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Research Article / Araştırma Makalesi

CHROMATOGRAPHIC AND COMPUTATIONAL APPROACHES TO THE PHARMACOKINETIC PROFILE OF PIROXICAM AND ITS SENSITIVE ASSAY IN RABBIT PLASMA

TAVŞAN PLAZMASINDA PİROKSİKAMİN FARMAKOKİNETİK PROFİLİNE VE HASSAS ANALİZİNE YÖNELİK KROMATOGRAFİK VE HESAPLAMALI YAKLAŞIMLAR

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ABSTRACT

Objective: *A simple, rapid, sensitive, and cost-effective high-performance liquid chromatographic (HPLC) method was developed for the pharmacokinetic study and determination of Piroxicam.*
Material and Method: *The chromatographic separation was performed with a Agilent ZORBAX reversed-phase C18 column (250 mm × 4,6 mm ID × 5 µm). Mobile phase composition was 0.04 M KH_2PO_4: acetonitrile:methanol (50:40:10) at pH 3.8. The flow rate was adjusted as 0.9 mL/min.*
Result and Discussion: *An isocratic method was used for the analysis. The UV detector was set at 360 nm, and a retention time of 7 minutes was obtained for piroxicam. The method showed linear performance over the concentration range of 0.1–4 mg/l.*
Keywords: *ADMET, chromatography, DFT calculation, pharmacokinetic analysis, piroxicam*

ÖZ

Amaç: *Piroksikam'ın farmakokinetik çalışması ve tayini için basit, hızlı, hassas ve maliyet etkin bir yüksek performanslı sıvı kromatografi (HPLC) yöntemi geliştirilmiştir.*
Gereç ve Yöntem: *Kromatografik ayrım, Agilent ZORBAX ters fazlı C18 kolon (250 mm × 4,6 mm ID × 5 µm) kullanılarak gerçekleştirilmiştir. Mobil faz bileşimi, pH 3.8'de 0.04 M KH_2PO_4:asetonitril:metanol (50:40:10) oranında hazırlanmıştır. Akış hızı 0.9 ml/dk olarak ayarlanmıştır.*

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Sonuç ve Tartışma: *Analiz için izokratik yöntem kullanılmıştır. UV dedektörü 360 nm'ye ayarlanmış ve piroksikam için 7 dakikalık bir alıkonma süresi elde edilmiştir. Yöntem, 0.1–4 mg/l konsantrasyon aralığında doğrusal performans göstermiştir.*
Anahtar Kelimeler: *ADMET, DFT hesaplaması, farmakokinetik analiz, kromatografi, piroksikam*

INTRODUCTION

Piroxicam (PRX) is used to treat inflammation caused by musculoskeletal and joint problems, including osteoarthritis, ankylosing spondylitis and rheumatological disorders [1]. Its chemical structure is 4-hydroxy-2-methyl-1,1-dioxo-N-pyridine-2-yl-1λ6,2-benzothiazine-3-carboxamide. PRX is a weak acid because of its tautomeric structure. [2].

Analytical techniques have been established to quantitatively measure plasma concentrations and conduct quality control analysis for each pharmaceutical agent. The increasing need for fast analysis to optimize time utilization in the pharmaceutical sector requires the advancement of rapid, high-throughput analytical methods. The identification of pharmaceuticals in biological matrices and in vitro drug development assays is a difficulty, frequently required by the need for rapid and sensitive results from samples. PRX has been analyzed by several analytical methods such as spectrofluorimetry [3,4], spectrophotometry [5,6], thin-layer chromatography (TLC) [7,8], capillary electrophoresis (CE) [7,9,10], and liquid chromatography (LC). The HPLC-based assays provide the rapid and immediate quantification of many analytes in a single run. Furthermore, the high sensitivity of HPLC facilitates the utilization of minimal sample volumes and the quantification of analytes in various matrices where drug concentrations may be low [6-8,11-19]. The pharmacokinetic profile of drugs could present the absorption, distribution, metabolism, and excretion of a drug under investigation in animals/healthy volunteers or patients. The results can be helpful in the clinical applications, efficiency, and safety usage of related drugs. Due to the fast changing of computer technologies, pharmacokinetic and toxicity assessments of preclinical drugs have great interest. In vitro and in vivo absorption, distribution, metabolism, excretion, and toxicity (ADMET) prediction methods have been commonly used [20,21]. At this point, in silico approaches used for predicting ADMET properties have become increasingly attractive due to their lower cost and higher throughput compared to experimental measurement methods. Currently, many in silico methods are successfully applied to predict ADMET parameters in vitro. Furthermore, in silico models have been developed that can replace in vivo models in the assessment of pharmacokinetics, toxicity, and other relevant parameters. [22]. To the best of our knowledge, the pharmacokinetic profile for PRX has been firstly identified by the computational approach in this study.

