

Identification of Acid Degradation Product of Saquinavir Mesylate by LC-MS: Molecular Docking and *In Silico* Toxicity studies.

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SUMMARY

Saquinavir mesylate (SQM) is subjected to forced degradation under conditions of hydrolysis, oxidation, dry heat, photolysis as recommended by International conference on Harmonization guideline Q1A (R2). In total, degradation products (I-V) were formed in acidic hydrolytic, alkaline hydrolytic and oxidative conditions. SQM and all degradation products were optimally resolved by isocratic elution over a C18 column. The HPLC method was found to be linear, accurate, precise, sensitive, specific, rugged, and robust for quantification of SQM as well as degradation products. The major degradation products (DP-1) formed in hydrolytic acid conditions was isolated through column chromatography and analyzed by LC-MS and mass spectral analysis. In Silico molecular docking studies on HIV protease (PDB: 4QGI) and toxicological properties of the major acid degradation product could be pharmacologically inactive than saquinavir mesylate.

Key Words: Saquinavir mesylate, Degradation Products, HPLC, mass spectra, Molecular Docking, Toxicity.

Saquinavir Mezilatin Asit Bozunma Ürününün LC-MS ile Belirlenmesi: Moleküler Docking ve in silico Toksikite çalışmaları

ÖZ

Saquinavir mesilat (SQM), Uluslararası Uyumlaştırma kılavuz Q1A (R2) konferansında önerildiği gibi hidroliz, oksidasyon, kuru sıcaklık, fotoliz koşulları altında zorla bozulmaya maruz kalır. Toplamda, asidik hidrolitik, alkalik hidrolitik ve oksidatif koşullarda bozunma ürünleri (I-V) oluşturulmuştur. SQM ve tüm bozunma ürünleri, bir C18 kolonu üzerinde izokratik elüsyon ile optimum şekilde çözülmüştür. HPLC yönteminin, bozulma ürünlerinin yanı sıra SQM miktarını ölçmek için doğrusal, doğru, kesin, hassas, spesifik, sağlam ve sağlam olduğu bulundu. Hidrolitik asit koşullarında oluşan ana bozunma ürünleri (DP-1), kolon kromatografisiyle izole edildi ve LC-MS ve kütle spektral analizi ile analiz edildi. Silico moleküler yerleştirme çalışmalarında HIV proteazı (PDB: 4QGI) ve ana asit bozunma ürününün toksikolojik özellikleri, saquinavir mezilattan farmakolojik olarak inaktif olabilir.

Anahtar Kelimeler: Saquinavir mesilat, Bozunma Ürünleri, HPLC, kütle spektrumları, Moleküler Yerleştirme, Toksikite.

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INTRODUCTION

Saquinavir mesylate (SQM) chemically (2S)-N-[(2S,3R)-4-[(3S,4aS,8aS)-3-(tert-butylcarbamoyl)-3,4,4a,5,6,7,8,8a-octahydro-1H-isoquinolin-2-yl]-3-hydroxy-1-phenylbutan-2-yl]-2-(quinoline-2-carboxylamino)butanediamide;methanesulfonic acid (Fidler, Bray, & Soerjomataram, 2018). Saquinavir mesylate is used to treat HIV infection by selectively binding to the protease enzymes and thus preventing its replication (Deeks, Smith, Holodniy, & Kahn, 1997). Proteases such as HIV protease, is an enzyme which hydrolysis newly formed peptide bond in HIV virus. It is commercially marketed as Invirase in antiviral therapy of HIV-1, HCV infected patients (Geronikaki, Eleftheriou, & Poroikov, 2016). It is the first drug to be available to HIV patients in United states as approved by USFDA in 2002 (Kim, Dintaman, Waddell, & Silverman, 1998). SQM a peptidomimetic HIV protease inhibitor and has been effective in reducing viral load and mortality and is substrate for multidrug resistance transporter P-glycoprotein (P-gp) (Roberts, 1995). SQM appears white crystalline powder, its melting point is 349.84°C and has an aqueous solubility of 0.22g/100ml and logP of 3.8 in *n*-octanol/water combination.

