

Validated Analytical Method for the Determination of Sorafenib in Dosage form and Human plasma in presence of its Degradation Products

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Abstract : Herein, the potency, bioavailability and purity of sorafenib can be easily investigated in the presence of different degradation products through the present work. The bioanalysis of sorafenib in tablets and human plasma was achieved by a simple chromatographic procedure. The separation was conducted at room temperature using a stainless steel Hibar C₁₈ (150 X 4.6 mm i.d). The analytes were detected with UV detector at 255 nm. A simple mobile phase of acetonitrile / phthalate Buffer / methanol (75: 24.5: 0.5, v/v) (pH 4) was eluted at a flow rate of 1.5 mL/ min. A rectilinear calibration curve was obtained over concentration range of 0.05 – 2.0 µg /mL, with a detection and quantification limits (LOD, LOQ) of 0.006 and 0.017 µg /mL respectively.

Key words : Bioanalysis, HPLC, Stability indicating , Kinetics, tablets, real plasma.

1. Introduction

Sorafenib (Fig 1) is 4-[4-[[4-chloro-3-(trifluoromethyl)phenyl] carbamoyl amino] phenoxy]-N-methylpyridine-2-carboxamide¹. Sorafenib is tyrosine protein kinases inhibitor. It is used for the treatment of hepatocellular, renal cell and thyroid carcinoma². There is no official method for sorafenib determination. It has been analysed by different HPLC methods using different mobile phases like acetonitrile / water (82.5 : 17.5, v/v)³, 20 mM potassium dihydrogen phosphate / acetonitrile (35:65, v/v)⁴ . 20mM ammonium acetate / acetonitrile / methanol (2.5:6.7:8.3%)¹ , acetonitrile / 10mM ammonium formate (54:46, v/v)⁵, gradient elution using formic acid in water / acetonitrile⁶, acetonitrile / 20mM ammonium acetate (40:60 v/v)⁷, acetonitrile / 0.1% formic acid in water (65/35 (v/v))⁸ , acetonitrile/10 mM ammonium acetate (65:35, v/v) containing 0.1% formic acid⁹. Our proposed method is more sensitive than the others as it can measure down to 0.05 µg/mL

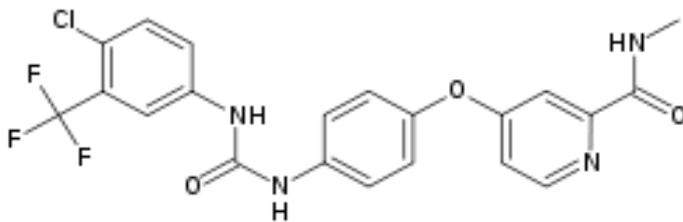


Fig 1: Structure formula of Sorafenib

Experimental

Reagents and Materials

All chemicals used were of Analytical Reagents grade, and the solvents were of HPLC grade. Acetonitrile 99.9% and methanol 99.9% were purchased from Sigma Aldrich, Germany. Potassium hydrogen phthalate was purchased from Fisher Chemical, USA. Hydrochloric acid and sodium hydroxide were purchased from Al-Nasr chemical company, Egypt. Sorafenib was purchased from Sigma Aldrich, Germany. The purity of the drug was established by applying the comparison method [7] and was found to be 100.10 ± 0.56 . Nexavar[®] tablets each labeled to contain 200 mg of sorafenib, Batch No.B.XFFN91. Produced by Bayer pharmaceuticals were obtained from the local pharmacy.

Instrument

The analysis was performed using Shimadzu[™] LC-20A Series Chromatograph equipped with a Rheodyne injector valve with a 20 μ L loop and a SPD-20A UV detector operated at 255 nm. LC Workstation (Nishinokyo-Kuwabaracho, Nakagyo-Ku, Kyoto 604-8511, Japan).

Chromatographic Condition

A stainless steel Hibar C₁₈ (150 X 4.6 mm i.d) was used. The mobile phase consisted of acetonitrile / phthalate Buffer / methanol (75%: 24.5%: 0.5%). The pH was adjusted by using HCl or NaOH to pH 4.0. The mobile phase was then sonicated for 30 min then filtered through a 0.45 μ m membrane filter (Millipore, Ireland). The first deviation of the base line was considered as the column hold up value.

