±1.40	-

IR: Immediate release, PK: Pharmacokinetic, PSD: Particle size distribution, SD: Solid dispersion, TICA: Ticagrelor

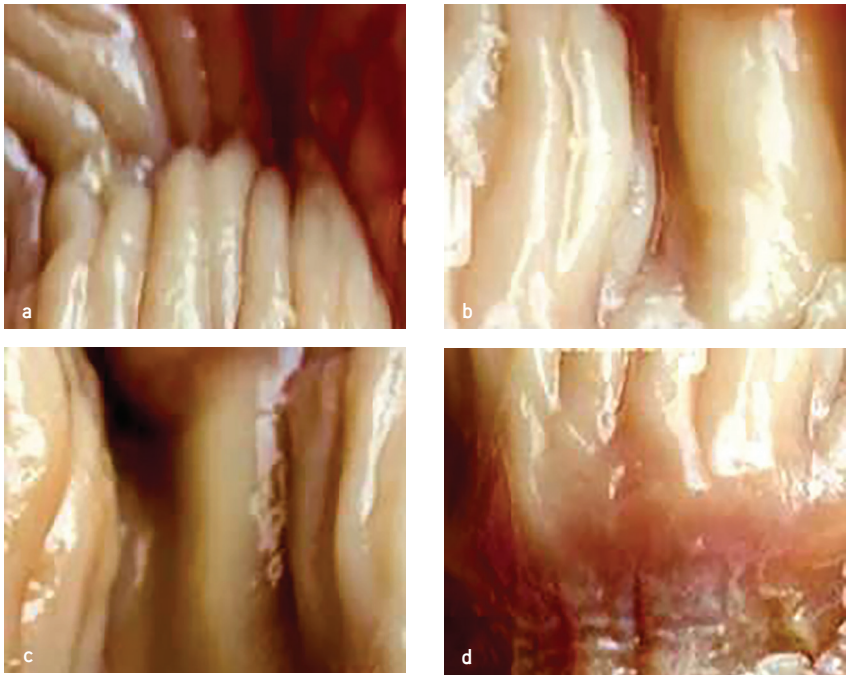


Figure 12. Gastric lumen of the Wistar rat after administration of (a) TICA-IR TABLET 90; (b) TICA-SD TABLET 90; (c) API 90 mg/kg, and (d) untreated

Parameters	TICA-IR TABLET 90	TICA-SD TABLET 90	API/90
Redness	*	*	*
Bleeding	*	*	*

*None; **Slight; ***Moderate; ****High; *****Severe. API: Active pharmaceutical ingredient, IR: Immediate release, SD: Solid dispersion, TICA: Ticagrelor

DISCUSSION

The analytical toolbox comprising optical microscopy (Olympus -BX 53), PXRD, DSC and DVS (surface management system), explored in this work, has proven to be very useful in characterizing, screening and monitoring the polymorphic stability of Ticagrelor formulations. The evaluation of the particle morphology and crystal properties of Ticagrelor using the polarized microscope (Olympus BX-53) reveals the crystalline nature of the API. It is clear from the PXRD report that the Ticagrelor API employed in this investigation is a mixture of various metastable polymorphs, necessitating careful attention while choosing the appropriate formulation and process technology. The DSC thermogram of Ticagrelor provides specific information, i.e., glass transition temperature (Tg), melting point (Tm) and crystallization temperature (Tc), which are critical for ensuring polymorphic stability during formulation and processing.

The ASD technique formulated with co-povidone VA 64 and vitamin E TPGS was found to be the most promising method to enhance *in vitro* dissolution performance and polymorphic stability of metastable Ticagrelor. Optical microscopy (Olympus BX 53) has proven to be an important tool to evaluate the impact of small traces of crystalline solids on the ASDs of Ticagrelor, providing an early indication of crystallization leading to a polymorphic transformation. Amorphization of crystalline Ticagrelor could be possible with all three carriers, i.e., Co-

povidone VA 64, Soluplus, and HPMCAS. However, faster dissolution and satisfactory stability of the ASD prepared with carrier co-povidone VA 64 could be attributed to its low CDF. Co-povidone VA 64 was found as an excellent crystallization inhibitor and could elevate the glass transition temperature (Tg) of Ticagrelor SD to 52.72 ˚C, thereby ensuring improved polymorphic stability. The RHg for the ASD, determined with the help of DVS, could provide important insights into the RH-induced polymorphic transformation.

Formulation of ASD with polymer co-povidone VA 64 [i.e., TICA-SD tablet with co-povidone (1:0.4:4)] showed significantly faster dissolution by 10.7-fold compared to the Ticagrelor tablet formulation prepared in line (similar Q1/Q2) with reference product BRILINTA® (AstraZeneca LP).

Surprisingly, the dissolution rate of a Ticagrelor SD formulation with soluplus [TICA-SD tablet with soluplus (1:0.4:4)] and with HPMCAS was increased only by three- to four-fold compared to that of the conventional immediate release Ticagrelor tablet formulation.

Despite complete amorphization of Ticagrelor with co-povidone VA 64, Soluplus and HPMC Acetate Succinate (Figure 6), the kinetics of dissolution of solid dispersion with Soluplus and HPMC Acetate Succinate were found to be significantly slower compared to SD with co-povidone VA 64. The dissolution of the ASD depends on various factors, such as hygroscopicity, hydrophobicity, glass transition temperature (Tg), and CDF. Although amorphization enhances solubility and bioavailability to a great extent, its CDF leads to a polymorphic transformation, thereby favoring one thermodynamically stable crystalline form during *in vitro* and *in vivo* dissolution. Also, increased molecular mobility leads to kinetic instability. Low molecular weight excipients are known to lower CDF and elevate the glass transition temperature (Tg). The lower dissolution rate of SD with Soluplus and HPMC Acetate succinate may be attributed to their higher CDF and hydrophobicity.[14,27] The impact of micronization on the dissolution of Ticagrelor was not found to be significant. An increase in 1.5-fold dissolution was observed for the conventional IR formulation with micronized Ticagrelor (D90:12.88 µ).

Formulation prepared with SMEDDS technology could increase the dissolution of Ticagrelor by 2.7-fold only, which could be attributed to the drug precipitation or crystallization during *in vitro* dissolution. Higher percentages of oil, surfactant, and co-surfactant may help to improve the dissolution further. However, a further increase in the percentage of components of SMEDDS would significantly increase capsule fill weight. Hence, capsule formulation is not feasible.

Ticagrelor is absorbed with a median t_{max} of 1.5 h (range 1.0-4.0). The faster rate of dissolution in the biorelevant media, i.e., more than 90% dissolution at 30 minutes in both FaSSGF (pH 1.6) and FaSSIF (pH 6.5), gives a high degree of assurance that the drug Ticagrelor would be in a highly dissolved state to be absorbed in the absorption window. Further, the presence of vitamin E TPGS, one known PG Efflux inhibitor, in the formulation would enhance the permeability of Ticagrelor and thereby increase its bioavailability.

