Establishment of Inherent Stability of Saquinavir Under Various Conditions Using a Stability-Indicating High-Performance Liquid Chromatographic Assay with Photodiode Array (PDA) Detection

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Summary

A novel stability-indicating high-performance liquid chromatographic assay method with photodiode array (PDA) detection was developed and validated for Saquinavir (SQV) in the presence of degradation products generated from forced degradation studies. Resolution of drug and the degradation products formed under different stress studies was successfully achieved using a Vydac (C_{18}) monomeric (250mm × 4.6mm, 5 µm particle size) column and a mobile phase composed of acetonitrile and 0.025 M potassium dihydrogen phosphate buffer in the ratio 40:60 (v/v) at the detection wavelength of 237 nm. The method was validated with respect to specificity, chromatographic parameters, linearity, precision, accuracy and limits of detection and quantitation. PDA peak purity test confirmed the specificity of the developed method.

The method was also successful in the analysis of drug subjected to stability testing under accelerated conditions of temperature, humidity and to thermal and photolytic stress. SQV was found to degrade significantly in alkaline and oxidative conditions and also in the presence of light. The alkaline medium had the greatest effect on degradation of SQV. The rate of hydrolysis in acid was low at room temperature as compared to the one that was obtained when more intensive stress studies were carried out by refluxing the drug in acid at accelerated temperature (80°C). The drug was stable to dry heat and under high relative humidity (RH) at room temperature (75% & 97.3% RH, 25 °C).

Keywords: Saquinavir, Stability, HPLC, Validation.

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Introduction

The treatment of the HIV infection improved at the end of 1995 with the appearance of the protease inhibitors [1]. SQV, the first of the HIV protease inhibitors to reach the market, remains one of the most widely prescribed agents which has markedly improved morbidity and mortality in HIV-infected patients. HIV protease is an enzyme required for the proteolytic cleavage of viral polyprotein precursors into individual functional proteins found in infectious HIV. SQV (Fig.1) is a peptide-like substrate analogue that binds to the protease active site and inhibits the activity of the enzyme. SQV inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature noninfectious virus particles [2, 3].

The parent drug stability test guideline Q1A (R2) issued by International Conference on Harmonization (ICH) [4] suggests that the stress studies should be carried out on a bulk active compound used in the manufacture of pharmaceuticals to establish its inherent stability characteristics, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule. Such stability-indicating assay can also be a key method, which would be used for the analysis of stability samples studied within pharmaceutical research and development.

The U.S. Food and Drug Administration (FDA) define the stability-indicating assay as: "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating assay accurately measures the active ingredients, without interference from degradation products, process impurities, excipients or other potential impurities".

A review of literature reveals a number of assay methods reported over the period of time for SQV. These include HPLC with UV–visible spectrophotometry [5-7], Capillary Electrophoresis [8], LC-MS/MS [9] and Radioimmuno assay [10]. Because of the very nature of requirement of separation of multiple components during analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis. Other than separation of multiple components, the advantage of chromatographic methods is that these possess high-resolution capacity, sensitivity and specificity. So far, to best of our present knowledge, none of the published works has reported a stability-indicating HPLC assay method for SQV. There is very limited information in literature on the stability profile of SQV [11]. Keeping into view the susceptibility of SQV degradation under various stress conditions (pH, hydrolysis, oxidation, photolysis and thermal stress), it was felt that a HPLC method of analysis that separates the drug from the degradation products would be of general interest.

The presented HPLC study was designed as an assay procedure, that is, the objective of the procedure was the accurate and precise determination of the active drug in presence of its degradation products. It was not an impurity test, which would focus the accurate and precise quantification of impurities and would be a different goal of an analytical test procedure.

Separation of formulation excipients, as well as their own degradation products, would be necessary for a formulation assay. Since this method is designed for the bulk drug substance, a separate study and method would be needed to be devised for the specific formulation. Also identification of the degradant peaks is outside the scope of this current study, which was to demonstrate the stability-indicating nature of the described method.

Degradation compounds generated under the stressed conditions did not interfere with the parent drug peak in the described chromatographic system.
Stability is important from a quality control perspective and therefore the focus in the present study was to develop a simple isocratic stability-indicating method for SQV by degrading the drug under various stress conditions [12]. The drug was separated from degradation products on a reversed-phase column and the developed HPLC assay method was validated as per ICH guidelines [13].

Experimental

Drugs and chemical reagents

Saquinavir mesylate, N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]amino] butyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide methanesulfonate (99.70%, on anhydrous basis) was a kind gift from Aurobindo Pharma Ltd. Research Centre (Hyderabad, India). HPLC grade reagents such as acetonitrile and methanol were purchased from Rankem Chemicals (Delhi, India). All other reagents used were of analytical grade. HPLC-grade water was produced in the laboratory by a Milli-Q purification system (Millipore Corp., Vienna, Austria).

Instrumentation

A Shimadzu LC-10 series chromatographic system (Shimadzu Corporation, Kyoto Japan) was used in this study. More precisely, the system consisted of a Model SCL-10A controller unit, a Model DGU-2A degasser unit, two LC-10AT piston pumps, SIL-10AD autosampler and a Model SPD-M10AVP photodiode array (PDA) detector. Chromatographic system operation and recording of data were performed with the use of Class-VP software (Version 6.12, SP1 Shimadzu). A glass vacuum-filtration apparatus (Alltech Associates) was employed for the filtration of the buffer solution using 0.2 µm filters obtained from Pall Life Sciences. Degassing of the mobile phase was performed by sonication in Oscar Micro clean-103 Ultrasonic bath. A Model Genie-2 Spinix vortex mixer and a REMI C24 refrigerated centrifuge (REMI, India) were employed for the sample pre-treatment. Standard substances were weighed on AY 220 Shimadzu analytical balance. All pH values were measured using a Systronics pH meter (Model 361, ASE ltd, India). Relative humidity was measured using Thermo-Hygro Clock (J412CTH, Globe, India) Thermal stability study was carried out in dry air oven (OSWORD^{TM}, Mumbai, India).

Chromatographic conditions

HPLC mobile phase consisting of acetonitrile and 0.025M potassium dihydrogen phosphate buffer (Phosphate buffer) in the ratio 40:60 (v/v) was premixed before use. Triethylamine (0.06 % v/v) was used as a peak modifier. The pH of the mobile phase was adjusted to approximately 3.1 with 85% ortho-phosphoric acid solution. Following its preparation, the mobile phase was passed through a 0.2µm filter (Pall Life Sciences) and was degassed using sonicator for 15 min. Chromatographic separation was achieved by a Vydac C_{18} monomeric column (250mm × 4.6mm, 5 µm particle size) from Grace Vydac, Hesperia, USA. Two different batch lots of C_{18} monomeric columns were used for intermediate precision studies. The stationary phase was thoroughly equilibrated with mobile phase each time before use. Reproducible retention factor (*k*) could be achieved after the passage of at least 150 ml mobile phase through the column. The LC system was

operated isocratically at a flow rate of 1 ml/min and the elution was performed at a temperature of 25°C.