International conference on harmonization (Guideline) guidelines of (Q1A) necessitate characterization of all the degradation products formed during forced degradation of any drug substances like hydrolysis, (acid and alkylation), oxidation, thermal and photolytic studies (Baertschi, Alsante, & Reed, 2016; Blessy, Patel, Prajapati, & Agrawal, 2014). ICH and FDA have provided guidelines for forced degradation studies for the investigation of degradation products of drugs and related substances (Procedures, 2000). A few HPLC methods reported for simultaneous estimation and identification of degraded products of SQM by LC-MS/MS (Thummar et al., 2017). A few analytical methods on SQM have reported like HPLC, LC-MS/MS in biological samples (Bickel et al., 2009; Ha, Follath, Bloemhard, & Krähenbühl, 1997; Remmel, Kawle, Weller, & Fletcher, 2000). Simultaneous determination of Saquinavir with other HIV protease inhibitor in human plasma by RP-HPLC have been reported by several researchers (Charbe et al., 2016; Droste, Verweij-van Wissen, & Burger, 2003; Markowitz et al., 1998; Sarasa-Nacenta et al., 2001; Simon, Thiam, & Lipford, 2001). Recently Gananadhamu et al., has reported on forced degradation products of SQM by UPLC-ESI-Q-TOF-MS/MS where major degradation is achieved with acid hydrolysis (Mohit et al., 2017).

The current study was to develop stability-indi-

cated assay method for SQM, to identify, isolate and characterize the degraded product produced during the stability studies of SQM using HPLC-UV method. The SQM and major degradation product in acid hydrolysis (DP-1) were also carried out for molecular docking and *In silico* toxicity studies.

MATERIALS AND METHODS

Drug and Chemicals

SQM procured from Hetero Bio Pharma Pvt Ltd (Hyderabad, India). Sodium hydroxide, hydrochloric acid, triethylamine, phosphoric acid was purchased from Standard reagents Pvt. Ltd. (Hyderabad, India). Methanol, acetonitrile, water (HPLC grade) purchased from Merck India Pvt. Ltd. (Mumbai, India). Hydrogen peroxide (H₂O₂) purchased from Alpha Pharma, Hyderabad, India.

Instruments and Software

Thermal forced degradation and hydrolytic degradation were accomplished in a highly precise hot air oven and water bath with digitally controlled temperature system respectively (Narang Scientific Works, New Delhi, India). Photo-degradation was performed in a UV-chamber (LAB India). The chamber was equipped with both fluorescent and near ultraviolet lamp with good illumination as described in ICH guidelines Q1B. The chamber was controlled at a temperature of 25°C and a relative humidity of 55%. The forced degradation samples were interpreted on a Shimadzu HPLC system consisting of isocratic pump with UV-detector and Rheodyne manual injector (Milford, USA). Empower2 software is used to collect and prepare the data. The chromatographic separation was done by Waters C₁₈ (250×4.6mm, 5µm) column. Isolated degradation compounds were studied on LC-MS-TOF studies were accomplished by positive mode of electron spray ionization (+ESI) on micro TOF-Q mass spectrometer with microTOF control software of ver. 2.0. The LC components encloses Agilent 1100 series LC system with Hystar software of 3.1 version. Waters C₁₈ column is used in LC-MS study.

In silico molecular docking was performed by using LeadIT with FlexX platform and toxicity studies by Osiris software and cytochrome inhibition by ephyschem.

Forced degradation study

The forced degradation of SQM was performed according to ICH guidelines Q1A(R2) (Guideline, 2012). A 25mg of the samples was dissolved in 5ml of methanol and remained in solution when acid or base to obtain a concentration of 25mg/ml. SQM was subjected to forced degradation study under acidic

conditions by refluxing with 25ml of 1N HCl, and under basic conditions with 0.5ml of NaOH at 75 °C for 3hrs respectively. Oxidative stress study was accomplished using 3% H₂O₂ for 15 days. The drug was placed in a thermally controlled oven at 75 °C up to 72hrs. A thin layer of drug solution was exposed to UV light of 320nm (200 watt-hour per square meter) for 72hrs and was kept at a distance of about 23 cm from the light source for 14 days with an exposure of 1.2 million lx h, for photolytic drug degradation. A set of solid drug and drug solution was kept in dark under same conditions of temperature and humidity for same period of time to serve as dark control(Shinde, Bangar, Deshmukh, Sulake, & Sherekar, 2013).