Standard solution

The standard solution of sorafenib 400 μ g/mL was prepared in methanol. The working solutions were prepared by diluting aliquots of the standard solution with the mobile phase to obtain concentration over the range (0.05-2.0 μ g/mL). The stock solution was found to be stable for at least one week when kept in the refrigerator.

Construction of Calibration Graph

Twenty microliters aliquots of the working solutions were injected (triplicate) and eluted with the proposed mobile phase at flow rate 1.5 mL/min. The eluted peak was UV detected at 255 nm. The calibration graph was constructed by plotting the obtained peak area versus concentration of the drug. The regression equation was also derived.

Acidic and Alkaline Degradation

The acidic and alkaline degradations were obtained by addition of five milliliters of both one molar Hydrochloric acid (1 M HCl) and two molar Sodium hydroxide (2 M NaOH) in ten milliliters volumetric flasks containing aliquots of sorafenib standard solution respectively. The resultant mixtures were heated at different temperature 60,70,80,90,100 $^{\circ}$ C using a water bath with thermostatic control for different time intervals 5,15,25,35,45 minutes then allowed to cool. The cooled mixtures were neutralized using five milliliters 1 M NaOH or 2 M HCl respectively, completed to the mark with methanol. Then aliquots of the obtained solutions were transferred to ten milliliters volumetric flasks and further diluted with the mobile phase to the mark to

obtain final concentrations (0.05-2.0 µg/mL) and treated as described under “Construction of calibration graph”. Complete degradation was tested by the disappearance of HPLC peak of the parent drug.

Photolytic Degradation

The photolytic degradation was performed over different intervals 30,40,50,60,70 h by exposing aliquots of sorafenib standard solution contained in ten milliliters volumetric flasks to UV-Lamp at a wavelength of 254 nm at a distance of 15 cm placed in a wooden cabinet. The solutions were diluted with methanol to the mark. Then aliquots of the obtained solutions were transferred to ten milliliters volumetric flasks and further diluted with the mobile phase to the mark to obtain final concentrations (0.05-2.0 µg/mL). Then the solutions were analysed using the proposed chromatographic method.

Oxidative Degradation

The oxidative degradation was performed by adding five milliliters of five percent Hydrogen peroxide (5 % H₂O₂) to aliquots of sorafenib standard solution contained in ten milliliters volumetric flasks. The obtained mixtures were refluxed in round bottom flasks at different temperature settings 60,70,80,90,100 °C using a water bath with thermostatic control for different time intervals 5,10,15,20,25 min, cool, completed to the mark with methanol. Then aliquots of the obtained solutions were transferred to ten milliliters volumetric flasks and further diluted with the mobile phase to the mark to obtain final concentrations (0.05-2.0 µg/mL). Then the solutions were analysed using the proposed chromatographic method.

Analysis of Sorafenib Tablets

Ten Nexavar tablets were accurately weighed and the average weight was calculated. The tablets were grinded and mixed well. Aliquot quantity of the powder equivalent to the average weight of the tablets (200 mg of sorafenib) were transferred to a hundred milliliters volumetric flask. Sorafenib was extracted by addition of eighty milliliters of methanol and the solution was then sonicated for 20 min, filtered into hundred milliliters volumetric flask and completed to the mark with the same solvent. Aliquots of the obtained solution were further diluted with the mobile phase to produce final concentrations (0.05-2.0 µg/mL). Then the solutions were analysed using the proposed chromatographic method.

Analysis of Sorafenib in Spiked Plasma and Urine

Calibration curves in spiked plasma and urine were constructed by adding the working concentrations of the drug to one milliliter aliquots of plasma and urine in a series of centrifuge tubes. One milliliter aliquots of acetonitrile was added. The solutions were centrifuged at 4000 rpm for 20 min at ambient temperature. The resultant supernatants were aspirated and filtered using a microfiter paper. Twenty microliters were injected (triplicate) and eluted with the mobile phase. Then the solutions were analysed using the proposed chromatographic method.

