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Identification of degradation products of Saquinavir mesylate by LC-MS: Molecular Docking and *In Silico* toxicity studies

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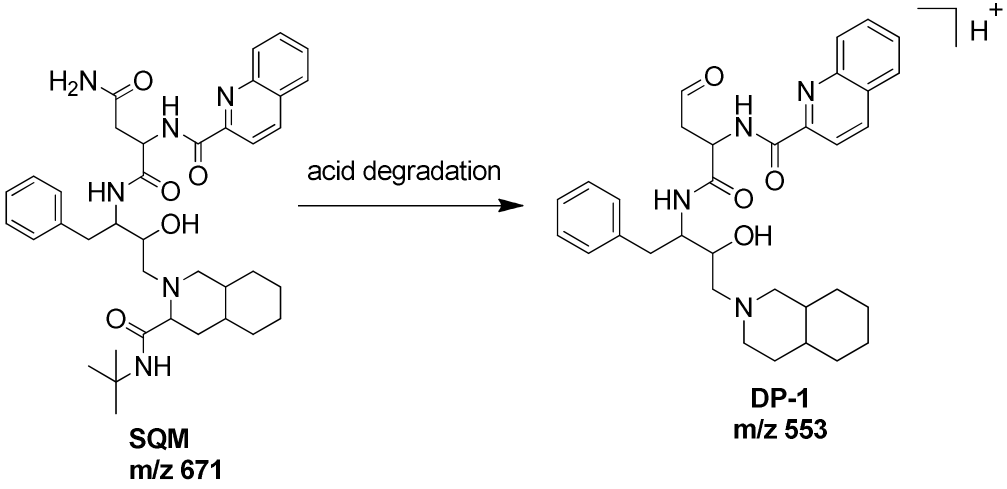
Graphical Abstract



SQM
m/z 671

DP-1
m/z 553

Abstract:

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, (I-V) degradation products (DPs) were formed in acidic hydrolytic, alkaline hydrolytic and oxidative conditions. Successful separation of SQM and its DPs was achieved on C_{18}(4.6mm×75mm) 3.5µg column at ambient temperature (30˚C) with mobile phase A (10mM ammonium acetate in water), B100% acetonitrile at 2.0ml/min flow rate in the gradient mode. The injection volume was fixed at 20µl and detection wavelength at 238nm. 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 identified and characterized by LC-MS/MS and proposed the fragmentation patterns by comparing with SQM. Further, DP-1 were isolated through column chromatography and analyzed by ^{1}H NMR. *In Silico* molecular docking studies on HIV protease (PDB: 4qgi) for DPs and SQM was estimated and found to be pharmacologically inactive than SQM. Prediction of Toxicity and ADME properities were performed for DP-1 and SQM and found to less toxic.

Keywords: Saquinavir mesylate; Degradation Products; HPLC; ^{1}H NMR; mass spectra; Molecular Docking; Toxicity.

Introduction

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). 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).

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).
- 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-indicated 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.

Experimental:

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(H_2O_2) purchased from Alpha Pharma, Hyderabad, India.

Forced degradation study:

The forced degradation of SQM was performed according to ICH guidelines Q1A(R2)(Guideline, 2012)

LC-MS Studies:

Sample was optimally analyzed on a X-Bridge C_{18}(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 180ºC; 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.

Forced degradation study

According to ICH guidelines Q1A(R2)(Guideline, 2012)

Acid/Base degradation study:

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% H_2O_2 for 15 days.

Thermal degradation study

Drug was placed in a thermally controlled oven at 75 ˚C up to 72hrs.

Photolytic degradation study

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.

Results and discussion

HPLC Method development

Optimized chromatographic conditions	
HPLC column	Waters C18 column
Mobile phase	A10mM ammonium acetate in water), B100% acetonitrile
Injection Volume	20µl
Detection wavelength	238 nm
Retention time	4.0 min
Flow rate	2.0 ml/min