Optimized HPLC Conditions

SQM and its products obtained on degradation were resolved on a waters C₁₈ column at a temperature of 30 °C with mobile phase acetonitrile, water, triethylamine (80: 19.5: 0.5 v/v) pH 6.5 adjusted with phosphoric acid mobile phase in an isocratic mode and the injector volume and detection wavelengths were fixed at 20µl and 238 nm, respectively and retention time of SQM was found to be 4.0 min (Figure 1). Each acid and alkali degraded drug solution were neutralized, filtered and diluted 10 times with diluent before the injection. Thermal and photolytic degraded solid drug solutions were made into solutions (1 mg/mL) and then diluted.

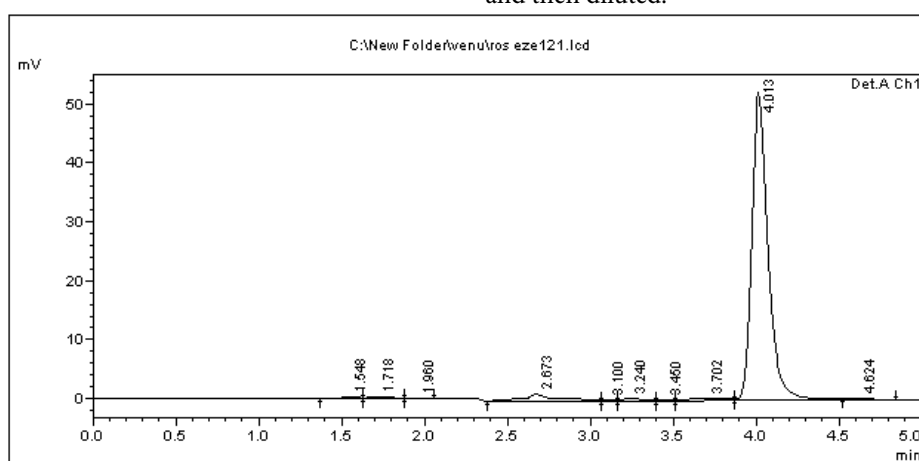


Figure 1. Chromatogram of SQM

Isolation and Characterization of Degradation Product I

Degradation Product (DP-1) was obtained as a major degradation product. It was isolated through preparative column chromatography. For its generation SQM(1.0g) in IM HCl in 100ml volumetric flask was heated at 70 °C. The solution was neutralized with NaOH and filtrate was collected and dried powder was collected. The later was chromatographed over a bed of silica gel with n-hexane and methanol in the gradient elution method. The elution was started with 100% n-hexane and gradually the polarity of the eluent was increased stepwise by adding a mixture of n-hexane and methanol in ratios of 99:1 followed by 98:2, 97:3 and so on going up to 90:10. The solvent was recovered under reduced pressure. The solid residue so obtained was analyzed by using mass spectral analysis.

LC-MS Studies

The isolated degradation compounds were performed for LC-MS study with different conditions than HPLC method. Sample was optimally analyzed

on a X-Bridge C₁₈ (4.6mm×75mm) 3.5µg column at ambient temperature (30 °C) with mobile phase A (10mM ammonium acetate in water), B100% ACN flowing at a rate of 2.0ml/min in the gradient mode. The injection volume was fixed at 20µl and detection wavelength at 238nm. The acid and alkali degraded drug solutions were neutralized and then diluted up to 10 times. The LC-MS studies were carried out using +APCI, ESI and modes of ionization with drug heated temperature of 180°C; 10L/min, capillary voltage of 4.8kv, end plate off set voltage of 65V. Nebulizing (40 psi) gas. All spectra were recorded under identical experimental conditions in the positive ESI mode and with an average of 20 scans.

In Silico Computational studies of SQM and DP-1

The docking studies were carried out for SQM and DP-1 on HIV Protease and structure DP-1 were built in Chem Bio Draw Ultra 12.0 (www.cambridgesoft.com), then converted into 3D structures using Chimera and the structures were energy minimized by using AM1 method. The docking experiment were carried out on HIV Protease variant G48T/L89M in complex with Saquinavir (PDB ID: 4QGI) extracted from RSC

Protein data bank (www.rcsb.org) (Volkova et al., 2012). The docking studies were carried out on LeadIT software using the default settings. The results of the docking scores for each SQM and DP-1 are shown in Table 1. Interactions of 3D and 2D poses between protein and ligands were obtained. *In silico* ADME and toxicity were assessed by Osiris software (Thipparapu, Ajumeera, & Venkatesan, 2017), Saquinavir is metabolized by cytochrome P450 and the calculation of CYP isoforms (CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2), logP, solubility and Ames test on ePhysChem (2015) was used (Zanger & Schwab, 2013).