No crystalline peaks were observed for the stability sample of Ticagrelor SD prepared with carrier Co-povidone VA 64, whereas two crystalline characteristic peaks at 2 theta 20.12 and 21.12, corresponding to the Ticagrelor polymorph form I, were observed for the Ticagrelor SD with carriers Soluplus and HPMCAS, indicating that Ticagrelor SD prepared with carrier Co-povidone VA 64 could provide superior polymorphic stability compared to those prepared with Soluplus and HPMCAS.

The bioavailability and peak plasma concentration (C_{max}) of Ticagrelor SD (TICA-SD TABLET 90) formulation were found to be 141.61±2.29% and 137.0±0.59%, respectively, compared to the conventional immediate release tablet formulation in line with Brilinta® (AstraZeneca LP) (TICA-IR TABLET 90). The bioavailability of Ticagrelor with micronization could not be enhanced significantly. The relative bioavailability of the Ticagrelor tablet with micronized API (TICA-IR TABLET 90M) was found to be 108.65±0.52% compared to the conventional immediate-release tablet formulation (TICA-IR TABLET 90). Based on the results of a dose-adjusted PKs study of Ticagrelor SD, the AUC_{0-24}, and peak plasma concentration (C_{max}) of 70 mg dose of Ticagrelor SD (TICA-SD TABLET 70) formulation were found to be similar to these parameters of conventional immediate release tablet formulation of Ticagrelor tablets (TICA- IR TABLET 90), indicating that 70 mg dose of Ticagrelor tablet with SD technique would be equivalent to 90 mg dose of conventional immediate release tablet formulation, prepared in line with reference product Brilinta® (AstraZeneca LP) for the desired therapeutic action.

Numerous works on increasing the oral bioavailability of Ticagrelor have been reported. Kim et al.[28] reported the 219.78±36.33% relative bioavailability of an SD formulation compared to pure Ticagrelor in a PK study. Na et al.[29] reported a 2.2-fold increase in relative bioavailability of one TPGS/PVA-based nanosuspension compared to the reference product Brilinta® 90 mg, (AstraZeneca LP) in rats. In another study, the oral relative bioavailability of Ticagrelor SMEDDS in rats increased by 6.3 times compared to a raw Ticagrelor suspension, as reported by Na et al.[30] A 5-fold increase in oral bioavailability for Ticagrelor SMEDDS compared to pure Ticagrelor was reported by Aparna et al.[31]

Ticagrelor is a third-generation oral antiplatelet drug with a higher risk of GI bleeding compared to clopidogrel. As per the USFDA approval package for Ticagrelor (Brilinta®, AstraZeneca), the most common adverse reactions (>5%) are bleeding and dyspnea. This includes getting out of breath, nose bleeds, heavier periods, bleeding gums, and bruising. Based on the above facts on Ticagrelor adverse reactions, there will always be an increased risk of GI bleeding associated with the administration of Ticagrelor formulations with significantly higher bioavailability compared to the reference product Brilinta® 90 mg (AstraZeneca LP). Hence, it is highly recommended to evaluate the impact of improved dissolution and bioavailability in the proposed formulations, which may lead to the adverse reaction of GI bleeding.[32]

The proposed SD formulation of Ticagrelor in the current study has a superior bioavailability compared to the conventional immediate release tablet formulation as offered by Brilinta® AstraZeneca LP. It is well-supported by a GI bleeding study in Wistar rats. The study reveals no GI redness or bleeding owing to the improved dissolution and bioavailability of the proposed formulation with SD technology.

In summary, the optimized Ticagrelor ASD formulation demonstrated enhanced bioavailability, minimal or low adverse reactions, and improved polymorphic stability. The adopted solvent evaporation manufacturing process would also enable a feasible and scalable process transition to spray drying and FBP technology.

CONCLUSION

TICA-SD, with co-povidone VA 64 and vitamin E TPGS, could be able to enhance the dissolution of Ticagrelor by 10.7 fold and have a relative bioavailability of 141.61±2.29% compared to a conventional immediate release tablet formulation prepared in line with Brilinta® (AstraZeneca LP). Based on a dose-adjusted PK study, the SD formulation of Ticagrelor allows a lower dose (70 mg) of Ticagrelor to achieve an equivalent therapeutic effect to that of the reference product Brilinta® 90 mg (AstraZeneca LP). No adverse reactions were noted in the GI bleeding study, which may be attributed to the enhanced dissolution and bioavailability of the SD formulation. The manufacturing process is feasible and is scalable to industrial-scale operation. PXRD and DSC data are in good agreement regarding the transformation of crystalline Ticagrelor to amorphous form. SD is found to be a superior technique in enhancing the bioavailability and stability of poorly soluble, metastable Ticagrelor and in enhancing the bioavailability and stability of poorly soluble, metastable, Ticagrelor, and could be extended to other BCS Class IV drugs.

The SD technique with carrier co-povidone VA 64, and vitamin E TPGS prepared by the solvent evaporation process could yield a Ticagrelor formulation with improved bioavailability and polymorphic stability.

Ethics

Ethics Committee Approval: Due approval obtained from ethics commitees such as CPCSEA and IAEC (approval number: CPCSEA/DIPS/0223/61, dated: 21.02.2023).

Informed Consent: Study conducted in Wistar rats hence patient informed consent not applicable.

Footnotes

Authorship Contributions

Concept: R.P., S.L., Design: R.P., S.L., Data Collection or Processing: R.P., S.L., Analysis or Interpretation: R.P., S.L., Literature Search: R.P., S.L., Writing: R.P., S.L.

Conflict of Interest: The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Synthesis, Characterization, and Antimicrobial Activity of Some New 2,4-Dihydro-3*H*-1,2,4-Triazole-3-thione Derivatives

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ABSTRACT

Objectives: Antimicrobial resistance is a major problem in the treatment of infectious diseases. Therefore, it is important to develop new and effective antimicrobial agents. For this purpose, a new series of compounds with a 2,4-dihydro-3*H*-1,2,4-triazole-3-thione structure was synthesized.

Materials and Methods: 2,4-dihydro-3*H*-1,2,4-triazole-3-thione compounds (T1-T8) were synthesized by heating thiosemicarbazide derivatives under alkaline conditions. Infrared (IR), ^{1}H-NMR, and ^{13}C-NMR spectroscopic methods were used to elucidate the chemical structures of the compounds. The antimicrobial activity of the compounds against eight bacterial strains (five Gram-negative and three Gram-positive) and two fungal strains was evaluated using the microdilution method.