System suitability

The peak retention factor (k) for the drug was calculated from $k = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the peak of interest and the solvent front, respectively. The separation factor (α) was estimated from $\alpha = k_2/k_1$, where k_1 and k_2 are the retention factors of two adjacent peaks. A useful and practical measurement of peak shape, the peak asymmetry factor, A_s, was calculated at 10% of peak height. The resolution factor (R_s) was calculated from $R_s = 1/4\ (\alpha\text{-}1)\ (N^{1/2})\ (k'/1+k')$, where N is the column plate number and k' is the average retention factor for the two bands. Column plate number was determined using the formula, $N = 5.54\ (t_R / w_h)^2$, where w_h is the bandwidth at 50% of peak height.

Standard and sample solutions

Primary stock solution of SQV was prepared by dissolving the analyte in methanol at a concentration of 5 mg/ml.Corrections to the theoretical concentrations were performed according to the degree of standard substance impurities. Intermediate stock solutions (500 and 50 µg/ml) in duplicate were prepared from the primary stock solution. Primary and intermediate stock solutions were kept at 4°C and remained stable for at least 14 days. Working stock solutions of SQV were prepared using a mixture of methanol-phosphate buffer, 50:50, v/v (diluent). Intermediate stock solutions were prepared weekly, while working stock solutions used for the calibration curves were prepared daily.

Degradation studies

The influence of acidic, alkaline and oxidative medium was tested on the sample solutions prepared from SQV primary stock solution (5mg/ml of Saquinavir mesylate in methanol). All the stress degradation studies were performed at an initial drug concentration of 1 mg/ml.

a) Five milliliter of stock solution was transferred to the 25 ml volumetric flask and made up to mark with Milli-Q water (control sample).
b) Five milliliter of stock solution was transferred to the 25 ml volumetric flask and made up to mark with 0.1M HCl solution.
c) Five milliliter of stock solution was transferred to the 25 ml volumetric flask and made up to mark with 0.1M NaOH solution.
d) Five milliliter of stock solution was transferred to the 25 ml volumetric flask and made up to mark with 3% H_2O_2 solution.

All the sample solutions were placed in an ultrasonic bath for 15 min and then stirred with magnetic stirrer for a duration of 3 h at room temperature. Samples were withdrawn at appropriate time intervals, centrifuged for 10 min at 5000 rpm and subjected to HPLC analysis after suitable dilution.

More intensive stress studies were carried out at an accelerated temperature by refluxing the drug in acid/oxidative medium of higher strength and for a longer duration of time.

a) For hydrolysis in water, the solution was refluxed at 80° C for 6 h.
b) Acid hydrolysis was performed in 0.1 and 1M HCl at 80° C for 6 h.
c) Oxidative studies were carried out at 80° C in 3 and 20% H_2O_2 for 6 h.

Degradation was also carried out in solid state by exposing the pure drug to dry heat at 80° C for 24 h in dark.

Photodegration studies

A 25 ml SQV solution at a concentration of 1mg/ml in a clear volumetric flask was exposed to natural sunlight during the daytime (60,000–70,000 lux) for 2 days. Suitable controls were kept in dark.

Humidity studies

The hygroscopicity and impact of moisture on the stability of the SQV was studied at different relative humidities. Humidity chambers of 75% and 97.3% were prepared using sodium chloride and potassium nitrate, respectively [14]. Saturated salt solutions remained in contact with the excess solid salt in a sealed desiccator. The chamber was equilibrated at room temperature for at least 24 hours before use and a relative humidity (RH) clock remained in the chamber during the sample incubation period for RH monitoring. Samples were withdrawn at appropriate time intervals and subjected to HPLC analysis after suitable dilution.

Additionally in order to study the impact of humidity on physical and chemical stability of SQV, differential scanning calorimetric analysis (DSC, Model TA 60, Instruments, Shimadzu Corporation, Kyoto, Japan) was performed for SQV by placing the drug substance into an open sample pan and heating at 10°C/min under a 50 ml/min stream of dry nitrogen. Sample weights of 2 mg to 5 mg were used.

All of the above stability sample solutions were kept for 1 h at laboratory temperature before injection. A minimum of four samples were generated for every stress condition, viz. the blank solution stored under normal conditions, the blank subjected to stress in the same manner as the drug solution, zero time sample containing the drug which is stored under normal conditions and the drug solution subjected to stress treatment. The comparison of these test results provided a real assessment of the changes.

Validation of the method

The strategy applied for the validation of assay method allowed us to confirm the linearity of SQV over a tested concentration range and to assess precision, accuracy and selectivity of the present analytical method. The validation range was selected on the basis of the preliminary experiments to cover the expected SQV concentration in the studies. These concentrations were between 1 and 25 µg/ml.

Specificity and selectivity

The specificity of the method was established through study of resolution factors of the drug peak from the nearest resolving peak, and also among all other peaks. The selectivity was determined by checking peak purity of all the peaks, including those of degradation products, using a PDA detector.

Linearity and range

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentration of the analyte in the sample. The linearity of the detector response for the test compounds was evaluated by injecting a total of seven calibration standard solutions of various concentrations (1, 2.5, 5, 10, 15, 20 and 25 µg/ml) covering the working range of the assay. The calibration curves were constructed by plotting peak area of SQV against the corresponding concentration. The linearity of

the calibration curve was tested and evaluated using both linear and polynomial regression model of calibration curve. Coefficient of calibration equation and the correlation coefficient were expressed.

Detection and quantification limits (Sensitivity)

LOD for the determination of SQV in the proposed method was established by signal-to noise ratio (S/N ratio) obtained by injecting samples with decreasing concentrations of SQV. Ratio of signal size to that of noise was calculated by the equation 2H/h, where H is the height of the peak corresponding to component concerned in the chromatogram obtained with the prescribed reference solution and h is the range of the background noise in a chromatogram obtained after the injection of a blank. Analyte concentration that produced S/N ratio greater than 3 was accepted as LOD.

LOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under stated experimental conditions. The limit of quantification (LOQ) was identified based on the criteria: a) the lowest concentration of the analyte that produced S/N ratio of greater than 10 and b) the analyte response that can be determined with sufficient precision i.e. precision ≤ 3 % of its nominal value [15].

Accuracy and precision

Accuracy was evaluated by fortifying a mixture of degraded solutions with the three known concentrations of the drug. The recovery of the added drug was determined. Six replicates of three different concentrations (4, 12 and 22 μg/ml), were injected on the same day and the values of relative standard deviation (R.S.D.) were calculated to determine intra-day precision. These studies were also repeated on different days to determine the inter-day precision. The intermediate precision of the method was determined by repeating the experiment on two different columns.

Results and discussion

HPLC method development

An isocratic method was found necessary to optimize the separation of major degradation products formed under various stress conditions. Different mobile phases comprising several combinations of phosphate buffer, ammonium acetate buffer, methanol, tetrahydrofuran and acetonitrile with different stationary phases were tested to provide sufficient resolution between analyte and the degradant peaks. The best results were obtained when acetonitrile was used as an organic component of the mobile phase. It is important to add buffer in the mobile phase to control the ionization of both, the analyte and the stationary phase, since the ionic state of both affects the acid–base equilibrium between analyte and stationary phase. The buffer molarity was changed and the choice of the optimal buffer strength was based on the theoretical plate number. Buffer strength of 0.025 M gave the highest plate number while higher the concentration resulted in a decrease in the plate number. Use of triethylamine, a peak modifier, helped in reducing the peak tailing by the suppression of silanols. The presence of triethylamine in the mobile phase provided tighter and well defined peaks.