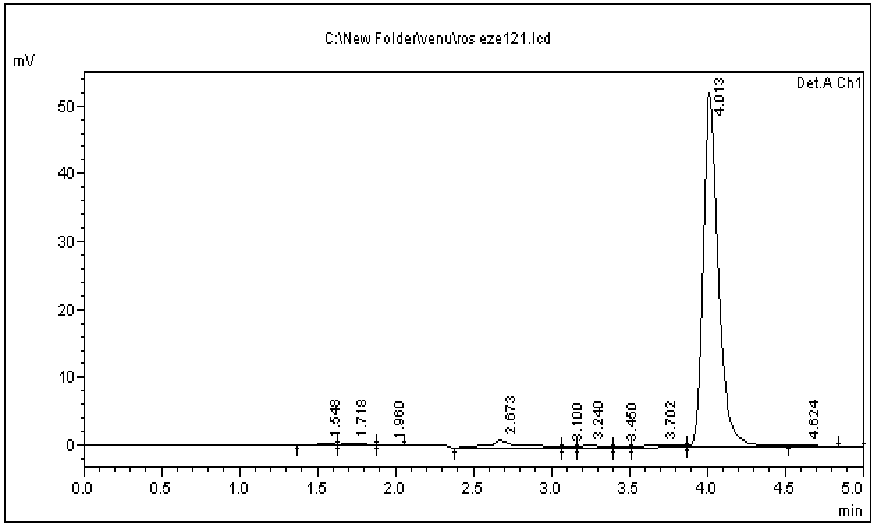


Fig 1: Chromatogram of SQM

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.

Linearity

linear for quantification of SQM and its acid degradation product in the concentration range of 5 to 30 μg/ml respectively.

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

- ## Accuracy study of SQM (n=3)

Spiked concentration(μg/mL)	Found concentration (μg/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

Precision data for SQM (n=3)

Concentration (μg/mL)	Intra-day precision		Inter-day precision	
	Found Concentration (μg/mL, Mean ± SD)	RSD(%)	Found Concentration (μg/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

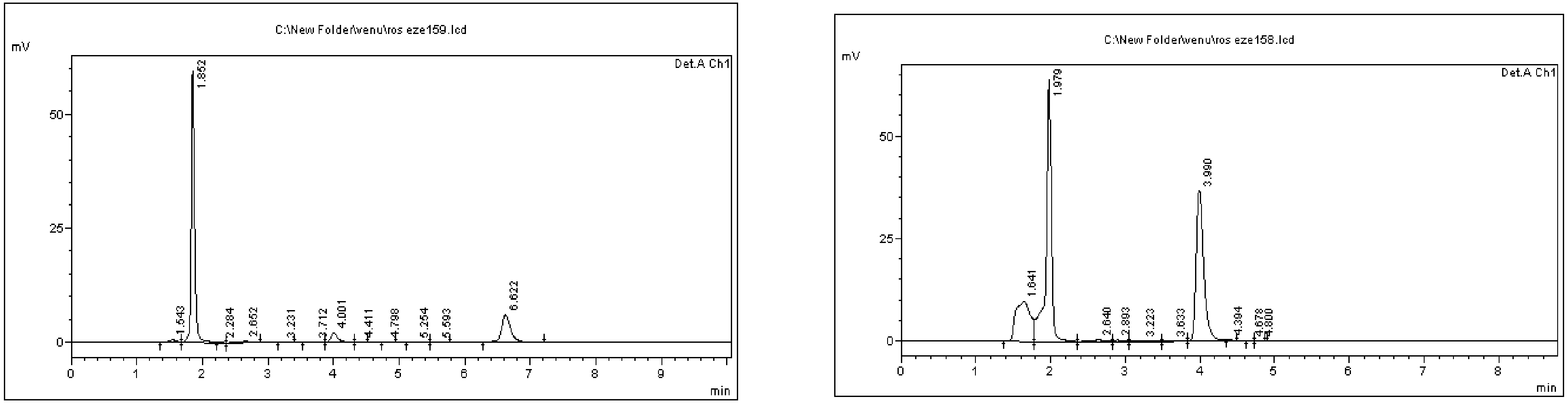


Fig 2 (A) Separation with acid degradation (B) Separation with alkali degradation of SQM