RESULTS AND DISCUSSION

Method Validation

The method was validated in unison with ICH (International Conference on Harmonization) guideline Q2 (R1) for SQM and degradation product for linearity, accuracy, precision, and specificity. This method was linear for quantification of SQM and its acid degradation product in the concentration range of 5 to 30 µg/ml respectively. The LOD values for SQM and its

acid degradation product are 1.4 and 1.3 respectively. The LOQ values for SQM and its acid degradation product are 4.5 and 4.1 respectively (Table 1).

For determination of accuracy standard addition method was used. A known quantity of SQM were spiked in triplicate to the previously degraded solution of SQM. The percentage recovery range is found to be 99.5 to 101.86 and RSD <2% (Table 2)

It is proposed that this method is sufficiently accurate for quantification of SQM and its degradation product. The method was found to be sufficiently precise with RSD being less than 2 for inter-day and intra-day precision. Significant variations were not observed in the calculated concentrations of SQM and DP-1 on any day. The method was found to be precise. No significant changes were found in percentage change in the calculated content and change in RT of SQM and its DP-1, on changing detection wavelength, composition of mobile phase, pH of mobile phase, flow rate and column. So, the method was found to be robust. Further, a resolution factor of > 2 indicates the method to be selectively stable-indicating (Table 2 and 3).

Table 1. Validation data and regression parameters of the determination of SQM by the proposed method

Parameter	HPLC method
Linearity range (µg/ml)	5-30
Slope	47364
Intercept	10273
Coefficient of determination (r ²)	0.997
LOD (µg/ml)	1.49420
LOQ (µg/ml)	4.52790

Table 2. Recovery data of the developed method for SQM (n=3)

Spiked concentration (mg/mL)	Found concentration (mg/mL, Mean ± SD)	RSD	% Recovery
15	15.28 ± 0.28	1.98	101.86
20	19.90 ± 0.81	1.90	99.5
25	25.20 ± 0.64	1.20	100.8

Table 3. Precision study of SQM (n=3).

Concentration (mg/mL)	Intra-day precision		Inter-day precision	
	Found Concentration (mg/mL, Mean ± SD)	RSD (%)	Found Concentration (mg/mL, Mean ± SD)	RSD (%)
20	19.35 ± 0.06	0.67	19.53 ± 0.56	1.03
40	39.65 ± 0.82	0.56	39.96 ± 0.25	0.61
80	79.15 ± 0.61	0.43	79.59 ± 0.02	0.06
100	99.78 ± 0.56	0.23	99.83 ± 0.21	0.19

Degradation profile of SQM

Figure 2A and 2B indicates SQM degradation with 1M HCl and 0.5M NaOH. A satisfactory separation of SQM and its degradation products is observed. Acid degradation chromatogram (Figure 2A) shows a complete degradation, whereas separation of base hydrolysis degradation products is satisfactory (Figure 2B)

Only 3.9% of SQM was observed by oxidative hydrolysis in presence of hydrogen peroxide. By photolytic and thermal degradation partial amount of SQM have been degraded. (Figures 3A–C). No formation of major degradation products has observed by oxidative, photolytic and thermal degradation.

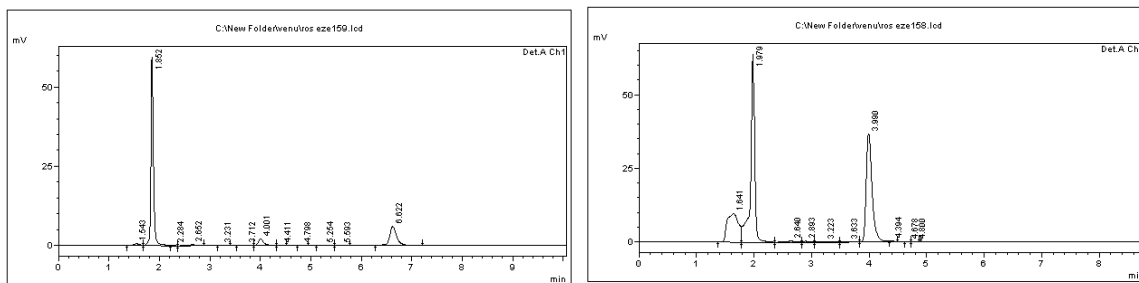


Figure 2. (A) Separation with acid degradation (B) Separation with alkali degradation of SQM