Determination of the drug concentration and/or its metabolites in biological samples should be validated with precision, accuracy, specificity, and detection limits. Therefore, we performed PRX quantification in rabbit plasma samples by the HPLC method. Gas chromatography and LC-MS methods are time-consuming and have an expensive instrumental setup. So, the HPLC method has been performed for the sensitive quantification and pharmacokinetic study of PRX. For the supporting of the chromatography results, a computational study is applied for pharmacokinetics profile identification.

MATERIAL AND METHOD

HPLC Analysis

Reagents

PRX standard, potassium dihydrogen phosphate, ortho-phosphoric acid, formic acid were supplied from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Deionized water was prepared with a Pure Flex water system (ELGA, Wycombe, UK). All chemicals were analytical reagent grade.

Instrumentation

Agilent Hewlett-Packard (Avondale, USA) Model 1100 series with UV detector was used in the HPLC system. The pH was measured using a Thermo Scientific Benchtop pH meter (Orion 3 Star Plus,

USA). All standard and spiked rabbit plasma samples were prepared using ultrasonic heating baths with Ultrasons J.P., S.A. (Barcelona) and the NF 200 Centrifuge (NUVE, Turkey).

Sample Preparation and HPLC Conditions

Preparation of Standart PRX Solution for Calibration Curves

Stock solutions were prepared by dissolving PRX in acetonitrile at a 10 µg/ml concentration. Working solutions for calibration curves were prepared by dilution with acetonitrile at 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 and 4 µg/ml. All solutions were stored (2–8°C) until use.

To prepare plasma standards for linearity studies by spiking PRX into blank rabbit plasma, 50 µl of the corresponding PRX solution was spiked into 180 µl of control rat blank plasma, and 270 µl of acetonitrile was added, vortexed for 5 minutes. Then it was centrifuged at 5000 rpm for 20 min, and the supernatant was collected. All solutions were stored (2–8°C) until use.

Preparation of Real Rabbit Plasma Sample

Real rabbit plasma sample (180 ul) was mixed with 320 ul acetonitrile and vortexed for 5 min. The mixture was centrifuged at 5000 rpm for 20 min. The supernatant was transferred to a 1.5 ml tube.

Chromatographic separations were performed at 25°C by using a C18 (250 mm × 4.6 mm ID × 5 µm, column. The flow rate was adjusted as 0.9 $ml.min^{-1}$. The mobile phase consisted of 0.04 M KH_2PO_4: acetonitrile: methanol (50:40:10) at pH 3.8. Samples were detected at 360 nm.

System Suitability and Validation

System suitability test parameters containing theoretical plate number, capacity factor and tailing factor, and RSD % for repetitive injections were evaluated according to USP criteria.

The method was validated with selectivity, sensitivity, linearity, precision, and accuracy parameters according to International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline [23]. The calibration curves of PRX were constructed in optimized conditions. The linearity of the method was analyzed by performing injections at different concentrations (0.1, 0.25, 0.5, 0.75, 1.5, 2 and 4 µg/ml) in acetonitrile and (0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 and 4 µg/ml) plasma spiked samples. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by formulas using the standard deviation (σ) of the peak areas of the lowest concentration and the slope of the calibration lines [23].

$$LOD = (\sigma/slope) \times 3.33$$

$$LOQ = (\sigma/slope) \times 10$$

On three consecutive days, four different concentrations within linear range were analyzed for intraday and interday precision and accuracy analysis. In this work, we used 0.25, 0.50, 1.00 and 2.00 µg ml^{-1} for standard and spiked solution analysis. Moreover, the recovery parameter was investigated in spiked samples. The selectivity of the assay was analyzed by using chromatograms of standard solution, spiked plasma solutions, and blank plasma solutions [23].