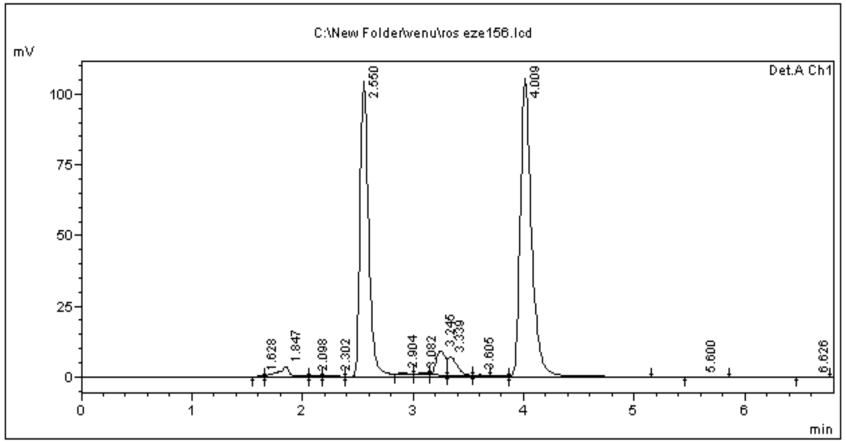


Fig 3A: Oxidative degradation separation

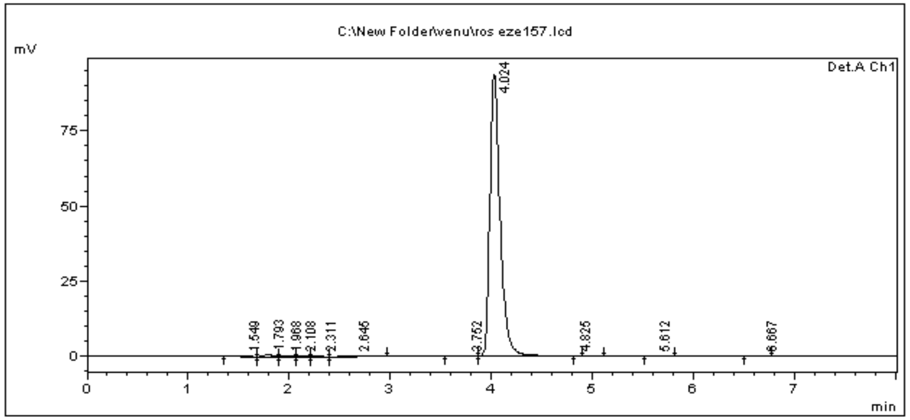


Fig 3B: Photolytic degradation separation

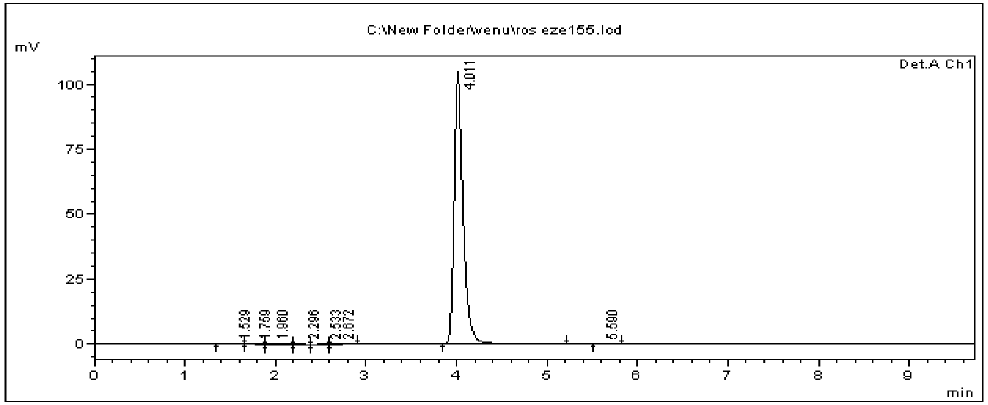


Fig 3C: Thermal degradation separation

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.

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 controlled 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 ^{1}H NMR and mass spectral data. The LC chromatogram of isolated DP-1 is shown to be 99% purity (Fig 4). The mass spectrum of $[M + H]^+$ions (m/z 553) of SQM acid degradation product (Fig 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.