Results: Compounds T4, carrying a benzoyl group, and T6, carrying a phenethyl group, showed the best antibacterial activity against *Enterococcus faecalis* ATCC 29212, with minimum inhibitory concentrations (MICs) of 41.79 mg/L and 81.25 mg/L, respectively. Compound T6 also demonstrated the strongest antibacterial activity against *Staphylococcus epidermidis* ATCC 12228, with an MIC of 40.62 mg/L. Antifungal activity assays revealed that compounds T4, T6, and T8 were the most potent against *Candida albicans* ATCC 90028, with MIC values of 40.62–83.59 mg/L, and that T6, T7, and T8 were the most potent against *Candida glabrata* ATCC 90030, with MIC values of 40.62–162.5 mg/L.

Conclusion: Among the compounds, T6 appears to exhibit significant antimicrobial activity against both Gram-positive bacteria (e.g., *E. faecalis* ATCC 29212 and *S. epidermidis* ATCC 12228) and fungi (e.g., *Candida strains*).

Keywords: Triazole, antibacterial, antifungal

INTRODUCTION

Antimicrobial resistance has become a significant threat to both animal and human health because of the misuse and overuse of antibiotics. Many countries have strengthened controls on antibiotic use, including eliminating or reducing such use. However, the emergence of antimicrobial-resistant microorganisms has intensified the search for novel antimicrobial agents.[1] Given the promising potential of chemical compounds in this context, it is essential to investigate their antimicrobial properties.

Five-membered heterocyclic rings, such as nitrogen-containing triazoles, thiadiazoles, and oxadiazoles, are common structural motifs in many drugs.[2,3] Among these five-membered rings, 1,2,4-triazole rings, which are bioisosteres of other five-membered rings, are frequently synthesized in studies of drug-candidate molecules.[4] They have different biological activities such as antibacterial, antifungal, antiviral, anticancer, antidepressant, and anti-inflammatory.[5,6] As a result of the reaction of thiosemicarbazides in a basic medium, ring cyclization occurs, and 2,4-dihydro-3*H*-1,2,4-triazole-3-thione

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compounds, which are important pharmacophore structures, are readily obtained.[7]

There are many drugs with antimicrobial activity on the market, such as tazobactam, fluconazole, voriconazole, and itraconazole, which contain a triazole ring (Figure 1). Numerous studies have demonstrated the potent antimicrobial activity of 2,4-dihydro-3*H*-1,2,4-triazole-3-thione compounds.[8,9] Onkol et al.[10] synthesized a group of 1,2,4-triazole compounds based on thiosemicarbazides. 3-[(1(2*H*)-phthalazinone-2-yl)methyl]-4-methoxyphenyl-1,2,4-triazole-5-thione showed the best MIC values of 64 and 32 mg/mL against *Candida albicans* and *Candida parapsilosis*, respectively. Similarly, another study synthesized a group of compounds with a 2,4-dihydro-3*H*-1,2,4-triazole-3-thione structure from thiosemicarbazides. Among these compounds, 5-(3-hydroxynaphthalen-2-yl)-4-(2-methoxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione exhibited strong bacteriostatic activity against Micrococcus luteus, with an MIC of 31.25 µg/mL.[11]

In this study, several new compounds containing 2,4-dihydro-3H-1,2,4-triazole-3-thione structures were synthesized. *In vitro* antimicrobial activity of the synthesized compounds against bacterial strains such as *Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 4352, Proteus vulgaris ATCC 13315, Enterococcus faecalis ATCC 29212, Staphylococcus epidermidis ATCC 12228, Staphylococcus aureus ATCC 29213, Acinetobacter baumannii ATCC 19606,* and fungal strains such as *C. albicans ATCC 90028, Candida glabrata ATCC 90030* was screened. In line with the need for new active pharmaceutical ingredients to combat antimicrobial resistance, this study aims to provide new data supporting the antimicrobial efficacy of 1,2,4-triazole structures reported in the literature.

MATERIALS AND METHODS

All solvents and other chemicals used in this study were purchased from Sigma-Aldrich. The melting points of the compounds were measured using a Schmelzpunktbestimmer SMP II apparatus. Infrared (IR) spectra of the compounds were recorded using a Shimadzu Fourier Transform Infrared Spectroscopy (FTIR)-8400S spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra of the compounds were recorded on a Bruker Avance III 600 MHz spectrometer. C, H, N, and S percentages in the compounds were measured using a Thermo Scientific Flash 2000 CHNS analyzer.

6-chloronicotinohydrazide and 2-(6-chloronicotinoyl)-*N*-substitutedhydrazine-1-carbothioamide derivatives (S1-S8) were obtained using the synthetic methods described in our previously reported study.[12]

N-Allyl-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (S1)

Yield: 80%; color: white powder; melting point: 164.3–165.3 °C. FTIR (cm^{-1}): 3223 and 3140 (N-H str.), 3088 (=C-H str.), 2974 and 2937 (C-H str.), 1678 (C=O str.), 1585, 1552, 1504, 1446 (C=N, C=C str., N-H b.), 1226 (C=S str.), 845 (=C-H b.). ^{1}H-NMR [dimethyl sulfoxide (DMSO)-d_6, 400 MHz, δ ppm]: 10.63 (s, 1H, NH), 9.47 (s, 1H, NH), 8.87 (dd, *J* = 2.5, 0.8 Hz, 1H, Ar-H), 8.38 (s, 1H, NH), 8.30 – 8.23 (m, 1H, Ar-H), 7.69 (d, *J* = 8.4 Hz, 1H, Ar-H), 5.80 (ddd, *J* = 22.2, 10.2, 5.0 Hz, 1H, allyl proton), 5.16 – 5.07 (m, 1H, allyl proton), 5.06 – 4.99 (m, 1H, allyl proton), 4.09 (s, 2*H*, allyl protons). ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 185.90, 164.34, 153.71, 150.15, 139.83, 135.59, 128.40, 124.80, 115.96, 46.60.

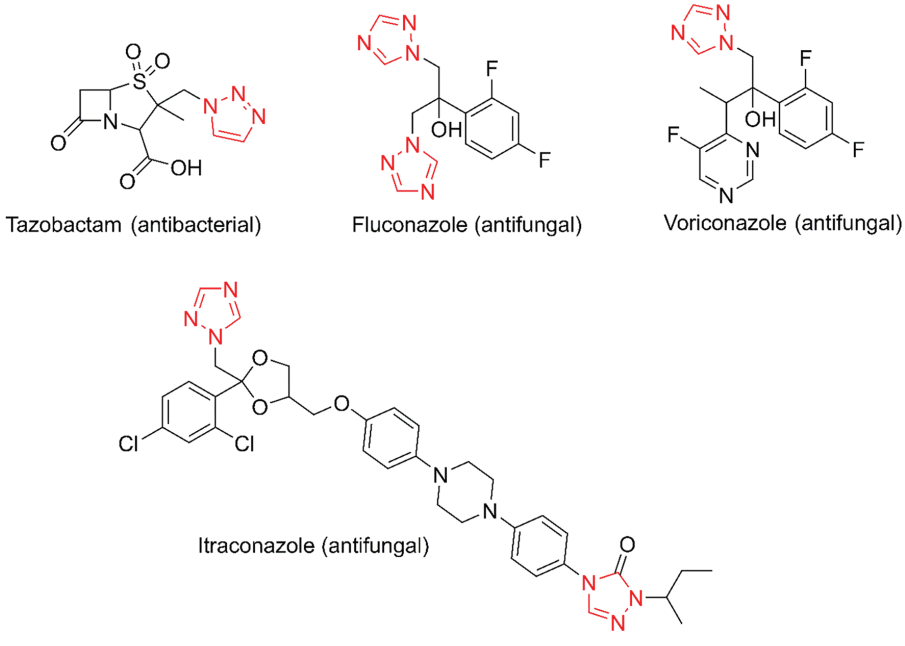


Figure 1. Some marketed antimicrobial drugs carrying a triazole ring

N-Butyl-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (S2)