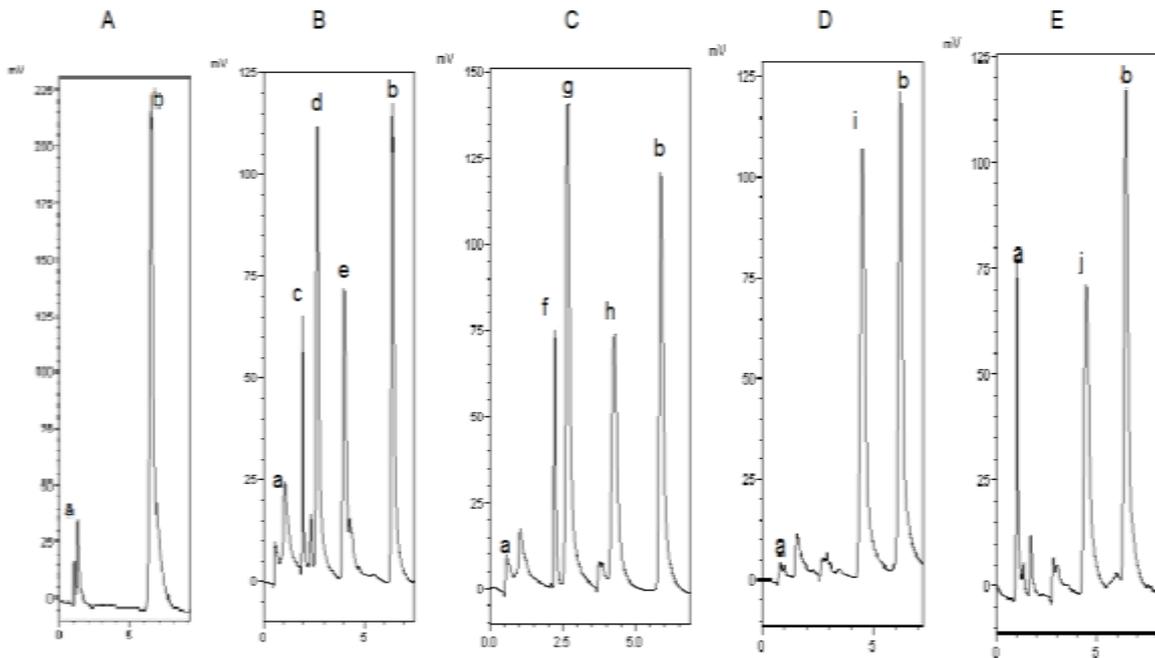
Analysis of Sorafenib in Patient Plasma

A healthy volunteer (male 30 years) was instructed to fast for eight hours, then administered a Nexavar[®] 200 mg tablet in different days, A blank blood samples were collected from the volunteer before each administration. After different time intervals; 0.5,1, 2, 3 and 4 h blood samples were collected into test tubes containing anticoagulant, sodium citrate. The samples were then centrifuged at 2000 rpm for 20 min. The resultant supernatants were aspirated and filtered using a microfiter paper. Twenty microliters were injected (triplicate) and eluted with the mobile phase. Then the solutions were analysed using the proposed chromatographic method.

Results and Discussion

The proposed method allows the separation of sorafenib from its acidic, alkaline, oxidative and photolytic degradation products. The optimum chromatographic condition was using a mobile phase composed of acetonitrile / phthalate buffer / methanol (75%: 24.5%: 0.5%) with a flow rate of 1.5 mL/min and the analytes were detected at 255 nm. The peaks were well separated from each other (Fig.2) . Two peaks of acidic and alkaline degradations were appeared at the same retention times, and one peak for each of the oxidative and photolytic degradation. The retention times were 6.3 min. for sorafenib and 2.7 min., 4.0 min. for both the

acidic and alkaline degradation products, 3.6 min, 5.1 min. for the oxidative and photolytic degradation products respectively.



- A: Sorafenib chromatogram
 B: Acidic degradation at 100 °C
 C: Alkaline degradation at 100 °C
 D: Oxidative degradation at 100 °C
 E: Photolytic degradation at 254 nm.
- a:solvent front
 b:sorafenib peak
 c,d,e:acidic degradation products
 f,g,h:alkaline degradation products
 i:oxidative degradation product
 j:photolytic degradation product

Fig.2: Chromatograms of sorafenib and its degradation products

UV. Detection

The UV absorption spectrums of the studied drug was scanned and the λ_{\max} 255 was selected for detection of peaks (Fig 3).