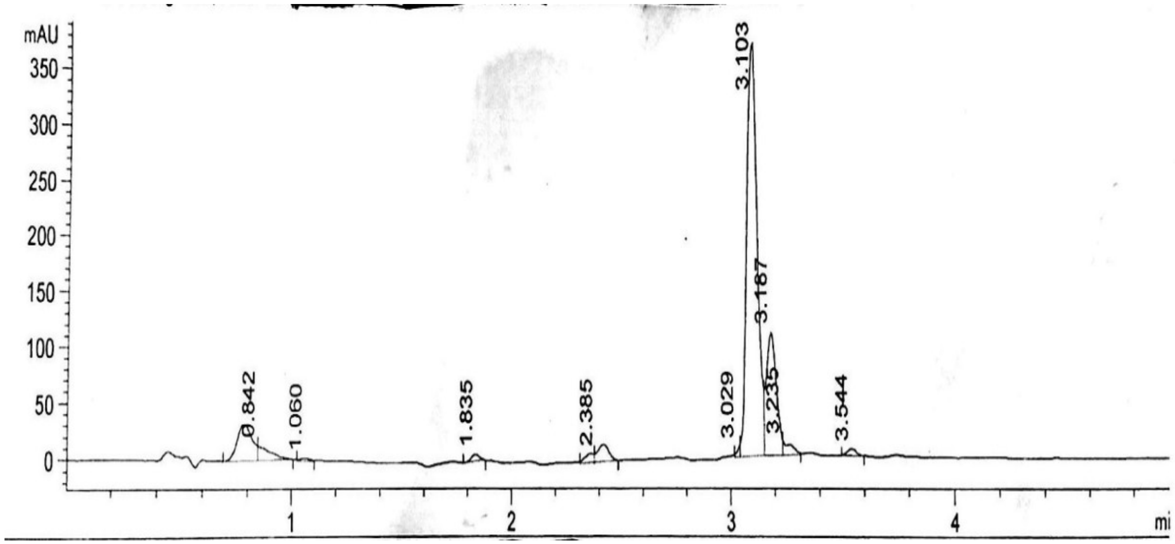


Fig 4: LC Chromatogram of isolated DP-1

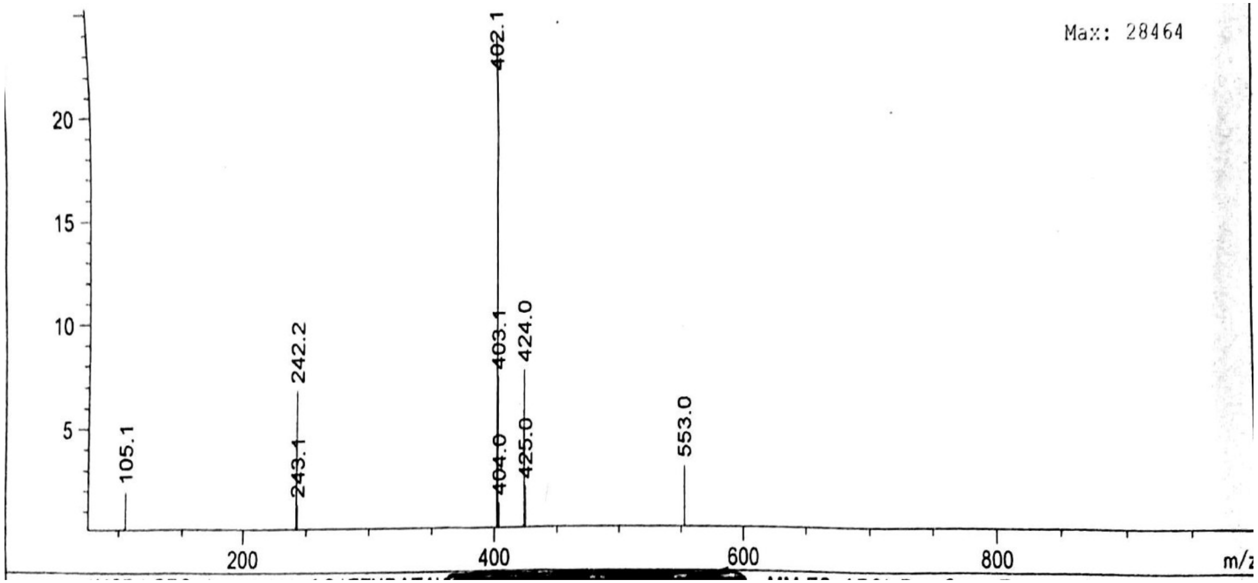


Fig 5: Mass Spectra of isolated DP-1