Yield: 80%; color: white powder; melting point: 149.3–149.8 °C. FTIR (cm^{-1}): 3330 and 3132 (N-H str.), 3037 (=C-H str.), 2960 and 2874 (C-H str.), 1662 (C=O str.), 1585, 1556, 1519, 1452 (C=N, C=C str., N-H b.), 1242 (C=S str.), 846 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm):10.57 (s, 1H, NH), 9.33 (s, 1H, NH), 8.87 (d, *J* = 2.3 Hz, 1H, Ar-H), 8.29 – 8.22 (m, 1H, Ar-H), 8.16 (s, 1H, NH), 7.72 – 7.65 (m, 1H, Ar-H), 3.41 (q, *J* = 8.0 Hz, 2*H*, $CH_2CH_2CH_2CH_3$), 1.49 – 1.42 (m, 2*H*, $CH_2CH_2CH_2CH_3$), 1.29 – 1.19 (m, 2*H*, $CH_2CH_2CH_2CH_3$), 0.85 (t, *J* = 8.0 Hz, 3*H*, $CH_2CH_2CH_2CH_3$). ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 185.90, 164.30, 153.70, 150.12, 139.81, 128.43, 124.83, 44.10, 31.58, 20.13, 14.51.

N-(Pyridin-3-yl)-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (S3)

Yield: 75%; color: yellow powder; melting point: 164.9–165.5 °C. FTIR (cm^{-1}): 3284 and 3211 (N-H str.), 3051 (=C-H str.), 1654 (C=O str.), 1629 (C=N str. pyridine), 1585, 1541, 1506, 1456 (C=N, C=C str., N-H b.), 1253 (C=S str.), 840 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 11.21 and 10.93 (2s, 1H, NH), 10.07 (s, 1H, NH), 9.94 (s, 1H, NH), 8.92 (d, *J* = 2.4 Hz, 1H, Ar-H), 8.53 (s, 1H, Ar-H), 8.39 – 8.26 (m, 2*H*, Ar-H, Ar-H), 7.84 (s, 1H, Ar-H), 7.71 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.37 (dd, *J* = 8.2, 4.7 Hz, 1H, Ar-H).13C-NMR (DMSO-d_6, 100 MHz, δ ppm): 182.17, 163.85, 153.86, 153.13, 150.20, 149.31, 139.87, 138.97, 136.55, 134.40, 128.95, 128.27, 124.89, 123.67.

N-[2-(6-Chloronicotinoyl)hydrazine-1-carbonothioyl] benzamide (S4)

Yield: 75%; color: green powder; melting point: 210.0–210.9 °C. FTIR (cm^{-1}): 3132 and 3113 (N-H str.), 3061 (=C-H str.), 2950 and 2890 (C-H str.), 1666 (C=O str.), 1583, 1558, 1521, 1435 (C=N, C=C str., N-H b.), 1247 (C=S str.), 829 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 13.29 (s, 1H, NH), 12.34 and 11.82 (2s, 1H, NH), 11.45 (s, 1H, NH), 8.99 (d, *J* = 2.6 Hz, 1H, Ar-H), 8.41 (ddd, *J* = 8.3, 2.6, 1.0 Hz, 1H, Ar-H), 8.12 (dt, *J* = 8.3, 1.2 Hz, 2*H*, Ar-H), 7.74 – 7.61 (m, 2*H*, Ar-H), 7.61 – 7.48 (m, 2*H*, Ar-H). ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 181.58, 168.44, 165.91, 160.83, 153.99, 152.32, 149.90, 148.45, 139.69, 133.88, 131.91, 129.29, 128.10, 126.66, 125.65.

N-Benzyl-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (CAS Registry number 1388757-91-2) (S5)

Yield: 80%; color: white powder; melting point: 167.8–168.0 °C. FTIR (cm^{-1}): 3421 and 3149 (N-H str.), 3032 (=C-H str.), 2976 and 2937 (C-H str.), 1681 (C=O str.), 1585, 1552, 1504, 1446 (C=N, C=C str., N-H b.), 1226 (C=S str.), 845 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 10.69 (s, 1H, NH), 9.56 (s, 1H, NH), 8.88 (d, *J* = 2.4 Hz, 1H, Ar-H), 8.74 (s, 1H, NH), 8.27 (dt, *J* = 8.5, 2.1 Hz, 1H, Ar-H), 7.71 – 7.65 (m, 1H, Ar-H), 7.33 – 7.24 (m, 4H, Ar-H), 7.21 (s, 1H, Ar-H), 4.73 (d, *J* = 6.0 Hz, 2*H*, CH_2). ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 182.73, 164.41, 153.73, 150.15, 139.95, 139.83, 128.75, 128.37, 127.68, 127.32, 124.81, 47.39.

N-Phenethyl-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (S6)

Yield: 82%; color: yellow powder; melting point: 153.9–154.5 °C. FTIR (cm^{-1}): 3350 and 3157 (N-H str.), 3039 (=C-H str.), 2943 and 2926 (C-H str.), 1681 (C=O str.), 1583, 1545, 1494, 1454 (C=N, C=C str., N-H b.), 1247 (C=S str.), 842 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 10.68 (s, 1H, NH), 9.21 (s, 1H, NH), 8.76 (s, 1H, Ar-H), 8.38 (s, 1H, NH), 8.17 (dd, *J* = 8.4, 2.5 Hz, 1H, Ar-H), 7.69 – 7.63 (m, 1H, Ar-H), 7.33 – 7.15 (m, 5H, Ar-H), 4.18 (s, 2*H*, CH_2), 2.88 (t, *J* = 7.3 Hz, 2*H*, CH_2). ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 181.68, 164.12, 155.41, 152.19, 146.89, 139.61, 136.75, 129.47, 129.05, 126.92, 125.69, 120.72, 44.79, 35.43.