Manipulation of mobile phase pH is a technique that works well for ionizable compounds, because the retention characteristics of ionizable compounds are a function of pH of mobile phase. SQV is a weak base and has pKa values of 1.1 and 7.1 [16]

corresponding to the quinoline and the octa –hydroiso-quinoline nitrogens (Fig. 1), respectively. Its ionization in solution is highly dependent on pH and percentage of ionization at a given pH can be calculated by the equation [17]:

$$\% \text{ SQV ionization} = 100 / [1 + \text{antilog } (pH - pK_a)]$$

For example, % ionization for SQV at pH 7.0 can be calculated as 100/ [1 + antilog (7.0−7.1)] = 100/ [1 + antilog (−0.1)] = 100/[1 + 0.79433] = 55.73%. Degree of ionization (% ionization) of SQV at different mobile phase pH is presented in Table 1.For such weakly basic samples, silanol interactions can lead to a poor band shape and reduced column efficiency. Hence, the mobile phase pH ~ 3.0 was preferred for initial developmental trials.

As can be seen in Fig. 2, at higher mobile phase pH (7.0), decreased retention and resolution were observed. This may be due to very small differences in the degree of ionization of SQV (55.73 %) and that of its degradation products obtained under different stress conditions. Further increase in mobile phase pH (7.5) resulted in reduced retention and overlapping of the peaks. Therefore, mobile phase pH was optimized in order to obtain appropriate retention in considerable run time. This was achieved at a mobile phase pH 3.1 that provided optimum selectivity with fair resolution and appropriate retention. The compounds at this pH showed adequate retention and resolution with acceptable peak shapes.

The proportion of acetonitrile and phosphate buffer was varied isocratically to obtain the best separation with $0.5 \leq k \leq 20$. Best results were obtained when the mobile phase composition was 60 % buffer and 40% acetonitrile. In the optimized conditions for the mobile phase, the resolution of all peaks was to the baseline and all the compounds were eluted in about 23.0 min. The good chromatographic characteristics obtained under established conditions are shown in Table 2, 3, 4. Both, system pressure and column performance, remained stable after analysis of a great number of validation samples. Degraded test solutions were examined under the same chromatographic conditions of analysis using PDA detector. Purity of analyte peaks was calculated and found to be close to 100%.

The drug substance dissolved in mobile phase was found to be very stable; after 48 hours of standing, solutions displayed no measurable loss of assay value nor developed any degradation peaks measured at any wavelength using a diode array detector.

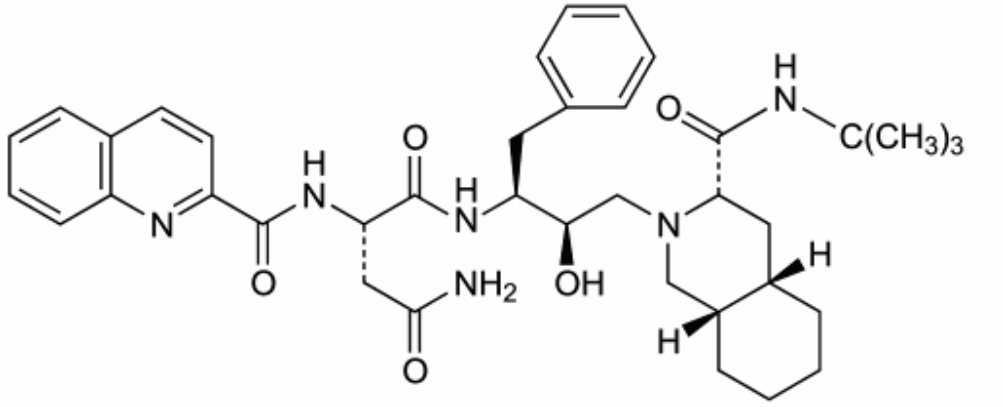


Fig. 1. The chemical structure of saquinavir.