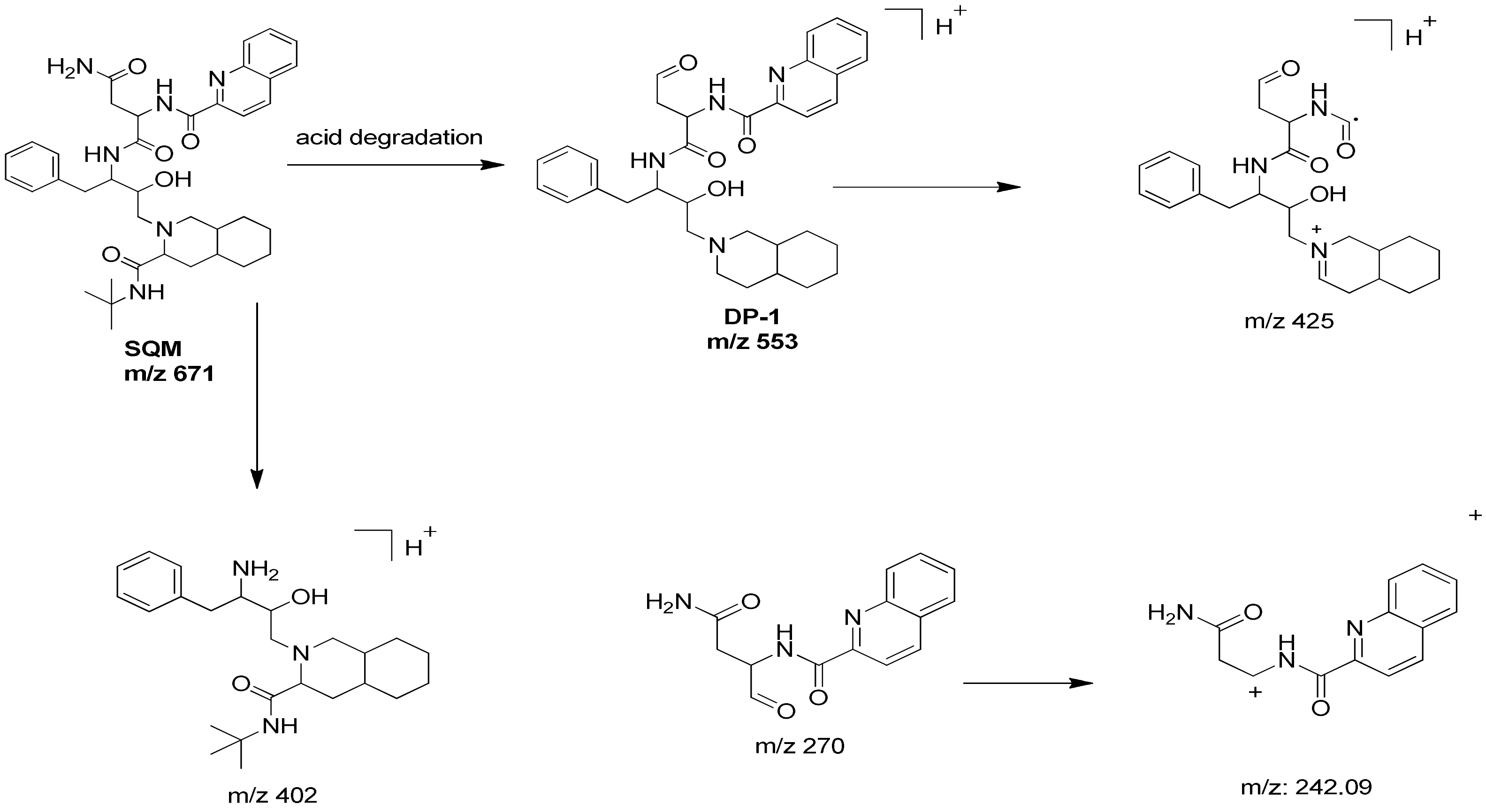


*Figure 6:*Mass fragmentation of DP-1

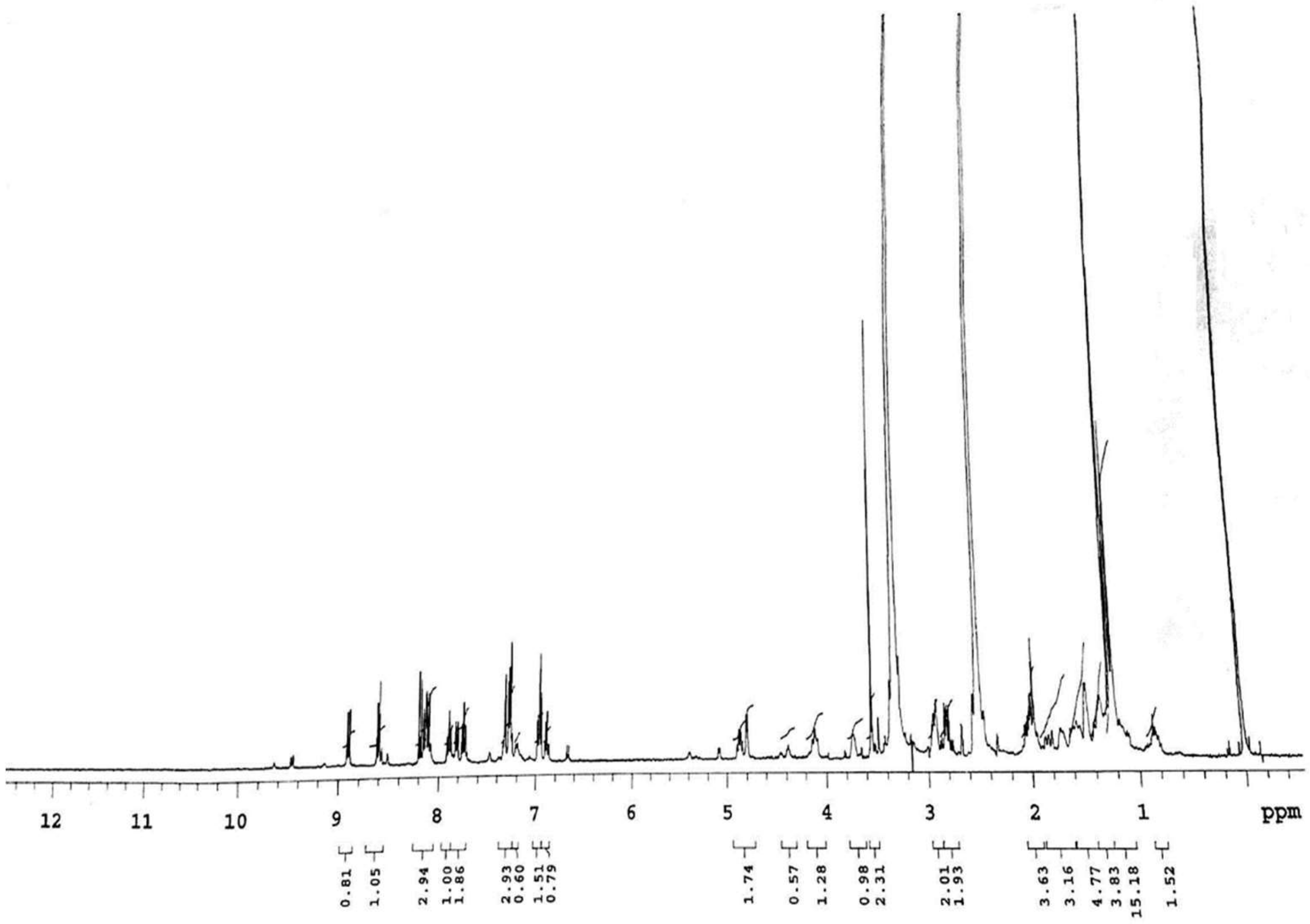


Figure 7: ^{1}H NMR data of degradation product (DP-1)

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.

The crystal ligand of SQM was redocked and calculated the binding affinity.