Animal Studies

Experimental Design

The four healthy New Zealand Rabbits (bodyweight 2-4 kg) used in this investigation were in optimal nutritional condition and had unlimited access to food and water during the trial. The Committee for the Care and Use of Laboratory Animals at the University of Health Sciences (Ankara, Turkey) authorized all animal-related experiments (Number 2021-23).

All rabbits were weighed, and blood samples were obtained from the ear vein for control purposes before PRX treatment. Blood samples were extracted from each rabbit's ear vein and placed in tubes which included heparin for use as an anticoagulant at 0.5, 1, 1.5, 2, 3, 4, 6, 10, 24, 36, and 48 hours following a single intramuscular injection of 20 mg PRX in situ forming a gel. Within an hour of collection, samples were centrifuged at 4000 rpm for 10 minutes to separate plasma, which was then

stored in a refrigerator at 20°C until analysis.

Pharmacokinetic Analysis

The pharmacokinetic variables defining the absorption, distribution, and elimination properties of PRX in rabbits were determined using each rabbit's plasma PRX concentration-time profile following intramuscular administration of PRX in situ gels. In addition, the non-compartmental pharmacokinetic model evaluated plasma drug concentration-time data to determine the different kinetic variables. Rabbits (n=4) were dosed intramuscularly in the hind leg.

A non-compartmental analysis (PK Solver) was used to compute the area under the drug concentration-time curve (AUC), the mean residence time (MRT), and the half-life ($t_{1/2}$) (2). The time it took to achieve the maximum plasma concentration (Tmax) and the maximum plasma concentration (Cmax) were calculated directly from the plasma data. For Tmax, all data are provided as mean standard deviation (S.D.) or median (ranges).

Computational Methods

Geometry Optimization, FMOs, and MEPS of PRX

As it is known, density functional theory (DFT) is one of the most widely used methods in quantum chemistry, which gives realistic results. In this part of the study, geometry optimization was performed on the DFT/B3LYP/6-311+G(d) basis set using Gaussian 09 [24], to determine the electronic states of the PRX molecule and its atoms and also to obtain accurate results in the study.

As a result of the calculation, the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), known as Frontier boundary molecular orbitals (FMOs), were determined. Then, we obtain information about the reactivity and kinetic stability of the compound under consideration by estimating the energy gap between both molecular orbitals. Afterward, the same level molecular electrostatic potential surface (MEPS) was computed for the relevant compound.

ADMET Prediction

Discovery Studio (DS) 3.5 [25] was performed to determine the Lipinski [26] and Veber [27] analyses for the related compound (PRX). The drug similarity properties of the compounds were assessed using ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analyses performed using the relevant software. These assessments are based solely on predictions based on the compounds' chemical structures. As part of the analysis, water solubility, intestinal absorption (HIA), blood-brain barrier penetration potential (BBB), cytochrome P450 2D6 binding affinity (CYP2D6), hepatotoxicity risk, and plasma protein binding (PPB) were calculated.

In addition, the OSIRIS Property Explorer [28] was used to determine the potential toxicity profiles of the compounds. Parameters such as mutagenicity, tumorigenicity, irritant effects, and potential effects on reproduction, drug similarity criteria, and drug score were examined in detail.