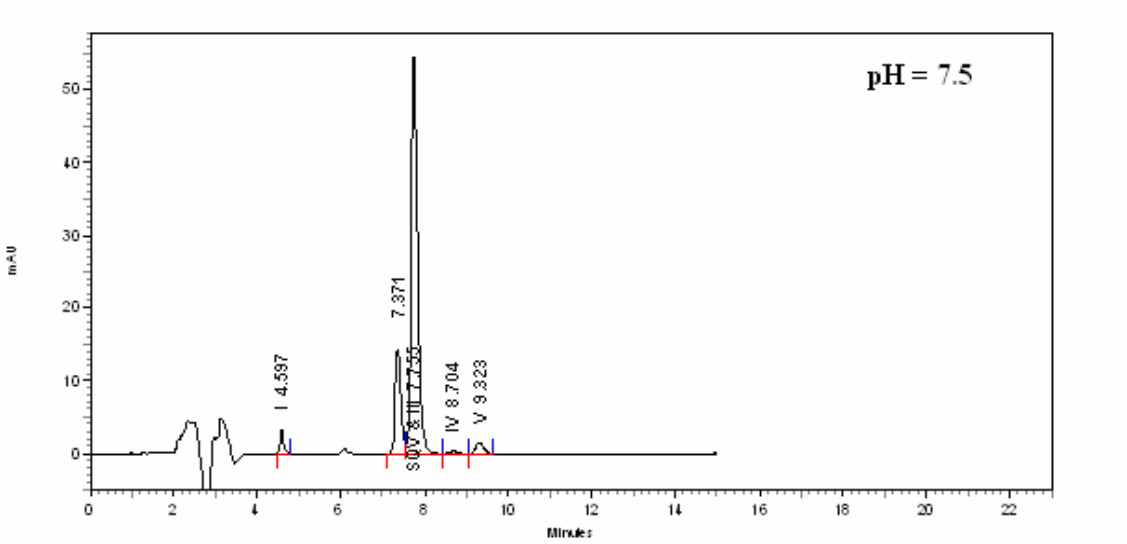


Fig. 2a

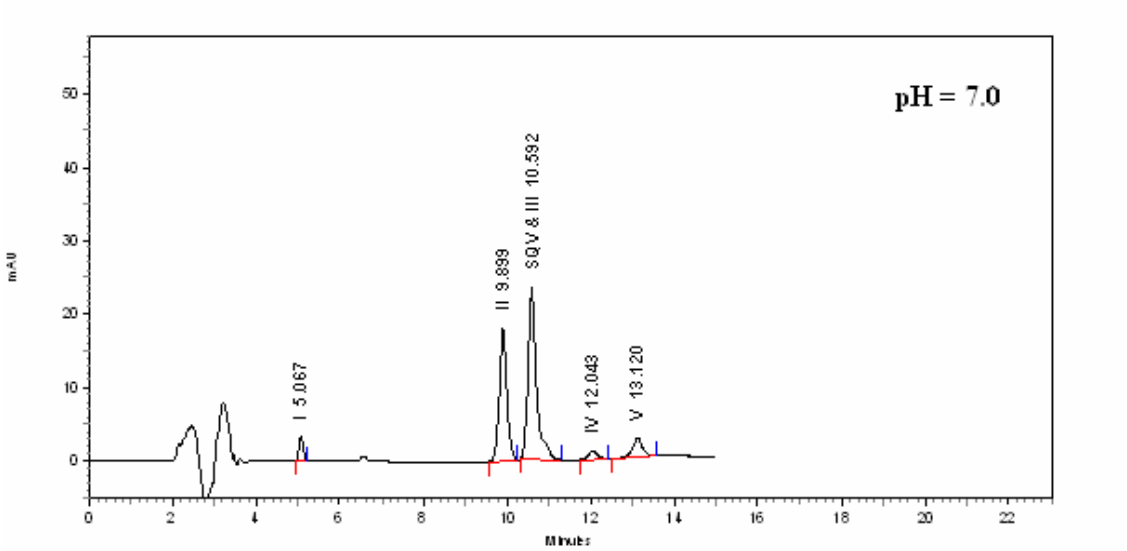
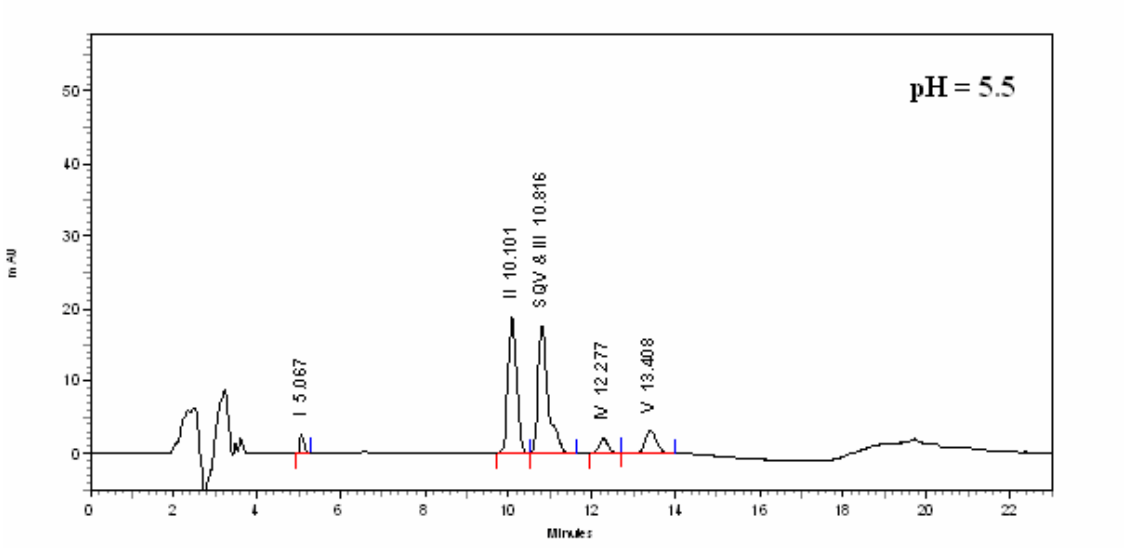

pH = 7.0
mAU
I 5.067
II 9.899
SQV & III 10.592
IV 12.043
V 13.120
Minutes

Fig. 2b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mAU
I 5.067
II 10.101
SQV & III 10.816
IV 12.277
V 13.408
Minutes

Fig. 2c

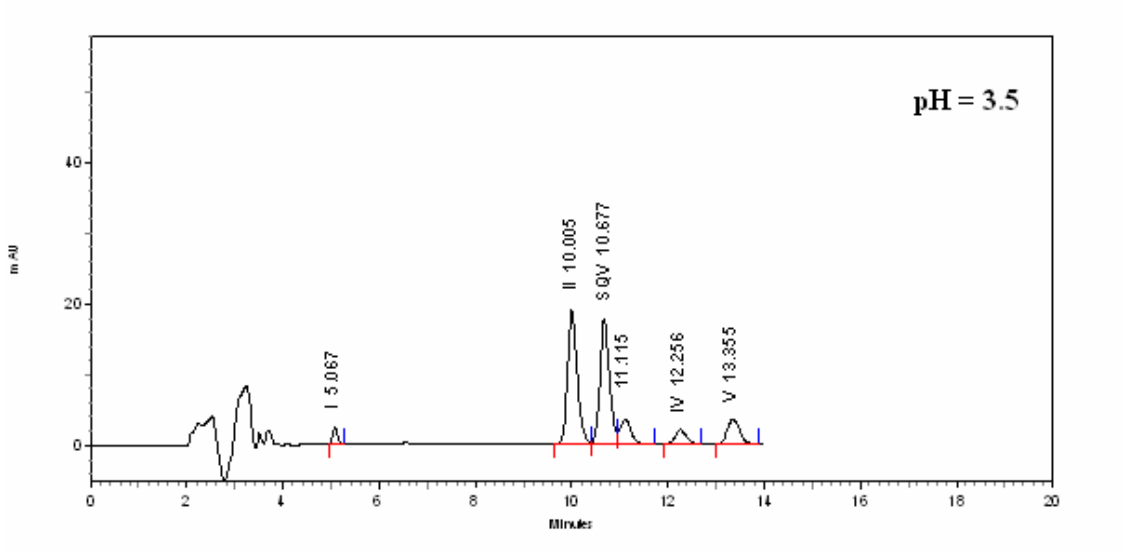

Fig. 2d

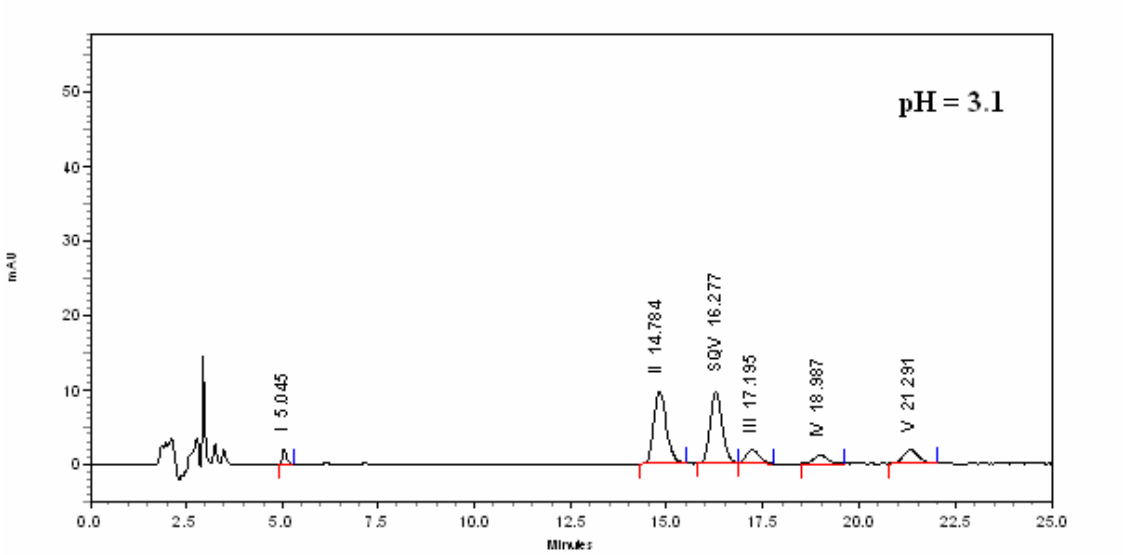

Fig. 2e

Fig. 2.
Representative chromatograms of test solution at different mobile phase pH showing their retention behavior. At Higher mobile phase pH (Fig. 2a, 2b, 2c) decreased retention and resolution was observed along with the overlapping of peaks. Appropriate retention in considerable run time was achieved at mobile phase pH 3.1 (Fig. 2e) that provided optimum selectivity with fair resolution and considerable retention.