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

Parameter	SQM (Dock 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

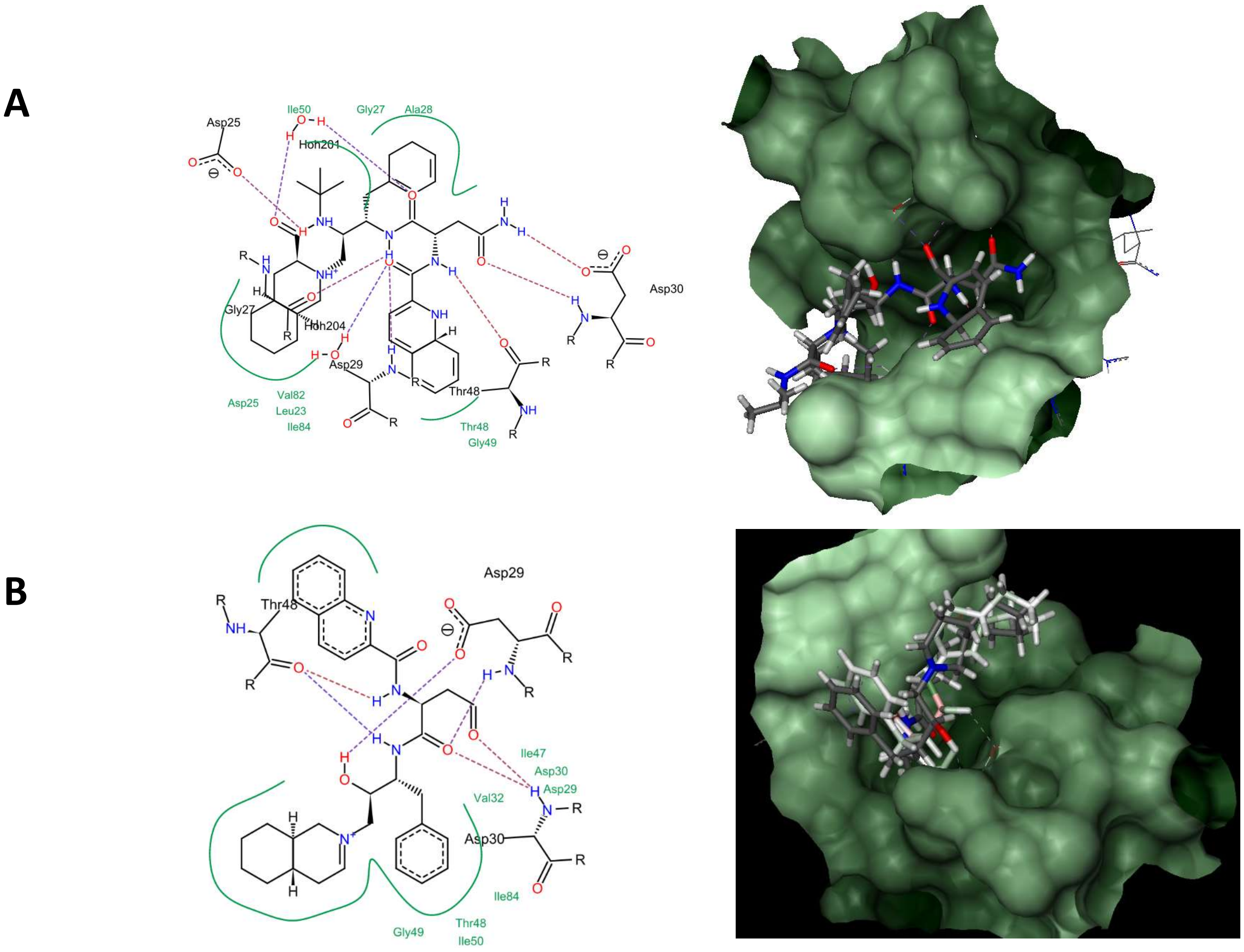


Figure 8: Molecular Docking studies 2D and 3D interactions of A)SQM and B)DP-1

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

Compound	**cLogP**	**Solubility**	**Druglikeness**	**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-I

Compound	**CYP2D6inhibition**	**CYP3A4inhibition**	**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 ^{1}H NMR and 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.

References

- Baertschi, S. W., Alsante, K. M., & Reed, R. A. (2016). *Pharmaceutical stress testing: predicting drug degradation*: CRC Press
- Bickel, M., Bodtländer, A., Knecht, G. K., Stephan, C., von Hentig, N., Kurowski, M., . . . Lutz, T. (2009). Once-daily treatment with saquinavir mesylate (2000 mg) and ritonavir (100 mg) together with a fixed-dose combination of abacavir/lamivudine (600/300 mg) or tenofovir/emtricitabine (245/200 mg) in HIV-1-infected patients. *Journal of Antimicrobial Chemotherapy, 64*(6), 1260-1264. doi:10.1093/jac/dkp348
- Blessy, M., Patel, R. D., Prajapati, P. N., & Agrawal, Y. (2014). Development of forced degradation and stability indicating studies of drugs—A review. *Journal of pharmaceutical analysis, 4*(3), 159-165.
- Charbe, N., Baldelli, S., Cozzi, V., Castoldi, S., Cattaneo, D., & Clementi, E. (2016). Development of an HPLC–UV assay method for the simultaneous quantification of nine antiretroviral agents in the plasma of HIV-infected patients. *Journal of Pharmaceutical Analysis, 6*(6), 396-403. doi:https://doi.org/10.1016/j.jpha.2016.05.008
- Deeks, S. G., Smith, M., Holodniy, M., & Kahn, J. O. (1997). HIV-1 protease inhibitors: a review for clinicians. *Jama, 277*(2), 145-153.
- Droste, J., Verweij-van Wissen, C., & Burger, D. (2003). Simultaneous determination of the HIV drugs indinavir, amprenavir, saquinavir, ritonavir, lopinavir, nelfinavir, the nelfinavir hydroxymetabolite M8, and nevirapine in human plasma by reversed-phase high-performance liquid chromatography. *Therapeutic drug monitoring, 25*(3), 393-399.
- Fidler, M. M., Bray, F., & Soerjomataram, I. (2018). The global cancer burden and human development: A review. *Scandinavian Journal of Public Health, 46*(1), 27-36. doi:10.1177/1403494817715400
- Geronikaki, A., Eleftheriou, P., & Poroikov, V. (2016). Anti-HIV Agents: Current Status and Recent Trends.
- Guideline, I. H. T. (2012). Stability Testing of New Drug Substances and Products Q1A (R2) 2003. *Current step, 4*.