N-(m-Tolyl)-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (S7)

Yield: 75%; color: white powder; melting point: 149.2–150.0 °C. FTIR (cm^{-1}): 3319 and 3184 (N-H str.), 3055 (=C-H str.), 2916 (C-H str.), 1670 (C=O str.), 1589, 1545, 1489, 1460 (C=N, C=C str., N-H b.), 1224 (C=S str.), 854 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 10.82 (s, 1H, NH), 9.78 (s, 2*H*, NH), 8.91 (d, *J* = 2.5 Hz, 1H, Ar-H), 8.30 (dd, *J* = 8.4, 2.6 Hz, 1H, Ar-H), 7.69 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.21 – 7.18 (m, 3*H*, Ar-H), 6.97 (d, *J* = 7.1 Hz, 1H, Ar-H), 2.26 (s, 3*H*, CH_3). ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 181.62, 164.37, 153.74, 150.19, 139.85, 139.64, 137.97, 128.55, 128.41, 127.28, 126.61, 124.81, 123.99, 21.62.

N-(3-Methoxyphenyl)-2-(6-chloronicotinoyl)hydrazine-1-carbothioamide (S8)

Yield: 80%; color: white powder; melting point: 150.5–151.5 °C. FTIR (cm^{-1}): 3279 and 3144 (N-H str.), 3037 (=C-H str.), 2972 and 2831 (C-H str.), 1670 (C=O str.), 1585, 1556, 1521, 1456 (C=N, C=C str., N-H b.), 1251 (C=S str.), 848 (=C-H b.).^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 10.83 (s, 1H, NH), 9.83 (s, 2*H*, NH), 8.91 (t, *J* = 2.4 Hz, 1H, Ar-H), 8.30 (dt, *J* = 8.4, 2.4 Hz, 1H, Ar-H), 7.69 (dd, *J* = 8.4, 1.9 Hz, 1H, Ar-H), 7.22 (td, *J* = 8.1, 2.0 Hz, 1H, Ar-H), 7.07 (s, 1H, Ar-H), 7.00 (d, *J* = 8.0 Hz, 1H, Ar-H), 6.77 – 6.65 (m, 1H, Ar-H), 3.72 (s, 3*H*, OCH_3).^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 181.50, 164.39, 159.63, 153.78, 150.19, 140.86, 139.85, 129.47, 128.39, 124.84, 118.88, 111.37, 55.79.

General synthesis method of 2,4-dihydro-3H-1,2,4-triazole-3-thione structures (T1-T8)

Add 5–6 mL of 2N NaOH solution to the 2-(6-chloronicotinoyl)-*N*-substituted hydrazine-1-carbothioamide derivatives. The mixture is heated in a water bath for 6 h. After reaction is confirmed by TLC, the mixture is neutralized with 1N HCl, and the solid is filtered and then crystallized from the appropriate solvent.[13]

4-Allyl-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T1)

Yield: 75%; color: yellow powder; melting point: 249.5–250.0 °C. FTIR (cm^{-1}): 3088 (=C-H str.), 2928 and 2839 (C-H str.), 2766 (S-H str.), 1647 (C=N str.), 1597, 1558, 1531, 1498 (C=N and C=C str.), 839 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 14.22 and 13.96 (3s, 1H, NH), 7.78 – 7.71 (m, 1H, Ar-H),

7.65 (dd, *J* = 9.6, 2.7 Hz, 1H, Ar-H), 6.52 – 6.36 (d, *J* = 9.0 Hz, 1H, Ar-H), 5.85 (ddq, *J* = 17.2, 10.5, 4.4 Hz, 1H, allyl proton), 5.15 (ddq, *J* = 11.9, 10.2, 1.5 Hz, 1H, allyl proton), 4.89 (ddq, *J* = 17.2, 7.2, 1.6 Hz, 1H, allyl proton), 4.72 (ddt, *J* = 18.1, 5.0, 1.9 Hz, 2*H*, allyl protons). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm):167.86, 162.19, 152.71, 149.70, 149.09, 140.32, 137.55, 132.39, 124.95, 122.47, 120.72, 117.71, 104.76, 46.28. Anal. calcd for $C_{10}H_9ClN_4S$: C 47.53, H 3.59, N 22.17, S 12.69 %. Found: C 49.05, H 3.40, N 22.68, S 12.98 %.

4-Butyl-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T2)

Yield: 80%; color: white powder; melting point: 262.8–263.5 °C. FTIR (cm^{-1}): 3107 (N-H str.), 3028 (=C-H str.), 2955 and 2872 (C-H str.), 1647 (C=N str.), 1602, 1568, 1533, 1496 (C=N and C=C str.), 1290 (C=S str.), 840 (=C-H b.). ^{1}H-NMR (DMSO-d6, 400 MHz, δ (ppm)): 7.82 (dd, J = 2.7, 0.8 Hz, 1H, Ar-H), 7.68 (dd, J = 9.5, 2.7 Hz, 1H, Ar-H), 6.47 (dd, J = 9.5, 0.7 Hz, 1H, Ar-H), 4.09 – 3.92 (m, 2*H*, CH2CH2CH2CH3), 1.63 – 1.43 (m, 2*H*, CH2CH2CH2CH3), 1.28 – 1.05 (m, 2*H*, CH2CH2CH2CH3), 0.79 (dt, J = 13.0, 7.3 Hz, 3*H*, CH2CH2CH2CH3). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm): 167.89, 162.21, 152.72, 149.91, 148.93, 148.41, 140.51, 140.22, 137.94, 125.22, 122.69, 120.76, 104.84, 43.77, 30.00, 19.55, 13.82. Anal. calcd for $C_{11}H_{13}ClN_4S$: C 49.16, H 4.88, N 20.85, S 11.93 %. Found: C 50.65, H 4.70, N 20.98, S 11.77 %.

4-(Pyridin-3-yl)-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T3)

Yield: 80%; color: yellow powder; melting point: 295.1–296.0 °C. FTIR (cm^{-1}): 3182 (N-H str.), 3080 (=C-H str.), 1645 (C=N str.), 1606, 1583, 1550, 1471 (C=N and C=C str.), 1286 (C=S str.), 831 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 14.20 and 12.24 (s, 1H, NH), 8.89 (dd, *J* = 2.4, 0.8 Hz, 1H, Ar-H), 8.29 (dd, *J* = 8.3, 2.4 Hz, 1H, Ar-H), 7.98 (ddd, *J* = 10.4, 2.7, 0.7 Hz, 2*H*, Ar-H), 7.82 – 7.64 (m, 2*H*, Ar-H), 6.35 (dd, *J* = 9.6, 0.6 Hz, 1H, Ar-H). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm): 165.85, 162.86, 162.27, 159.11, 154.50, 151.30, 140.96, 140.85, 140.19, 137.19, 126.68, 124.99, 119.81, 109.47. Anal. calcd for $C_12H_8ClN_5S$: C 49.75, H 2.78, N 24.17, S 11.07 %. Found: C 51.50%, H 2.99%, N 23.86%, S 11.35%.