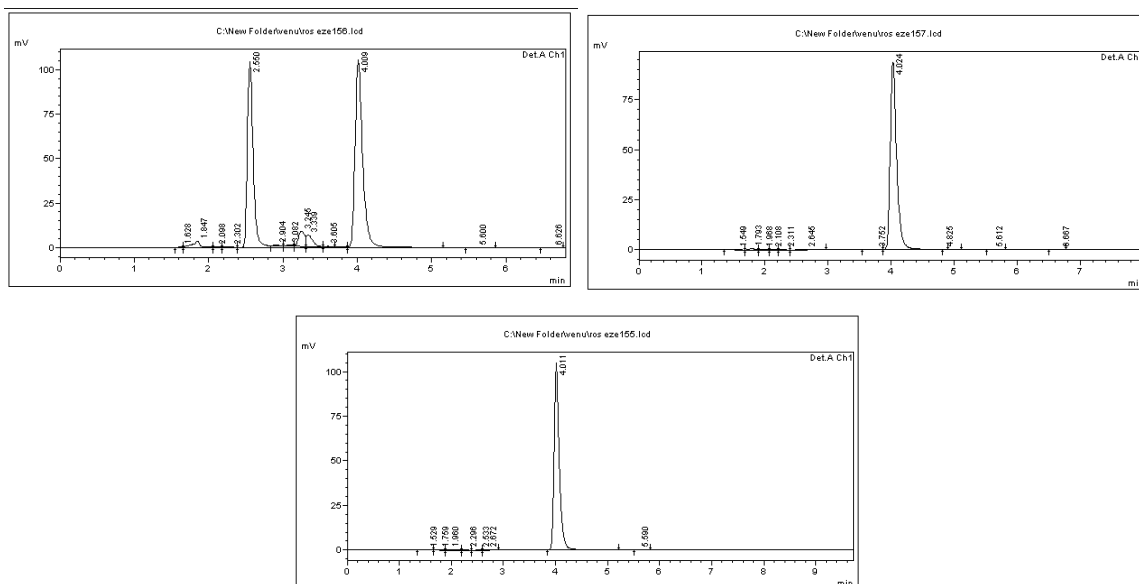


Figure 3. HPLC chromatogram A) Oxidative Degradation B) Photolytic C) Thermal Degradation

Degradation Product Identification

SQM was subjected to acid degradation using 1M HCl. After refluxing with 1M HCl, a complete degradation was observed. This reaction was monitored by RP-HPLC where complete fading of SQM peak was observed indicating complete degradation. It was also observed that one peak appeared at different time indicating the presence of only one degradation product. Structure elucidation of this degradation product was done by using mass spectral data. The LC chro-

matogram of isolated DP-1 is shown to be 90% purity (Figure 4). The mass spectrum of $[M + H]^+$ ions (m/z 553) of SQM acid degradation product (DP-1) (Figure 6) shows the productions of m/z 424 (loss of $C_{14}H_{16}N_2O$ from the parent ion at m/z 553), m/z 420 with base peak (loss of $C_{14}H_{12}N_3O_3$ from the parent ion at m/z 553), m/z 270 (loss of $C_{24}H_{39}N_3O_2$ from the parent ion at m/z 553), m/z 242 (loss of CO from the ion at m/z 242). The proposed fragmentation pathway of the mass spectrum of the degradation product is shown in Fig 5.