Validation of the developed stability-indicating method

Specificity and selectivity

The results of the stress studies indicated the specificity of the developed method. After the exposure of SQV solution to stress conditions, an assay of SQV was performed on the resultant solutions.

As shown in Fig. 3, the method had sufficient specificity and selectivity as the drug and degradation products were well separated from each other, with the resolution factor of >2 in all cases. Online peak purity was determined using PDA analysis, the principle of which is the comparison of the spectra of the analyte peak, taken upslope, at the apex and on the downslope. If these spectra do not match then the peak is non-homogeneous. Data of peak purity index values under different stress condition are listed in Table 2, 3, 4.

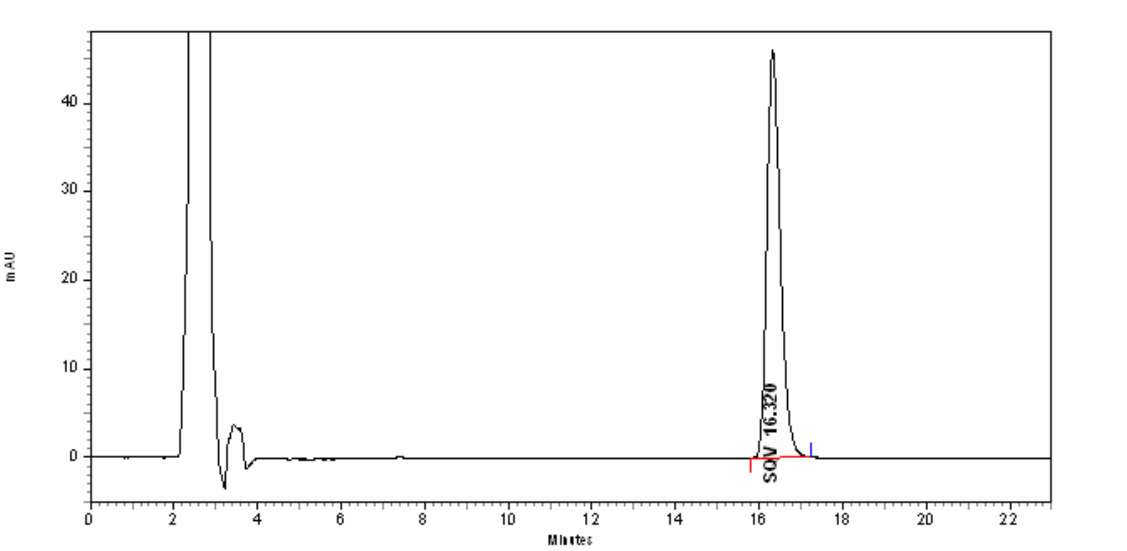

Fig. 3.
Typical chromatogram showing the separation of SQV.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

Linear calibration plot for the developed method was obtained over the calibration range of 1 µg/ml to 25 µg/ml, with coefficient of correlation (r^2 value) ≥ 0.998. Further evaluation was conducted to check the model's adequacy using both linear and polynomial regression model of calibration curve. Though the correlation coefficient using linear regression model of calibration curve was acceptable (r^2 = 0.99872), there was a bias error at estimating the value of lowest two levels from calibration curve (Table 5). Cubic regression with correlation coefficient r^2 = 0.99997 gave closer approximation of the calibration curve. Results are given in Table 6 .The LOD for SQV was found to be 0.5 µg/ ml and LOQ as 1 µg/ml.

Accuracy and precision

Table 7 lists the relative standard deviation (R.S.D.) data obtained on analysis of the samples on the same day (n = 6) and on consecutive days (n = 6). As evident, the R.S.D. values were <0.87% and <0.88% for intra- and inter-day studies, respectively, demonstrating that the method was sufficiently precise. Even intermediate precision was established for the method as almost similar resolution was observed on repeating the

experiment on two different batch lots of C_{18} monomeric columns. The method appeared to give similar results between columns of different production lots (Table 8). The recovery of SQV obtained from the difference between peak areas of unfortified samples and fortified samples ranged from 97.68 to 100.92% (Table 9).

Table 1: % Ionization of SQV at different mobile phase pH.

Mobile Phase pH	*pH - pK_a (pk_a= 7.1)*	*% Ionization*
3.1	-4.0	99.99
5.0	-2.1	99.21
7.0	-0.1	55.73
7.5	0.4	28.47

Table 2: Chromatographic separation characteristics of SQV and the degradation products formed under alkaline degradation*

Analyte	**Retention time (min)**	**Resolution Factor (USP)**	**Width at 50 % peak height (min)**	**Plate number (USP)**	**Asymmetry 10% peak height**	**Peak Purity index**
I	4.789	0.00	0.10	12705	1.26	1.0000000
II	14.560	24.63	0.35	9587	1.26	0.9999910
SQV	16.075	2.61	0.32	13980	1.12	0.9999910
III	17.013	1.63	0.35	13089	1.02	0.9999564
IV	18.763	2.86	0.37	14246	1.00	0.9998725
V	21.056	3.42	0.42	13923	1.17	0.9998854

* Alkaline degradation was performed in 0.1 M NaOH at room temperature.

Table 3: Chromatographic separation characteristics of SQV and the degradation products formed under oxidative condition*

Analyte	**Retention time (min)**	**Resolution Factor (USP)**	**Width at 50 % peak height (min)**	**Plate number (USP)**	**Asymmetry 10% peak height**	**Peak Purity index**
I	8.416	0.00	0.18	12110	1.10	1.0000000
SQV	16.363	17.81	0.33	13621	1.22	0.9999967
II	17.515	1.17	0.36	13113	1.17	0.9998672
III	20.885	1.22	0.49	10064	1.37	0.9999654

* Oxidation was carried out in 30 % of H_2O_2 at 80° C for 6 h.