4-Benzoyl-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T4)

Yield: 77%; color: green powder; melting point: 294.4–294.8 °C. FTIR (cm^{-1}): 3196 and 3117 (N-H str.), 3045 (=C-H str.), 1668 (C=N str.), 1583, 1558, 1521, 1433 (C=N and C=C str.), 1247 (C=S str.), 829 (=C-H b.). ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm): 13.34 (s, 1H, NH), 8.99 (dd, J = 2.6, 0.7 Hz, 1H, Ar-H), 8.41 (dd, J = 8.4, 2.5 Hz, 1H, Ar-H), 8.20–8.10 (m, 2*H*, Ar-H), 7.74–7.60 (m, 2*H*, Ar-H), 7.60–7.50 (m, 2*H*, Ar-H). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm): 166.46, 162.09, 157.95, 151.80, 148.07, 137.99, 133.19, 132.80, 129.03, 128.91, 126.83, 125.39. Anal. calcd for C14H9ClN4OS: C, 53.09; H, 2.86; N, 17.69; S, 10.12%. Found: C, 51.47; H, 3.01; N, 17.97; S, 9.75%.

4-Benzyl-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T5)

Yield: 70%; color: white powder; melting point: 222.2–223.2 °C. FTIR (cm^{-1}): 3066 (=C-H str.), 2933 (C-H str.), 2750 (S-H str.), 1660 (C=N str.), 1597, 1541, 1494, 1448 (C=N and C=C str.), 833 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 14.14 and 11.99 (s, 1H, NH), 7.68 – 7.55 (m, 1H, Ar-H), 7.49 (dd, *J* = 9.6, 2.7 Hz, 1H, Ar-H), 7.37 – 7.17 (m, 3*H*, Ar-H), 7.13 – 6.98 (m, 2*H*, Ar-H), 6.35 (d, *J* = 9.6 Hz, 1H, Ar-H), 5.40 and 5.33 (2s, 1H, CH_2). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm): 168.42, 162.06, 152.67, 149.54, 149.13, 140.19, 137.54, 136.07, 129.16, 128.08, 127.03, 125.01, 122.41, 120.66, 47.02. Anal. calcd for C14H11ClN4S: C 55.54, H 3.66, N 18.50, S 10.59%. Found: C 54.66%, H 3.76%, N 18.33%, S 10.83%.

4-Phenethyl-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T6)

Yield: 77%; color: yellow powder; melting point: 289.3–289.9 °C. FTIR (cm^{-1}): 3028 (=C-H str.), 2926 (C-H str.), 2760 (S-H str.), 1624 (C=N str.), 1600, 1558, 1494, 1454 (C=N and C=C str.), 837 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm):8.35 – 8.02 (m, 1H, Ar-H), 7.87 – 7.54 (m, 2*H*, Ar-H), 7.37 – 7.06 (m, 3*H*, Ar-H), 7.06 – 6.83 (m, 1H, Ar-H), 4.27 (dt, *J* = 17.2, 7.5 Hz, 2*H*, CH_2), 3.07 – 2.73 (m, 2*H*, CH_2). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm):164.32, 155.26, 149.17, 146.62, 139.40, 138.07, 136.48, 129.23, 129.21, 129.16, 128.91, 128.87, 128.82, 127.03, 126.68, 125.45, 124.72, 44.55, 35.28. Anal. calcd for $C_{15}H_{13}ClN_4S$: C 56.87, H 4.14, N 17.69, S 10.12 %. Found: C 58.22, H 4.37, N 18.01, S 9.85 %.

4-(m-Tolyl)-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T7)

Yield: 82%; color: yellow powder; melting point: 239.1–240.0 °C. FTIR (cm^{-1}): 3292 (N-H str.), 3049 (=C-H str.), 2910 (C-H str.), 1635 (C=N str.), 1595, 1554, 1587, 1456 (C=N and C=C str.), 1247 (C=S str.), 846 (=C-H b.). ^{1}H NMR (DMSO-d6, 400 MHz): δ (ppm) 8.29 (dd, J = 2.5, 0.8 Hz, 1H, Ar-H), 7.68 (dd, J = 8.4, 2.5 Hz, 1H, Ar-H), 7.53 (dd, J = 8.4, 0.8 Hz, 1H, Ar-H), 7.48–7.08 (m, 4H, Ar-H), 2.32 (s, 3*H*, CH3). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm): 161.80, 151.70, 149.07, 139.49, 139.19, 134.89, 130.58, 129.66, 129.46, 129.01, 126.15, 124.72, 122.74, 118.89, 115.56, 21.24. Anal. calcd for C14H11ClN4S: C 55.54, H 3.66, N 18.50, S 10.59%. Found: C 54.20, H 3.74, N 18.88, S 10.02%.

4-(3-Methoxyphenyl)-5-(6-chloropyridin-3-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (T8)

Yield: 80%; color: yellow powder; melting point: 203.2–203.9 °C. FTIR (cm^{-1}): 3063 (=C-H str.), 2895 (C-H str.), 2735 (S-H str.), 1653 (C=N str.), 1604, 1564, 1539, 1489 (C=N and C=C str.), 835 (=C-H b.). ^{1}H-NMR (DMSO-d_6, 400 MHz, δ ppm): 14.25 (s, 1H, NH), 8.36 (dd, *J* = 2.4, 0.8 Hz, 1H, Ar-H), 7.74 (dd, *J* = 8.4, 2.5 Hz, 1H, Ar-H), 7.58 (dd, *J* = 8.4, 0.8 Hz, 1H, Ar-H), 7.53 – 7.34 (m, 1H, Ar-H), 7.34 – 7.09 (m, 1H, Ar-H), 7.09 – 7.00 (m, 1H, Ar-H), 6.94 (ddd, *J* = 7.8, 1.9, 1.0 Hz, 1H, Ar-H), 3.76 (s, 3*H*, OCH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz, δ ppm): 169.29, 160.24, 160.19, 152.18, 149.49, 148.39, 148.00, 139.62, 135.42, 130.76, 124.75,

122.29, 121.16, 115.67, 55.96. Anal. calcd for C14H11ClN4OS: C 52.75, H 3.48, N 17.58, S 10.06%. Found: C 51.34, H 3.57, N 17.35, S 10.42%.

All IR, ^{1}H-NMR and ^{13}C-NMR spectra of the compounds were given in the supplementary file (Figure S1-S48).