RESULT AND DISCUSSION

HPLC Method Optimization and Validation

Various parameters were examined to optimize chromatographic conditions for the analysis of PRX. Different compositions of acetonitrile, methanol, and phosphate buffer were tested for optimal conditions. Moreover, we analyzed the effect of different pH values. As a result, The chromatographic separation was performed with a Agilent ZORBAX reversed-phase C18 column (250 mm × 4.6 mm ID × 5 µm). The mobile phase composition was acetonitrile: methanol: 0.04 M potassium dihydrogen phosphate (50:40:10) at pH 3.8. The column temperature was set at 25°C. The isocratic gradient was used for analysis. The optimum flow rate was 0.9 ml/min. The detection wavelength was determined from absorption spectra as 360 nm.

System Suitability Parameters

System suitability parameters, a capacity factor, tailing factor, and theoretical plate number, were

evaluated using standard PRX solutions (Table 1). The optimized condition gives satisfactorily good results in terms of chromatographic metrics.

Table 1. System suitability parameters for the optimized method of PRX (n=6)

Compounds	**Parameters PRX (Mean±SD)**	**Recomended Values**
Capacity Factor (k')	1.73±0.01	1 < **k'** < 10
Tailing Factor	0.9±0.04	<2
Theoretical Number of Plates [Plates/Column]	4464±140.2	>2000

Method Validation

Selectivity

The selectivity of a method describes its capacity to identify specific analyte(s) within a complex mixture, free from interference by other components of the mixture. Selectivity of the method was evaluated by blank plasma, spiked plasma, and real plasma samples (Figure 1).

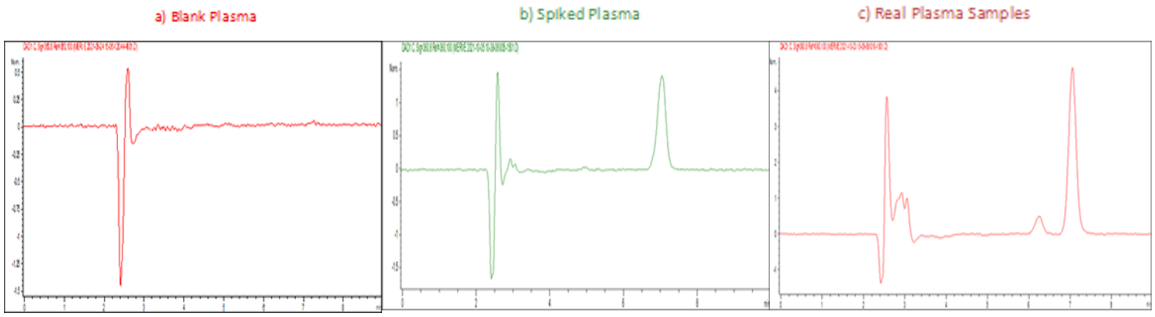


Figure 1. Chromatograms of blank plasma, spiked plasma, and real plasma samples

In an optimized system, validation parameters were evaluated with intra- and inter-day experiments [23]. In Figure 2, chromatograms of PRX spiked plasma samples were given at different concentrations.