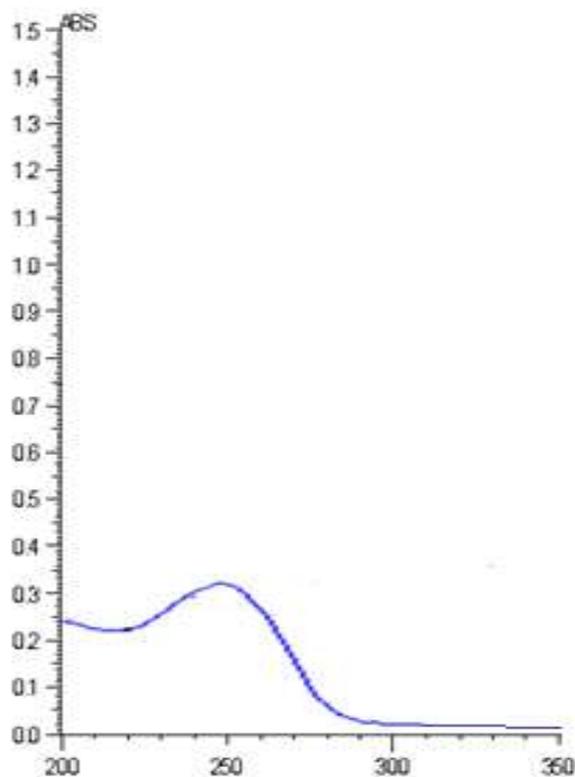


Fig 3: Spectrum of sorafenib 1.0 µg/mL

Experimental Parameters

Different experimental parameters affecting the separation of sorafenib and its different degradation compounds were studied. The studied variables includes mobile phase composition, flow rate. The variables were studied and optimized by changing each variable separately while keeping all others constant. The bases of optimization were obtaining highest number of theoretical plates and good resolution.

Mobile phase

The mobile phase composition was studied and optimized by making several modifications including the pH, the ratio of acetonitrile, methanol and phthalate buffer.

pH

The pH was studied over the pH range 3.0 to 7.0. The pH was changed using increasing volumes of 0.1 M sodium hydroxide or hydrochloric acid. The highest number of theoretical plates was obtained at pH 4 Table.1.

The ratio of acetonitrile

The effect of different ratios of acetonitrile were studied over the range from 50 to 90 % (v/v). The optimum ratio that gave the highest number of theoretical plates was 75 %, v/v .Table 1.

The ratio of methanol

The effect of different ratios of methanol were studied over the range from 0.25 to 1% (v/v). The optimum ratio that gave the highest number of theoretical plates was 0.5 %, (v/v) Table 1. This small ratio of methanol was needed to separate the oxidative degradation product and the drug peaks, also, to resolve the

acidic and alkaline degradation products. It is noticed that increased ratio of methanol increases the retention time of sorafenib.

The ratio of phosphate buffer:

Different ratios of phthalate buffer were studied over the range from 9.5 to 45.5% (v/v). A ratio of 24.5 %, v/v revealed the highest number of theoretical plates Table 1.

Elution flow rate

The influence of flow rate on resolution and column efficiency was studied over the range of 0.75-2.0 ml/min. The optimum resolution was achieved at a flow rate of 1.5 mL/min Table 1.

Table 1:Effect of different experimental parameters on the column efficiency of the proposed method:

Experimental Parameters		K'	N
pH	3.0	8.10	3750
	4.0	8.00	3970
	5.0	8.15	3890
	6.0	8.12	3820
	7.0	8.11	3720
Ratio of acetonitrile	50	10.11	2860
	60	9.20	3560
	75	8.00	3970
	80	5.30	3870
	90	4.50	3775
Ratio of methanol	0.25	6.22	3910
	0.5	8.00	3970
	0.75	11.22	3880
	1.0	13.40	3550
Ratio of phosphate buffer	9.5	4.50	2540
	19.5	5.30	3150
	24.5	8.0	3970
	39.5	9.20	2670
	45.5	10.11	2110
Flow rate	0.75	11.3	2120
	1.0	9.60	2950
	1.5	8.00	3970
	2.0	5.60	3820

K':Capacity factor for Sorafenib

N :The number of theoretical plates for sorafenib

Analytical Validation

The international conference on harmonization (ICH) guidelines were considered for the validation study of the proposed method ¹⁰.