Table 4: Chromatographic separation characteristics of SQV and the degradation products formed under forced acid degradation*

Analyte	Retention time (min)	Resolution Factor (USP)	Width at 50 % peak height (min)	Plate number (USP)	Asymmetry 10% peak height	Peak Purity index
I	3.221	0.00	0.08	8980	1.15	0.9999910
II	6.315	15.43	0.14	11272	1.07	1.0000000
III	14.994	21.82	0.31	12960	1.13	0.9998932
SQV	16.072	2.14	0.32	13975	1.20	0.9998640
IV	17.323	2.28	0.35	13571	1.03	0.9999654
V	19.339	2.00	0.50	8288	1.51	1.0000000
VI	27.781	2.27	0.44	22085	1.14	0.9999998

* Acid degradation was performed in 0.1 M and 1M HCl at 80° C for 6 h.

Table 5: Expression of the % bias of actual (spiked) concentration and concentration calculated from calibration curve. % bias = (Cactual-Ccalculated)/Cactual (%).

Conc.µg/ml	1	2.5	5	10	15	20	25
Linear Regression model	-16.22	-6.22	-1.92	2.82	3.39	-2.48	-1.51
Cubic Regression model	0.49	0.18	-0.52	0.28	-0.08	-0.89	0.00

Table 6: Calibration model, regression equation coefficients and correlation of coefficient

	Calibration equation	r^2
Linear model	y = 129605 x – 29080.6	0.9987
Cubic model	$y = 53.8483\ x^3 – 1457.02\ x^2 +133570\ x - 9973.27$	0.9999

Table 7: Reproducibility and precision data evaluated through intra-day and inter-day studies (n=6)

Actual Concentration (µg/ml)	Intra day precision		Inter day precision	
	Concentration measured ± S.D. (µg/ml)	R.S.D. (%)	Concentration measured ± S.D. (µg/ml)	R.S.D. (%)
4	4.1132 ± 0.01	0.24	4.0166 ± 0.02	0.58
12	11.8812 ± 0.10	0.87	11.9192 ± 0.11	0.88
22	21.4889 ± 0.06	0.26	21.5489 ± 0.15	0.71

Table 8: Intermediate precision studies.

Column Used	Retention time of SQV
Grace Vydac-C_{18} (1)	~ 16.520
Grace Vydac-C_{18} (2)	~ 16.070

Table 9: Recovery studies

Actual Concentration (µg/ml)	Concentration measured ± S.D. (µg/ml)	Mean % Recovery
5	5.0461 ± 0.09	100.92
10	9.7677 ± 0.03	97.68
20	19.6452 ± 0.20	98.23

Results of forced degradation studies

The results of the stress studies indicated the specificity of the method that has been developed. HPLC studies on the SQV under different stress conditions suggested the following degradation behavior.

Acidic condition

The drug concentration rapidly decreased with time on heating at 80° C in 1M HCl, forming degradation products at RRT 0.20,0.39,0.93,1.08,1.20 and 1.31. The rate of hydrolysis in acid was slower at room temperature as compared to that of hydrolysis at elevated temperature. More intensive stress studies that were carried out at accelerated temperatures by refluxing the drug in acid of higher strength (1 M) and longer duration of

time (6 h) showed sufficient degradation within 1 h. The peaks of the resultant degradants were well resolved from the SQV peak as shown in Fig. 4. In comparison the stress samples with blank samples, it was possible to determine peak areas and volume of impurities as % from all peak area. Peak purity test results confirmed that the SQV peak is homogeneous and pure in all the stress samples, analyzed under PDA (Table 4).

Degradation in alkali

SQV was found to be highly labile to alkaline condition in 0.1M NaOH at room temperature and most of the drug decomposed within 3 h with only a small peak being visible in the chromatogram as shown in Fig. 5. The stress samples so obtained were subjected to preliminary analyses to study the number and type of degradation products formed under alkaline condition. The major degradation products appeared at RRT 0.30, 0.91, 1.06, 1.17 and 1.31 (Table 2).

The results were critically compared with the blank solutions injected in a similar manner. It was observed that the fall in drug peak was quantitatively followed by a corresponding rise in the degradation product peaks.

European Medicine Evaluation Agency (EMEA) reports indicated that the oxidative decomposition of SQV mediated by molecular oxygen is responsible for the drug instability [18]. It is difficult to reproduce these oxidative reactions and to establish the mechanism for them. Consequently, many of these reports dealing with oxidation-reduction reactions are qualitative in nature rather than quantitative.

Many oxidation reactions are catalyzed by the hydrogen and hydroxyl ions. This can partly ascribed to the fact that the redox potential for many of the reactions depends upon the pH. The oxidation potential may be expressed by the following simplified version of the Nernst equation:

$$E = E_0 + \frac{0.0592}{n} \log \frac{a_{H+} \cdot a_{\text{Oxidised form}}}{a_{\text{Reduced form}}}$$

Where E_0 is the so called standard potential, E is the actual potential. While a_{H+} is the activity of H^+ ions and n is the number of electrons taking place in the change from the reduced-form to oxidized -form. It can be seen from the equation that an increase in the concentration of hydrogen ions causes increase in the value of E. In other words the reduced form of the system is less readily oxidized when pH is low. At pH above 4, the mechanism of degradation may involve an oxidative process triggered by the atmospheric oxygen on the unprotonated nitrogens. At lower pH values, these nitrogens are protonated, thus disfavouring oxidation [11].

SQV has low solubility and a base pK_a a little over 7 (pK_{a2} = 7.1) corresponding to the octa-hydroiso-quinoline nitrogen that (in our experiment) resulted in its precipitation at higher pH 7.0 and 9.0.

Neutral (water) condition

No major degradation product was observed after refluxing the drug in water for 6 h, indicating that hydrolysis had no effect on the degradation of the drug. Minor degradation products at RRT 0.26, 0.29 and 0.34 were formed (Fig. 6). Corresponding rate of hydrolysis degradation in water was much lower.

Oxidative conditions

No considerable degradation was observed in 3% Hydrogen peroxide at room temperature. Hence more intensive stress studies were carried out at accelerated temperatures by refluxing the drug in oxidative medium of higher strength and for longer duration of time. SQV degraded in the presence of 3% and 20% hydrogen peroxide when refluxed at 80° C for 6 h and the resultant drug peak was resolved from that of the degradation products as seen in Fig. 7. The major degradation products (Table 3) appeared at RRT 0.51, 1.07 and 1.28. The rate of degradation in oxidative condition was found to be much lower at room temperature as compared to that at elevated temperature.

Photolytic condition

SQV, in solution, degraded when exposed to natural sunlight during the daytime (60,000–70,000 lux) for 2 days to a major degradant at RRT 0.51, along with the two minor degradation products at RRT 0.41 and 0.46 (Fig. 8). The colour of the test solution was found to be changed to faint yellow with no appreciable change in the sample volume. The sample exposed to sunlight showed 50% degradation as compared to the control stored in dark conditions.

Humidity conditions

The drug was stable in equilibrium humidity chambers (75% and 97.3%) at controlled room temperature for 1 month. No considerable physical changes were observed during the study period and the exposed samples neither absorbed moisture (Hygroscopic) nor turned to liquid state (Deliquescent). A sharp peak with temperature around 354.78°C was observed in the DSC thermograms of both, the exposed and standard SQV samples. Also, similar values for onset temperature, melting point and melting enthalpy were observed for both the samples as shown in Fig. 9. Moreover, no additional peaks were observed in the test thermograms.