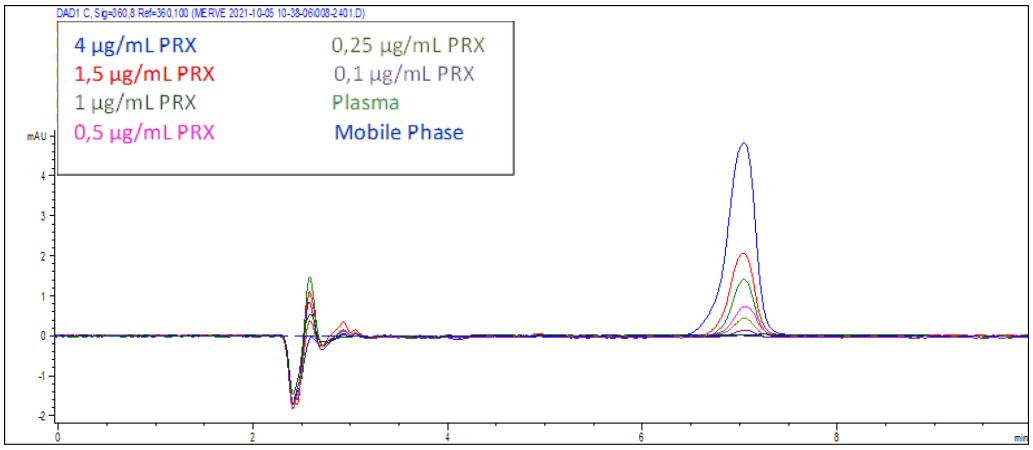


Figure 2. Chromatograms of PRX spiked plasma samples

Linearity

We investigated linearity for standard solutions and spiked plasma samples in the present work. In Table 2, linearity range, slope, and intercept values were given for two datasets. Sing the optimized chromatographic method, compounds were determined in the linear range of 0.1-4 µg/ml for PRX in ACN with a correlation coefficient(r) of 0.9998. For spiked rabbit plasma studies, the calibration curve was linear in the range of 0.1– 4 µg/ml for PRX and with a correlation coefficient (r) of 0.9996. Moreover, LOD and LOQ values were calculated as described before and given in Table 2. The LOD and LOQ values of PRX in rabbit plasma the HPLC technique were established as 0.013 and 0.039 µg ml^{-1}, respectively.

Table 2. Linearity results of developed method (n=6)

	PRX in ACN	**PRX in Rabbit Serum**
Retention Time(min)	7.042	7.042
Linearity Range (µg ml^{-1})	0.1- 4	0.1- 4
Slope (mAU $µg^{-1}$ ml)	25.354	25.071
Intercept (mAU)	0.0722	0.318
Regression Coefficient (R^2)	0.9998	0.9996
LOD (µg ml^{-1})	0.008	0.013
LOQ (µg ml^{-1})	0.024	0.039

Precision and Accuracy

The accuracy and precision of the method were calculated with intraday and interday experiments for standard solutions and PRX spiked plasma samples. Three different concentrations of standard RIV solutions (within the linear range) were analyzed on six consecutive days (inter-day precision) and six times within the same day (intra-day precision). The obtained values for relative standard deviation (RSD) and Bias of intra- and inter-day studies indicated that the precision and accuracy of the method were satisfactory. The results are summarized Table 3 and Table 4. Intra-day precision studies, the obtained values for relative standard deviation (RSD) were calculated to be less than 2%.

Table 3. Accuracy and precision for standard PRX solution in acetonitrile (n=6)

Added (µg ml^{-1})	**Found (µg ml^{-1})**	**Precision RSD%**	**Accuracy Bias%**
	Intraday		
0.25	0.24±0.002	1.88	-2.69
0.50	0.51±0.004	1.96	1.83
1.00	1.00±0.011	0.64	-0.27
4.00	3.99±0.005	0.12	-1.03
	Interday		
0.25	0.24±0.001	1.64	-3.22
0.50	0.49±0.000	0.46	-1.08
1.00	1.02±0.004	3.57	-4.07
4.00	4.01±0.002	0.21	0.34

Recovery

Recovery values were also calculated and found as percent 100, 98, and 96 for 0.25, 0.50, and 1.00 µg.ml^{-1} spiked samples. Method selectivity was analyzed, and no peak was detected in blank

plasma and standard solutions.

Table 4. Accuracy and Precision for determination of spiked PRX solutions on rabbit plasma (n=6)

Added (μg ml^{-1})	Found (μg ml^{-1})	Precision RSD%	Accuracy Bias%
Intraday			
0.25	0.28±0.002	1.51	5.59
0.50	0.51±0.002	0.78	1.46
1.00	0.96±0.002	0.64	-4.07
4.00	3.97±0.003	0.25	-1.27
Interday			
0.25	0.25±0.002	1.58	0.8
0.50	0.49±0.003	1.69	-1.47
1.00	0.98±0.005	0.43	-8.19
4.00	4.01±0.003	0.20	1.04