Antimicrobial Activity

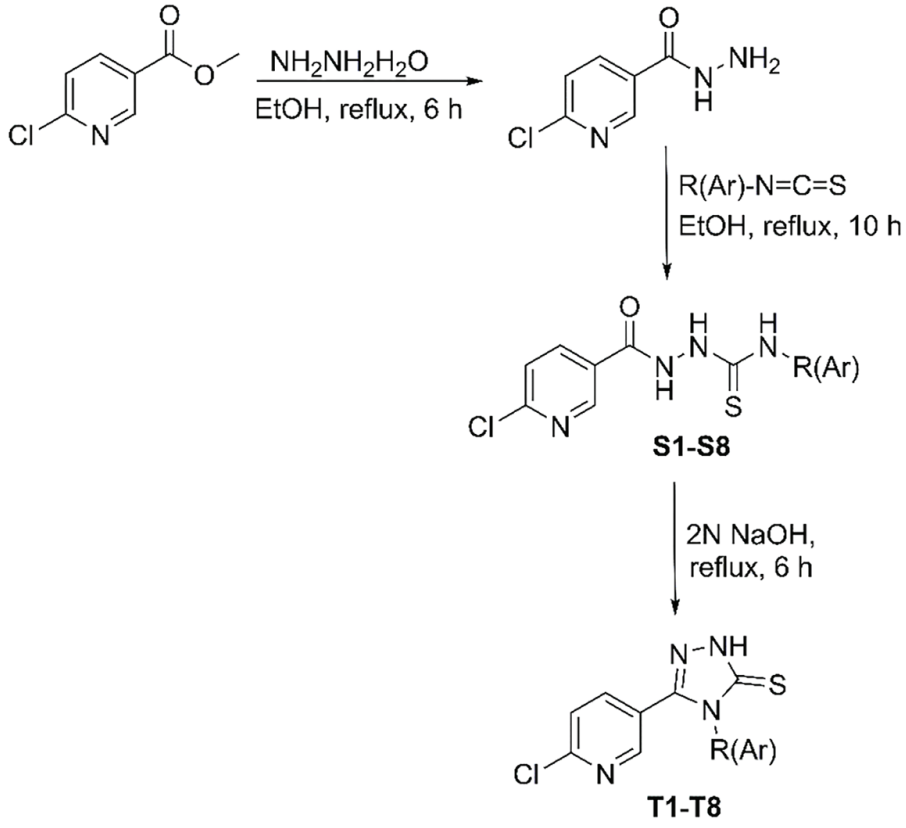
The minimum inhibitory concentration (MIC) values of the compounds were confirmed using reference strains following the guidelines established by the Clinical Laboratory Standards Institute.[14,15] Five Gram-negative bacteria (*P. aeruginosa ATCC 27853, K. pneumoniae ATCC 4352, E. coli ATCC 25922, P. vulgaris ATCC 13315, A. baumannii ATCC 19606)*, three Gram-positive bacteria *(E. faecalis ATCC 29212, S. aureus ATCC 29213, S. epidermidis ATCC 12228)*, and two yeasts *(C. albicans ATCC 10231, C. glabrata ATCC 90030)* were tested to determine the antimicrobial activity of the compounds.

All microorganisms tested in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The synthesized compounds were formulated in DMSO at a concentration of 10.000 mg L–1. Two-fold serial dilutions were prepared in the medium, ranging from 5000 mg/L to 2.4 mg/L. The inoculum of each strain was prepared from a broth culture incubated for 4–6 hours, while the broth cultures of yeast strains were incubated for 24 hours. The bacterial inocula were adjusted to a turbidity of 0.5 McFarland standard and diluted in Mueller-Hinton broth to a final concentration of 5×10^{5} colony-forming units per milliliter (colony-forming unit/mL). To prepare the yeast inocula, they were diluted in RPMI-1640 medium buffered with 0.165 M MOPS to pH 7.0. This dilution resulted in a final concentration of $0.5–2.5\times10^{3}$ in the test tray. The trays were securely covered and sealed in plastic bags to prevent evaporation. Incubation was performed at 35 ˚C: trays containing Mueller-Hinton broth for 18–24 hours and those with RPMI-1640 medium for 46–50 hours. The MIC was defined as the minimum concentration of fractions that completely inhibited detectable growth. Reference antimicrobial agents, such as meropenem and amphotericin B, were used. All experiments were performed in triplicate.

RESULTS

Chemistry

The target compounds, 4-substituted-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (T1-T8), were synthesized in three steps as shown in Figure 2. In the first step, the starting material, methyl 6-chloropyridine-3-carboxylate, was treated with hydrazine monohydrate to afford 6-chloronicotinohydrazide, a hydrazide. In the second step, thiosemicarbazide derivatives (S1–S8) were obtained by reacting this hydrazide compound with various isothiocyanates. In the last step, 2,4-dihydro-3*H*-1,2,4-triazole-3-thione compounds (T1–T8) were synthesized via ring cyclization of thiosemicarbazides by heating with 2N NaOH. The structures of the compounds were elucidated by IR, ^{1}H-NMR and ^{13}C-NMR spectroscopic methods.



Comp.	R(Ar)
T1	allyl
T2	butyl
T3	pyridin-3-yl
T4	benzoyl
T5	benzyl
T6	phenetyl
T7	*m*-tolyl
T8	*m*-(methoxy)phenyl

Figure 2. The synthesis pathway of 4-substituted-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones

IR spectra revealed that the synthesized compounds exist as thione and thiol tautomers. The IR spectra of the synthesized compounds T1, T5, T6 and T8 show the presence of thiol (S-H) absorption bands in the range 2735–2766 cm^{-1} and the absence of N–H stretching bands, indicating that these compounds are predominantly in the thiol form. Compounds T2, T3, T4, and T7 have N-H and C=S absorption bands in the ranges 3107-3292 cm^{-1} and 1247-1290 cm^{-1}, respectively, indicating that these compounds may also be primarily in the thione form.

The NH protons were detected in the range of 11.99–14.25 ppm in the 1H-NMR spectra of compounds T1, T3, T4, T5, and T8 from the series. The NH protons could not be detected because they were replaced by deuterium in the ^{1}H-NMR spectra of compounds T2, T6 and T7. Moreover, the carbons of the triazole ring were detected in the 13C NMR spectra of the compounds at 160.24–169.29 ppm.

The ^{13}C-NMR APT spectrum of Compound T1 was recorded. With this technique, carbon atoms bearing different numbers of attached protons (CH3, CH2, CH, C) can be readily distinguished, and their chemical shifts determined. Accordingly, carbon atoms with an even number of protons produced peaks above the baseline, whereas those with an odd number of protons produced peaks below the baseline. The methylene protons of the allyl substituent in compound T1 resonated above the baseline at 46.28 ppm and 104.76 ppm, whereas the methine carbon resonated below the baseline at 140.32 ppm. The carbon atoms that form the triazole ring and do not bear protons were detected above the baseline at 162.19 ppm and 167.86 ppm. Additionally, the aromatic carbon atoms bearing a single proton gave peaks below the baseline, whereas the ipso and imine carbons (C=N) gave peaks above the baseline.