Solid-state study

The solid-state studies showed that SQV was stable to the effect of temperature. When the drug powder was exposed to dry heat at 80° C for 24 h, no decomposition of the drug was seen.

Conclusion

This study presents a simple and validated stability-indicating HPLC method for the estimation of SQV in the presence of degradation products. All the degradation products formed during forced decomposition studies were well separated from the analyte peak. This demonstrates that the developed method was specific and stability-indicating.

SQV was found to be highly labile to alkaline hydrolysis in 0.1M NaOH at room temperature and most of the drug decomposed within 3 h. pH was the major determinant of SQV stability. SQV degradation increased in the alkaline conditions possibly because of base-catalyzed oxidation. The method proved to be simple, accurate, precise, specific and selective. Hence, it is recommended for the analysis of the drug and degradation products in stability samples by the industry.

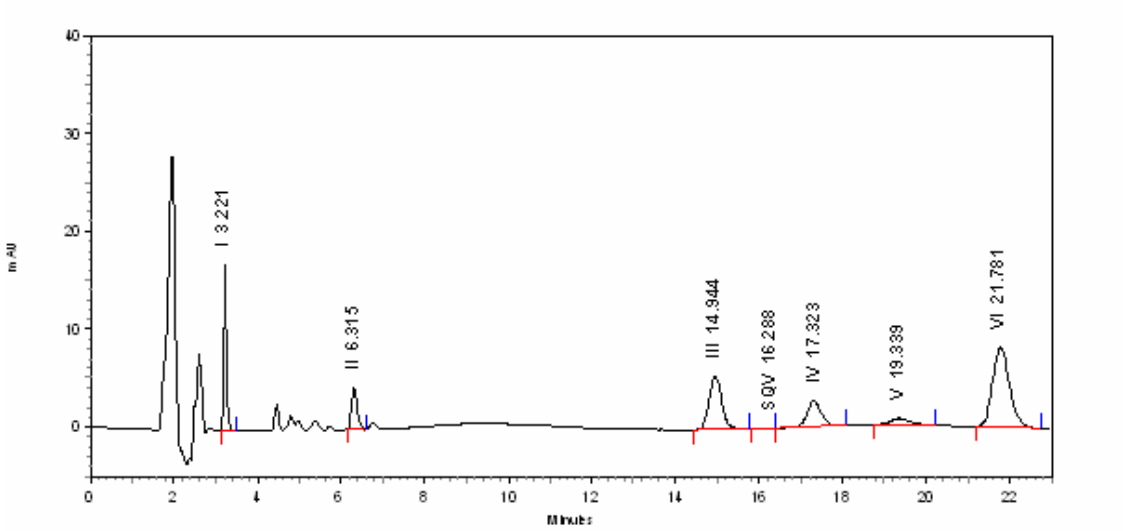

Fig. 4. Representative chromatogram obtained following the exposure of drug solution to 0.1 M HCl at 80° C. Most of the drug decomposed within 2 h with only a small peak being visible in the chromatogram.

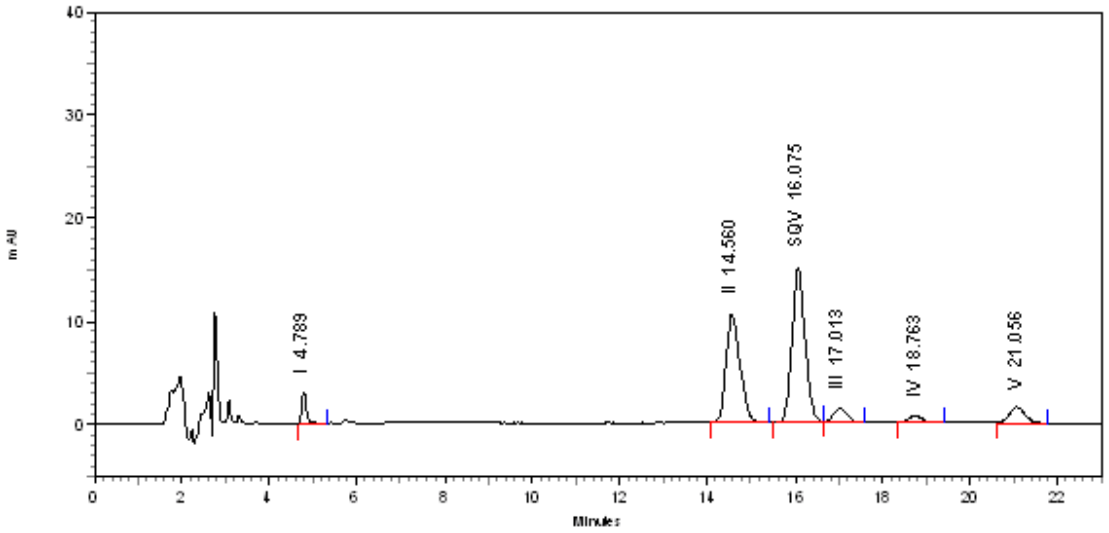

Fig. 5. Resultant chromatogram obtained following the exposure of drug solution to 0.1 M NaOH at room temperature, showing the separation of SQV and degradation products.

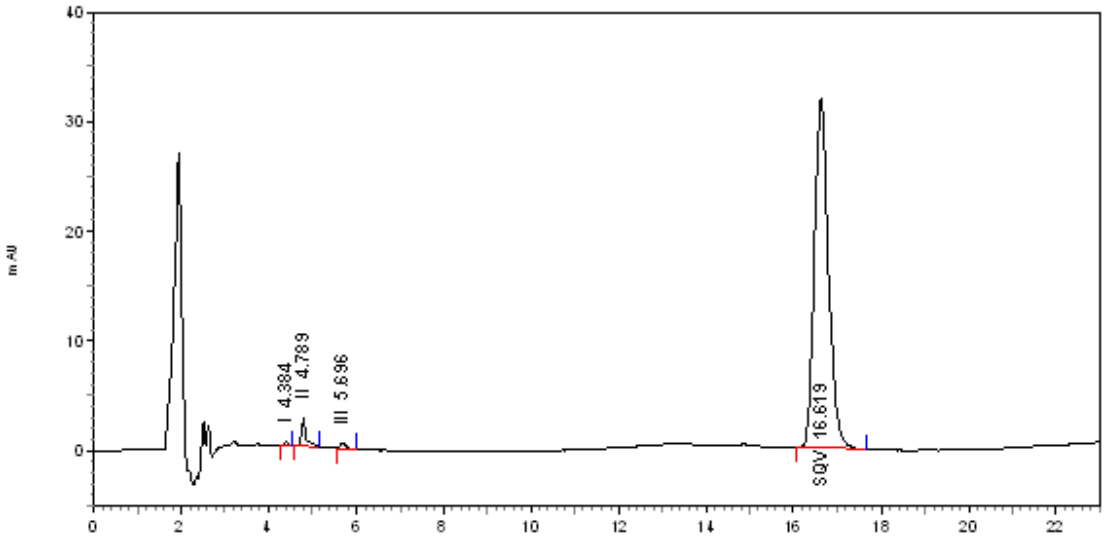

Fig. 6. Resultant chromatogram obtained following the refluxing of the drug in neutral (water) condition at 80° C, showing the separation of SQV and degradation products.