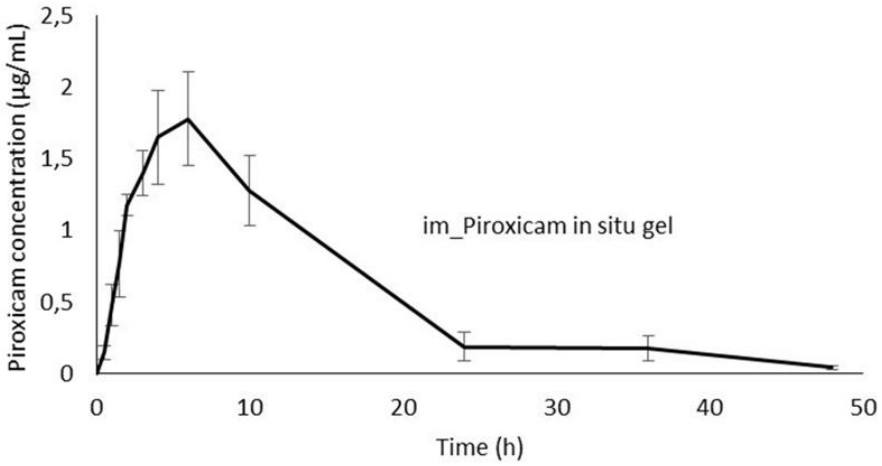


Figure 3. The pharmacokinetic profile of PRX

Pharmacokinetic Studies

Figure 3 and Table 5 show the plasma concentration-time data of the PRX injectable in situ forming gel formulation, as well as the pharmacokinetic characteristics [29,30]. In the days following injection, no adverse reactions or symptoms of PRX -related toxicity were identified.

Table 5. Pharmacokinetic study results

Parameter	Unit	Value
$t_{1/2}$	h	7.88±0.87
T_{max}	h	6.00±0.00
C_{max}	μg/ml	1.78±0.33
AUC $_{0-48}$	μg/ml*h	26.99±4.7
AUC 0-∞	μg/ml*h	27.48±4.85
MRT 0-∞	h	12.06±1.86

In this study, blood concentrations of PRX in situ gel were monitored over 48 h. The blood concentrations and pharmacokinetic parameter results of intramuscular in situ PRX gels were similar to the Xian et al [31]. Moreover, in situ gels prepared in this study can be loaded with higher PRX amounts as the drug has better solubility in both ethanol and propylene glycol than water.

Computational Study Details

As a result of calculations, the geometry optimized form, the energy levels of the HOMO–LUMO distribution plot, and the MESP map of the PRX compound are presented in Figure 4. This figure shows positive, negative and neutral charge distributions on the studied compound, such as PRX, in color scales. The red color on PRX indicates negative areas and is the suitable region for electrophilic attacks such as hydroxy, sulfur-bonded oxygens, and the oxygen atoms of the carboxamide in the PRX compound. In contrast, positive areas on the aforementioned compound are shown in blue and are suitable for nucleophilic attacks. The main structure contains the nitrogen atoms of pyridine, benzothiazine, and carboxamide. Consequently, green represents neutral regions. Table 6 also presents detailed data obtained based on quantum chemical descriptors. In this context, the global hardness (η), chemical potential (μ), electrophilicity index (ω) and electronegativity (χ), parameters are reported.