Antimicrobial activity

The *in vitro* antimicrobial activity of synthesized compounds against five Gram-negative bacteria, three Gram-positive bacteria, and two fungi was evaluated by the broth microdilution technique, following Clinical and Laboratory Standards Institute recommendations.[14,15] The MIC values were compared with those of commonly used commercial antibiotics employed as standard drugs, as shown in Tables 1 and 2. Based on the antibacterial activity results for the compounds used in this study, the test cultures *E. coli ATCC 25922, K. pneumoniae ATCC 4352,* and *P. vulgaris ATCC 13315* appeared to be resistant to all tested compounds.

While T3 and T4 did not show any antibacterial activity against *P. aeruginosa ATCC 27853*, the other compounds showed moderate activity against this bacterium. Only compound T8 showed antibacterial activity against *A. baumannii ATCC 19606*.

While T4 and T6 have the best antibacterial activity against *E. faecalis ATCC 29212* with an MIC value of 41.79 mg/L and 81.25 subsequently. T1, T3, T5, T7, and T8 showed moderate antibacterial activity against this bacterium. All compounds showed moderate activity against *S. aureus ATCC 29213*. T6 has the highest antibacterial activity against *S. epidermidis ATCC 12228*, with an MIC of 40.62 mg/L.

Based on the antifungal results, T4, T6, and T8 exhibited the greatest antifungal activity against *C. albicans* ATCC 90028, with MIC values of 83.59, 40.62, and 82.81 mg/L, respectively. T6, T7, and T8 exhibited antifungal activity against *C. glabrata* ATCC 90030, with MIC values of 162.5, 159.37, and 82.81 mg/L, respectively, whereas the other compounds showed no activity against this fungus.

DISCUSSION

Increasing antibiotic resistance among microorganisms necessitates developing novel compounds that effectively function as antibiotics. Recently, there has been increased research into compounds with antimicrobial properties.[16] Many compounds with a 2,4-dihydro-3*H*-1,2,4-triazole-3-thione structure have been reported to exhibit antimicrobial activity.[17,18] Beyzaei et al.[19] investigated the potent antifungal activity of their synthesized 1,2,4-triazole-3-thione compounds and reported that the N1 nitrogen in the triazole ring plays a critical role in hydrogen bonding with target enzymes. Ezelarab et al.[20] reported that hybrid compounds synthesized based

Table 1. MICs values of the tested compounds for antibacterial activity

Compunds	*P. aeruginosa ATCC 27853*	*E. coli ATCC 25922*	*K. pneumoniae ATCC 4352*	*P. vulgaris ATCC 13315*	*E. faecalis ATCC 29212*	*E. faecalis ATCC 29212*	*S. aureus ATCC 29213*	*A. baumannii ATCC 19606*
T1	631.25	-	-	-	631.25	-	1262.5	-
T2	662.5	-	-	-	-	662.5	662.5	-
T3	-	-	-	-	656.25	656.25	656.25	-
T4	-	-	-	-	41.79	668.75	668.75	-
T5	637.5	-	-	-	637.5	-	637.5	-
T6	650	-	-	-	81.25	40.62	325	-
T7	637.5	-	-	-	318.75	159.37	318.75	-
T8	662.5	-	-	-	331.25	165.62	165.625	331.25
Reference	0.5[a]	0.06[a]	0.5[a]	0.125[a]	2[a]	0.25[a]	0.06[a]	0.5[a]

[a]Meropenem, MICs: Minimum inhibitory concentrations

Table 2. MICs values of the tested compounds for antifungal activity

Compounds	*C. albicans ATCC 90028*	*C. glabrata ATCC 90030*
T1	-	-
T2	-	-
T3	164.06	-
T4	83.59	-
T5	-	-
T6	40.62	162.5
T7	-	159.37
T8	82.81	82.81
Reference	0.5[b]	1[b]

[b]Amphotericin B, MICs: Minimum inhibitory concentrations

on the structures of ciprofloxacin and 1,2,4-triazole-3-thione showed stronger antifungal activity against *Candida strains* than itraconazole.

In this study, the antimicrobial activities of synthesized 2,4-dihydro-3*H*-1,2,4-triazole-3-thione compounds were evaluated. Some compounds demonstrated notable antimicrobial activity (Tables 1 and 2). T4 and T6 demonstrated potent antimicrobial effects against *E. faecalis ATCC 29212* and *S. epidermidis ATCC 12228*, and all compounds demonstrated antimicrobial effects against *S. aureus* is a significant human pathogen that can cause both hospital-associated and community-acquired infections.[21,22] It can rapidly acquire resistance to antimicrobial agents.[23,24] Additionally, methicillin-resistant *S. aureus* became a global pandemic, causing over 100,000 deaths in 2019.[25] Therefore, it is crucial to develop novel, effective antimicrobials against *S. aureus* and other staphylococci for both animal and human health.

The newly synthesized compounds were assessed for their antifungal activity against *C. albicans* and *C. glabrata*. T6 exhibited the strongest antifungal activity against *C. albicans*. Besides, T8 exhibited potent fungistatic activity against both *C. strains*. *Candida* species are significant fungal pathogens that can cause infections. Systematic surveillance conducted by the Centers for Disease Control and Prevention in the United States indicates that these infections are the fourth-leading cause of bloodstream infections and the third-leading cause of bloodstream infections in intensive care units.[26] *C. albicans* is a highly pathogenic fungal species and is responsible for more than 250,000 deaths and millions of recurrent infections annually.[27] *C. glabrata* is a significant pathogen due to its rising incidence and emerging resistance to various antifungal agents.[28] Therefore, it is significant to develop effective prevention and treatment strategies against *Candida* species.

Among Gram-negative bacteria, only compound T8 had antibacterial activity against *A. baumannii*, while other compounds, except T3 and T4, also had antibacterial activity against *P. aeruginosa* and *A. baumannii* are common causes of hospital-acquired infections. They survive extended periods in healthcare settings and are associated with multidrug resistance due to prolonged exposure to antibiotics. Therefore, identifying effective new compounds with antibacterial activity and demonstrating their efficacy are important parts of solving the problem of antibiotic resistance.[29]

CONCLUSION

The rapid development of resistance to existing antimicrobial agents in the fight against infections has encouraged medicinal chemists to develop new antimicrobials. In this context, we synthesized novel compounds bearing a 2,4-dihydro-3*H*-1,2,4-triazole-3-thione structure and assessed their antimicrobial activities. The compounds T4 and T6 in this series exhibited significant antibacterial activity against Gram-positive bacteria, except for *S. aureus*. Additionally, T4, T6, and T7 have potent antifungal activity. The MIC values of T6 against Gram-positive bacteria (*E. faecalis* and *S. epidermidis*) and fungal strains (*C. albicans* and *C. glabrata*) indicate that compound T6 may represent an important chemical scaffold for the development of novel antimicrobial agents.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: F.T., Design: F.T., D.D.Ç., Data Collection or Processing: F.T., D.D.Ç., Analysis or Interpretation: F.T., D.D.Ç., Literature Search: F.T., D.D.Ç., Writing: F.T., D.D.Ç.

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