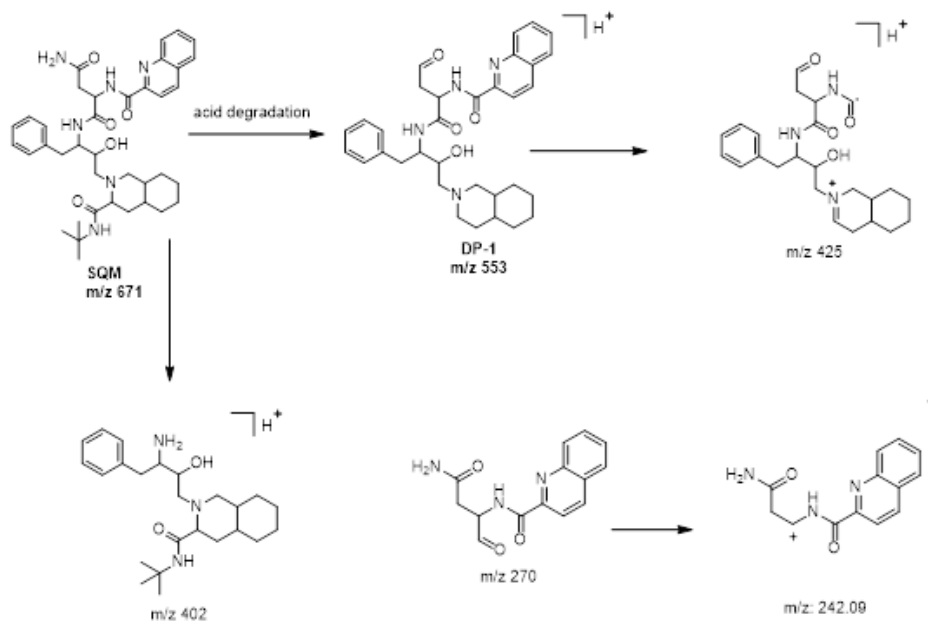


Figure 4. LC Chromatogram of isolated DP-1

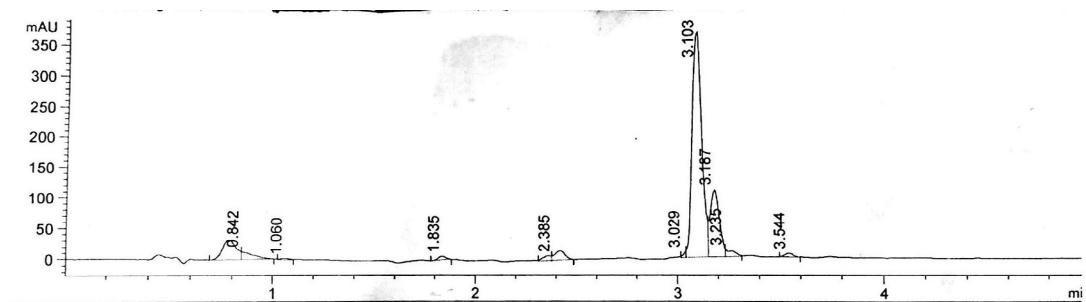


Figure 5. Mass fragmentation of DP-1

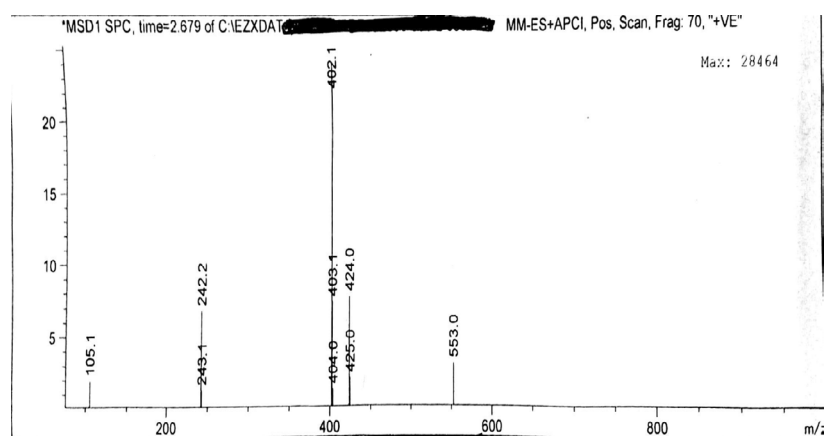


Figure 6. Mass Spectra of isolated DP-1

¹H-NMR Study

The isolated major degradation product (DP-1) was characterized by $^1\text{H-NMR}$ and data shown as follows (Figure 7):

$^1\text{H NMR}$ (400 MHz, DMSO-d_6): 9.02 (d, $J = 8.0$ Hz, 1H), 8.59 (d, $J = 8.4$ Hz, 1H), 8.09 - 8.18 (m, 4H), 7.89 (t, $J = 8.0$ Hz, 1H), 7.74 (t, $J = 7.6$ Hz, 1H), 7.36

(t, $J = 6.4$ Hz, 2H), 7.14 (t, $J = 8.0$ Hz, 2H), 7.02-7.06 (m, 1H), 4.64-4.70 (m, 1H), 4.41 (s, 1H), 4.07 (t, $J = 9.6$ Hz, 1H), 3.81 (s, 1H), 3.5 (s, 3H), 2.99 (t, $J = 12$ Hz, 2H), 2.68 (s, 1H), 2.5 (s, 3H), 1.99-2.07 (m, 2H), 1.64-1.74 (m, 2H), 1.49 (s, 2H), 1.39 (m, 2H), 1.25 (broad singlet, 12H).