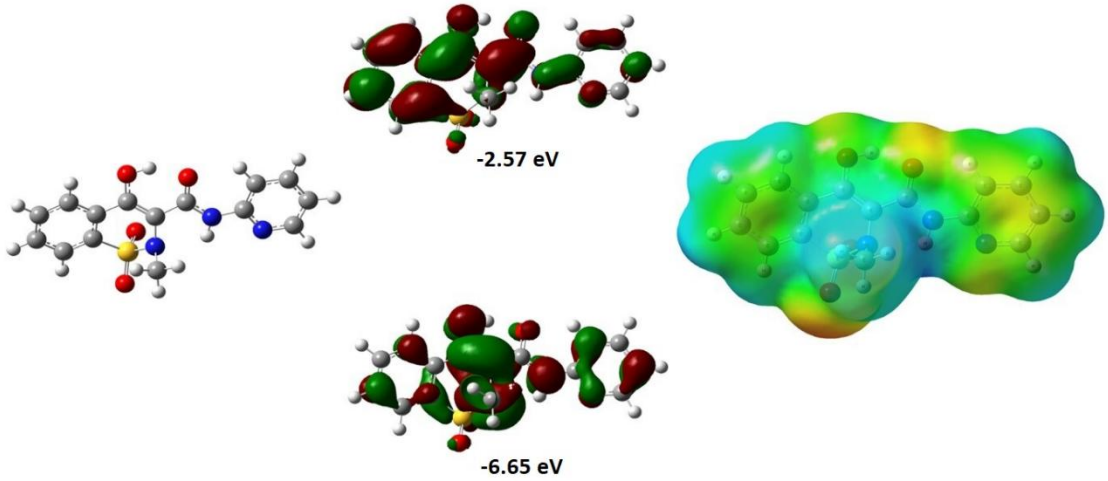


Figure 4. Frontier molecular orbitals (in the middle) and MEPS map (right side) of the geometry optimized compound PRX (1) (left side) at DFT/B3LYP/6-311G+(d) basis set in Gaussian09

ADMET Study Details

The pharmacokinetic properties of the potential candidate compound (PRX) were evaluated based on ADMET predictions. Table 7 gives the Lipinski and Veber rules test of the potential compound. It was shown that the compound is located within the borders of Lipinski and Veber rules. After, the pharmacokinetic properties of the relevant compound in the biological system were estimated. Its ADMET properties are shown in Table 8. In addition, the ADMET graph given in Figure 5 was generated for each compound. This graph summarizes the 95% and 99% confidence intervals for the absorption and BBB models. The two-dimensional polar surface area (PSA_2D) was evaluated in comparison with the logarithm of the partition coefficient between n-octanol and water (AlogP98) (PSA < 140 Å^2 and AlogP98 < 5). In Table 8, the absorption level of the compounds is 0, indicating good human intestinal absorption, while the calculated aqueous solubility value of 3 indicates high solubility.

The BBB penetration level of 3 (low) for PRX indicates that this compound has limited potential to penetrate the central nervous system. Furthermore, the compounds' inhibitory properties against the CYP2D6 enzyme are demonstrated in Table 8. However, the hepatotoxicity values suggest that PRX has potential to be toxic to the liver.

Finally, plasma protein binding (PPB) value shows that PRX has a strong binding affinity to carrier proteins in the blood.

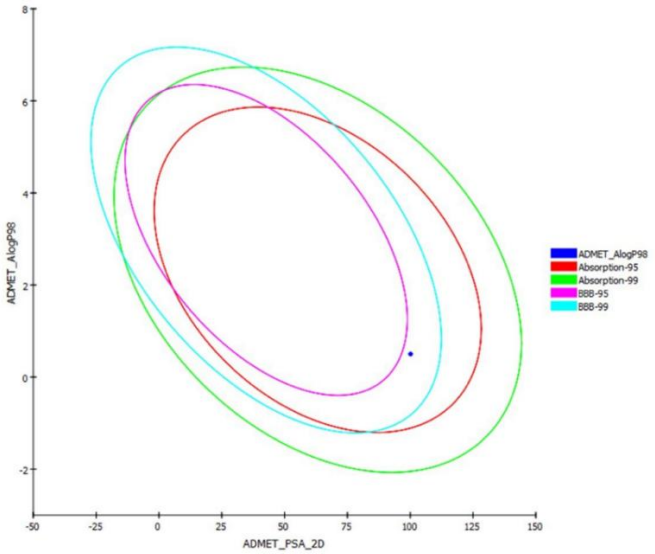


Figure 5. ADMET plot for the potential compound.

Table 6. The quantum chemical descriptors reckoned for the compound

Comp	εHOMO (eV)	εLUMO (eV)	Dipole M. (Debye)	(η)	(χ)	(μ)	ω
1	-6.65	-2.57	4.12	4.08	4.61	-4.61	2.61

Table 7. Drug-likeness properties of the potential compound based on the Lipinski and Veber rules.