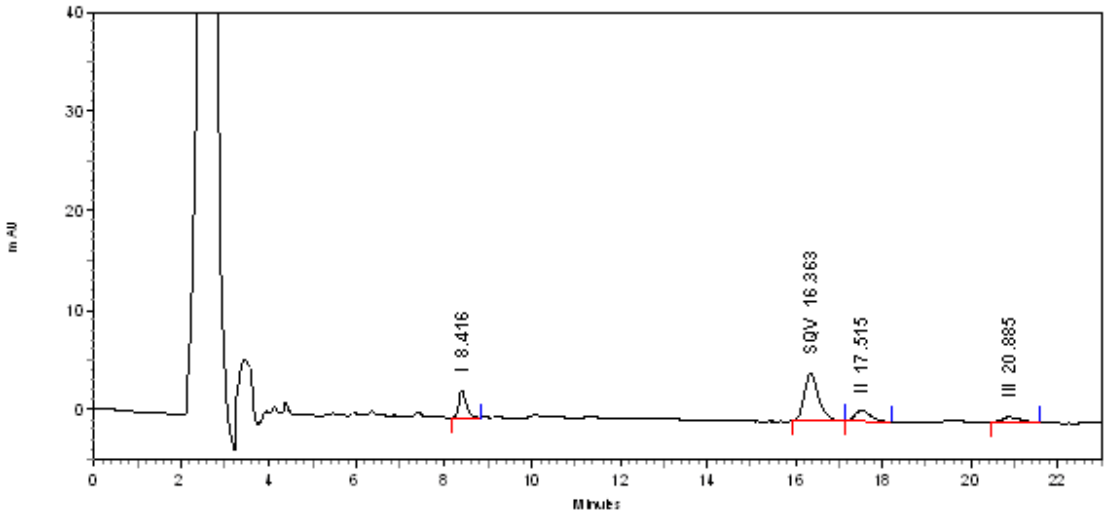

Fig. 7. Resultant chromatogram obtained following the exposure of drug solution to 20 % hydrogen peroxide solution at 80° C showing the separation of SQV and degradation products.

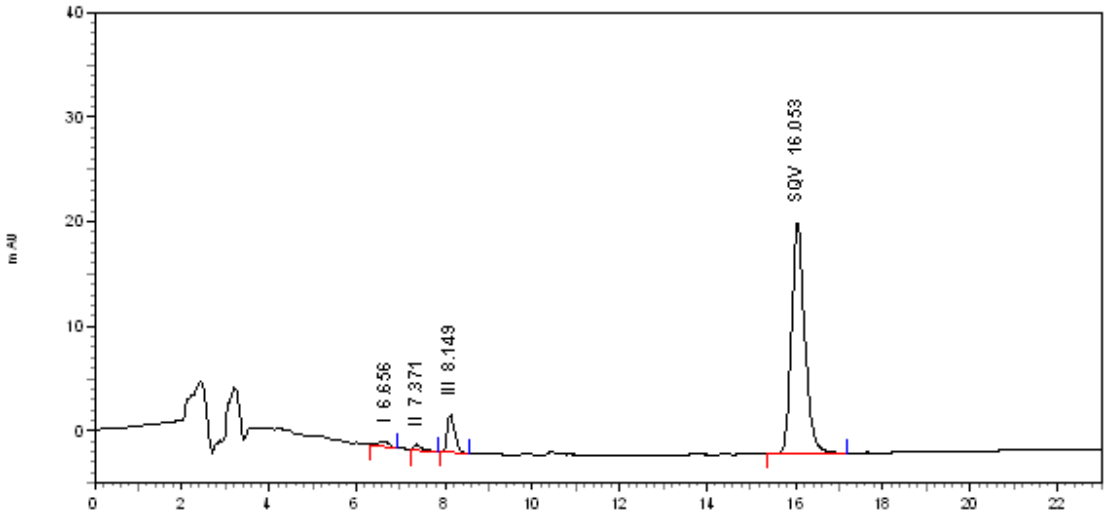

Fig. 8. Representative chromatogram obtained following the exposure of drug solution to natural sunlight during the daytime for 2 days showing the separation of SQV and degradation products.

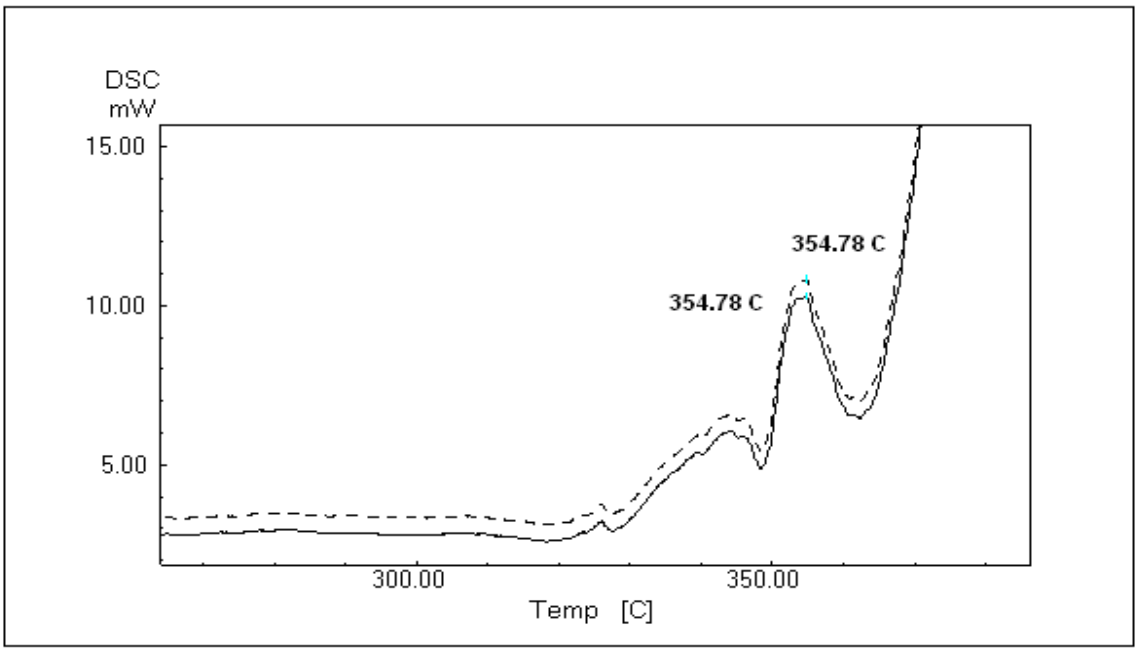

Fig. 9. DSC thermogram of SQV standard (solid line) with an onset temperature, melting point, and melting enthalpy at 349.38° C, 354.78° C, and 49.45 J/g respectively. DSC thermogram of SQV after 1 month storage at 97.3 % relative humidity (---) with an onset temperature, melting point, and melting enthalpy of 349.80° C, 354.78° C, and 47.02 J/g respectively.

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