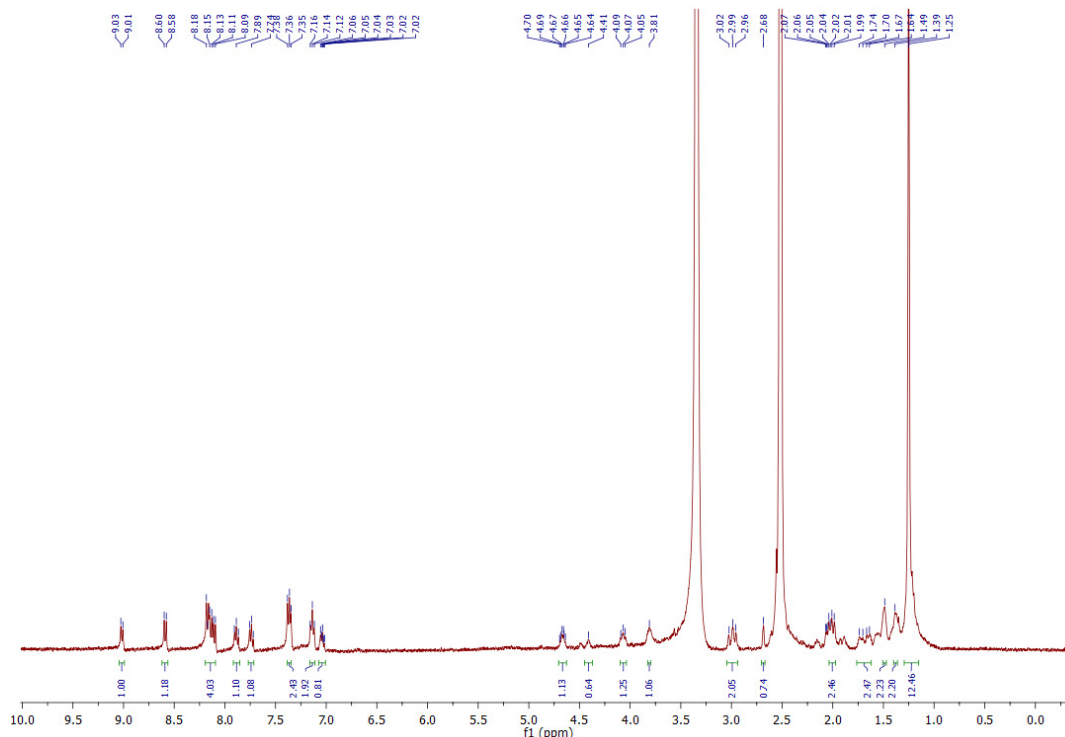


Figure 7. $^1\text{H NMR}$ data of degradation product (DP-1)

Molecular Docking, ADMET and Toxicity Studies

Saquinavir mesylate and its major acid degradation product (DP-1) were subjected on HIV Protease (PDB ID: 4QGI) which is having a co-crystal saquinavir. The docking was done using FlexX module in LeadIT 2.1 software (Slynko, Rognan, & Kellenberger, 2017). The crystal ligand of SQM was redocked and calculated the binding affinity. The SQM and DP-1 were docked in order to explore the possible interactions on biological system. SQM have shown highest binding affinity than DP-1 with FlexX score -21.44 and -15.10 respectively and 2D and 3D poses were shown in Figure-8. SQM forms hydrogen bonding interactions with Asp25, Asp29, Asp30 and hydrophobic interactions with Gly27, Ala28, Thr48, Gly49, Val82 and Ile84. Moreover, DP-1 has shown lesser

binding affinity and weak interactions compared to SQM and having hydrogen bonding interactions with Asp29, Asp30, Thr48 and hydrophobic interactions with Val32, Thr48, Gln49 (Table 4).

Toxicity risk assessment was carried out using Osiris software on SQM and DP-1 where SQM has shown drug likeness and has no mutagenic, tumorigenic, irritant and reproductive effect whereas DP-1 has less drug likeness property and having high irritant effect and no mutagenic, tumorigenic and reproductive effects. SQM and DP-1 showed no mutagenicity in the Ames test and have shown non-inhibition on CYP2D6 and inhibition on CYP3A4 and have same aqueous solubility and environmental toxicity (Table 5 & 6).