References

- Ha, H. R., Follath, F., Bloemhard, Y., & Krähenbühl, S. (1997). Determination of saquinavir in human plasma by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications, 694*(2), 427-433. doi:https://doi.org/10.1016/S0378-4347(97)00165-5
- Kim, A. E., Dintaman, J. M., Waddell, D. S., & Silverman, J. A. (1998). Saquinavir, an HIV protease inhibitor, is transported by P-glycoprotein. *Journal of Pharmacology and Experimental Therapeutics, 286*(3), 1439-1445.
- Markowitz, M., Conant, M., Hurley, A., Schluger, R., Duran, M., Peterkin, J., . . . Ho, D. D. (1998). A Preliminary Evaluation of Nelfinavir Mesylate, an Inhibitor of Human Immunodeficiency Virus (HIV)-1 Protease, to Treat HIV Infection. *The Journal of Infectious Diseases, 177*(6), 1533-1540. doi:10.1086/515312
- Mohit, T., N., P. P., L., P. A., Debasish, S., R., S., & Gananadhamu, S. (2017). Identification of degradation products of saquinavir mesylate by ultra-high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry and its application to quality control. *Rapid Communications in Mass Spectrometry, 31*(9), 771-781. doi:doi:10.1002/rcm.7842
- Procedures, A. (2000). Methods Validation, Draft guidance, US Department of Health and Human Services. *Food and drug Administration, CDER, CBER*.
- Remmel, R. P., Kawle, S. P., Weller, D., & Fletcher, C. V. (2000). Simultaneous HPLC Assay for Quantification of Indinavir, Nelfinavir, Ritonavir, and Saquinavir in Human Plasma. *Clinical Chemistry, 46*(1), 73-81. Remmel, R. P., Kawle, S. P., Weller, D., & Fletcher, C. V. (2000). Simultaneous HPLC Assay for Quantification of Indinavir, Nelfinavir, Ritonavir, and Saquinavir in Human Plasma. *Clinical Chemistry, 46*(1), 73-81.

References

- Roberts, N. A. (1995). Drug-resistance patterns of saquinavir and other HIV proteinase inhibitors. *AIDS (London, England), 9*, 27-S32.
- Sarasa-Nacenta, M. a., López-Púa, Y., Mallolas, J., Blanco, J. L., Gatell, J. M., & Carné, X. (2001). Simultaneous determination of the HIV-protease inhibitors indinavir, amprenavir, ritonavir, saquinavir and nelfinavir in human plasma by reversed-phase high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications, 757*(2), 325-332. doi:https://doi.org/10.1016/S0378-4347(01)00172-4
- Shinde, N. G., Bangar, B. N., Deshmukh, S. M., Sulake, S. P., & Sherekar, D. P. (2013). Pharmaceutical forced degradation studies with regulatory consideration. *Asian J. Res. Pharm. Sci, 3*(4), 178-188.
- Simon, V. A., Thiam, M. D., & Lipford, L. C. (2001). Determination of serum levels of thirteen human immunodeficiency virus-suppressing drugs by high-performance liquid chromatography. *Journal of Chromatography A, 913*(1), 447-453. doi:https://doi.org/10.1016/S0021-9673(00)01092-X
- Slynko, I., Rognan, D., & Kellenberger, E. (2017). Protein-Ligand Docking. *Tutorials in Chemoinformatics*, 355.
- Thipparapu, G., Ajumeera, R., & Venkatesan, V. (2017). Novel dihydropyrimidine derivatives as potential HDAC inhibitors: in silico study. *In silico pharmacology, 5*(1), 10.
- Thummar, M., Patel, P. N., Petkar, A. L., Swain, D., Srinivas, R., & Samanthula, G. (2017). Identification of degradation products of saquinavir mesylate by ultra-high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry and its application to quality control. *Rapid Communications in Mass Spectrometry, 31*(9), 771-781.

References

- Volkova, M. S., Jensen, K. C., Lozinskaya, N. A., Sosonyuk, S. E., Proskurnina, M. V., Mesecar, A. D., & Zefirov, N. S. (2012). Synthesis of novel MT3 receptor ligands via an unusual Knoevenagel condensation. *Bioorganic & Medicinal Chemistry Letters, 22*(24), 7578-7581. doi:https://doi.org/10.1016/j.bmcl.2012.10.005
- Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & therapeutics, 138*(1), 103-141

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