Name	MW (≤500 g/mol)	AlogP (≤5)	MPSA (≤140 A2)	NRB (≤10)	HA_Lipinski (≤10)	HD_Lipinski (≤5)	HA_Veber (≤12)	HD_Veber (≤12)
PRX	**331.346**	**0.501**	**107.97**	**2**	**7**	**2**	**5**	**2**

[MW, molecular weight; ALogP, octanol/water partition coefficient, a measure for lipophilicity; MPSA, molecular polar surface area; nRB, Number of Rotatable bonds; Num_H_Acceptors_Lipinski, Number of hydrogen bond acceptors; Num_H_Donors_Lipinski, Number of hydrogen bond donors; Num_H_Acceptors, Number of hydrogen bond acceptors based on Veber; Num_H_Donors, Number of hydrogen bond donors based on Veber]

Table 8. ADMET analysis of the potent compound

Compound	PSA_2D(<140 Å2)	AlogP98 (<5)	HIA	Solubility	BBB	CYP2D6	Hepatotoxic	PPB
PRX	**100.141**	**0.501**	**0 (good)**	**3 (good)**	**3 (low)**	**NI**	**T**	**true**

[PSA_2D, 2D polar surface area; AlogP98, the logarithm of the partition coefficient between n-octanol and water; HIA: Human intestinal absorption; BBB blood-brain barrier; CYP2D6 cytochrome P450 2D6 binding, NI: non-inhibitor; for CYP2D6: non-inhibitor; NT: non-toxic for Hepatotoxic; PPB plasma protein binding, more than 90% for PPB value is true: Chemicals strongly bound. Less than 90% for PPB value is false: Chemicals weakly bound]

Furthermore, as shown in Table 9, the relevant compound is classified as low-risk in terms of toxicity risk assessment. Based on these estimates, drug similarity and drug score values were calculated. The results indicate that the potential candidate compound (PRX) examined in this study exhibits more favorable pharmaceutical properties.

Table 9. The toxicity risk assessment of the potential compound

Toxicity Risks	Mutagenic	Tumorigenic	Irritant	Reproductive effective	cLogP	Solubility	MW	TPSA	Druglikeness
PRX	NM	NT	NI	LR	1.2.	-2.18	331	107.9	5.6

[NM: non-mutagenic; NT: non-tumorigenic; NI: non-irritant; LR: low risk for reproductive efficacy]

Drug similarity is defined as a positive parameter that evaluates whether a compound possesses pharmaceutically acceptable properties. Drug score, on the other hand, is a numerical indicator that indicates the candidate compound's suitability for development as a drug, and a value close to 1 represents the desired level.

As a conclusion, a fast, simple, accurate, and inexpensive method has been presented for the pharmacokinetic profile identification of PRX in rabbit plasma samples. Furthermore, the method has been controled in terms of linearity, precision, accuracy, quantification limits, and specificity according to the requirements of the ICH guideline. In silico approaches have been widely studied to see chemical and physicochemical features of compounds. To save time and cost, in silico studies are preferred, and the reliability of theoretical models is dependent on the information included in databases. The pharmacokinetic study results presented PRX behavior in silico studies. ADMET features of PRX have been explained in the biological matrix.

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AUTHOR CONTRIBUTIONS

Concept: T.T.T., N.K.B., O.E.; Design: M.Z.C., N.K.B.; Control: A.S., S.A.O., Y.O.; Sources: O.A.A., O.E., A.S., S.A.O., Y.O.; Materials: T.T.T., N.K.B., S.A.O.; Data Collection and/or Processing: E.K., B.A.G.; Analysis and/or Interpretation: O.A.A., M.Z.C.; Literature Review: M.Z.C., E.K., B.A.G.; Manuscript Writing: O.A.A., M.Z.C., T.T.T.; Critical Review: O.A.A., T.T.T., M.Z.C., N.K.B., E.K., B.A.G., O.E., A.S., S.A.O., Y.O.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Animals at the University of Health Sciences (Ankara, Turkey) authorized all animal-related experiments (Number 2021-23).

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