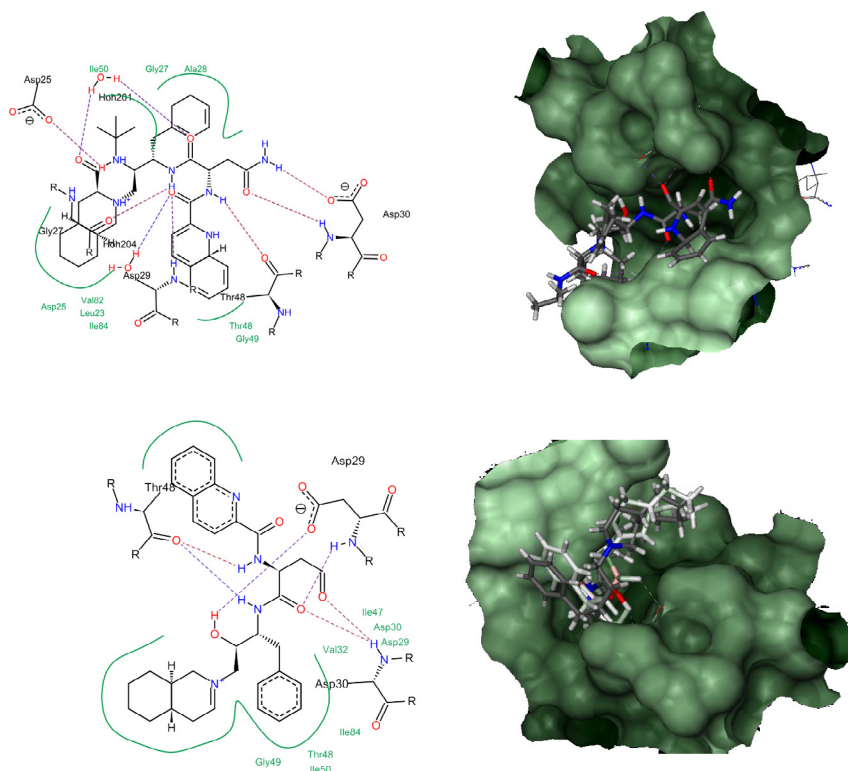


Figure 8. Molecular Docking studies 2D and 3D interactions of SQM and DP-1

Table 4. Molecular docking results of SQM and DP-1

Parameter	SQM (Flexx Score)	DP-1
Flexx Score	-21.44	-15.10
Ligand Interactions	Leu23, Asp25, Gly27, Ala28, Asp30, Thr48, Gly49, Ile84, Val82	Asp29, Asp30, Val 32, Thr48, Ile47, Gly49, Ile50, Ile84
No of Hydrogen Bonds	05	04
No of Hydrophobic Bonds	04	02

Table 5. Toxicity risk assessment of SQM and DP-1.

Compound	cLogP	Solubility	Drug likeness	Drugscore	Mutagenic	Tumorigenic	Irritant	Reproductive effect
SQR	2.84	-5.66	1.56	0.32	No	No	No	No
DP-1	3.22	-5.18	-0.69	0.18	No	No	High	No

Table 6. ADME/Toxicity calculations for SQM and DP-1.

Compound	CYP2D6 inhibition	CYP3A4 inhibition	logP o/w	Aqueous solubility	Environmental toxicity	Ames test
SQM	Noninhibitor (93%)	Inhibitor (74%)	4.7 Log unit ± 0.38	4.68 -log(mol/L) ± 0.70*	-0.74-log(mmol/L) ± 0.53*	Inactive (71%)
DP-1	Noninhibitor (57%)	Noninhibitor (64%)	3.59 Log unit ± 0.38	4.99 -log(mol/L) ± 0.70*	-0.07 -log(mmol/L) ± 0.53*	Inactive (72%)

CONCLUSION

A validated forced degradation study was established to study the degradation product of SQM under acid, base hydrolysis, oxidation, photolysis and thermal stress conditions. The major acid degradation product (DP-1) were identified, isolated and characterized by Mass spectra data. *In Silico* molecular docking studies have revealed that DP-1 has shown weak interactions than SQM on HIV protease. Toxicity were assessed by using Osiris software and the results shown DP-1 has high irritant effect compared with SQM.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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