Linearity and range

After optimizing of different conditions, the linearity of the method was investigated and it was found that the peak area and the final concentration of the drug was linear over the range 0.05-2.0 µg/ml for sorafenib. A good correlation coefficient (r) of 0.9997 was obtained and the regression equation was as follows

$$\text{Peak area} = 7.4 + 2410 C \quad r = 0.9997$$

where

C = drug concentration in µg/mL

The regression statistical study provides small values of standard deviations of the slope (s_b) intercept (s_a) and residuals (Sy/x) which indicate low scattering of the points around the calibration. The calculated values were 50.4, 4.2, 18.30 respectively.

Accuracy and precision

The accuracy and precision were investigated as percent relative error (%Er) and percent relative standard deviation (% RSD) respectively¹¹. Small values of percent relative error (%Er) and percent relative standard deviation (% RSD) were obtained which indicate well accuracy and precision. The results were summarized in Table 2.

Table 2: Intra day and inter day precision for the proposed method

Conc.added µg/mL	Conc.found (µg/mL)	% Found	% RSD	% Er
Intraday				
0.05	0.04970	99.40±0.34	0.34	0.20
1.00	0.10020	100.20±0.42	0.42	0.25
2.00	1.99700	99.81±0.40	0.40	0.23
Interday				
0.05	0.05021	100.41±0.35	0.35	0.20
1.00	0.09962	99.62±0.31	0.31	0.17
2.00	2.0030	100.15±0.42	0.42	0.24

Each result is the average of three separation determinations.

Intra- day precision

The repeatability of method was achieved through three replicate analysis of pure sample of different concentrations (0.05-2.0 µg /mL) on three successive times. The results are listed in table 2. The calculated %RSD values were small which indicate well repeatability.

Inter-day precision

The reproducibility of the method was performed through three replicate analysis of pure sample on three successive days. The calculated %RSD values were small which indicate well reproducibility. The results are listed in Table 2.

Robustness

Small variations in the chromatographic conditions like pH, mobile phase composition and flow rate were induced to study the robustness. The pH was changed over (3.5-4.5), acetonitrile ratio over (60- 80%), methanol over (0.4-0.6%) and flow rate over (1.2-1.7 mL/min). The study revealed no significant effect on the performance data Table 3

Table 3: Results of method robustness study

Compound	Parameters	Retention time	Theoretical plates	Capacity factor	Tailing factor
sorafenib	60% acetonitrile	7.2	3560	9.20	1.22
	75% acetonitrile	6.3	3970	8.0	1.05
	80% acetonitrile	4.4	3870	5.3	1.11
	0.4% methanol	6.0	2106	7.6	1.12
	0.5% methanol	6.3	3970	8.0	1.05
	0.6% methanol	7.1	2272	9.2	1.12
	pH3.5	6.2	2259	7.9	1.21
	pH 4.0	6.3	3970	8.0	1.05
	pH4.5	6.3	1954	8.0	1.12
	1.2 mL min ⁻¹	7.0	2285	9.0	1.21
	1.5 mL min ⁻¹	6.3	3970	8.0	1.05
	1.7 mL min ⁻¹	5.8	1816	7.3	1.13

System suitability

The system performance was verified through three injections of the drug reference solution and calculating the % RSD of the retention time, tailing factor, number of theoretical plates, and capacity factor. Small values of % RSD were obtained which indicate well system performance. The calculated values were 0.15, 0.24, 0.13, and 0.15 for % RSD of the retention time, tailing factor, number of theoretical plates, and capacity factor respectively.

Limits of detection and quantification

Both limits of detection (LOD) and quantification (LOQ) were calculated according to the formula $LOD = 3.3 S_a/b$ and $LOQ = 10 S_a/b$ ¹¹. where b is the calibration graph slope. The calculated values were 0.006 and 0.017 for (LOD) and (LOQ) respectively .

Specificity

Different forced degradation procedures were performed to provide a stability indicating method of sorafenib. The obtained peaks of sorafenib and the different degradation products were well resolved figure 2. Two different degradation compounds were produced in both alkaline and acidic media with retention times of 2.7, 4.0 min., one oxidative degradation product at 3.6 min and one photolytic degradation product at 5.1 min.

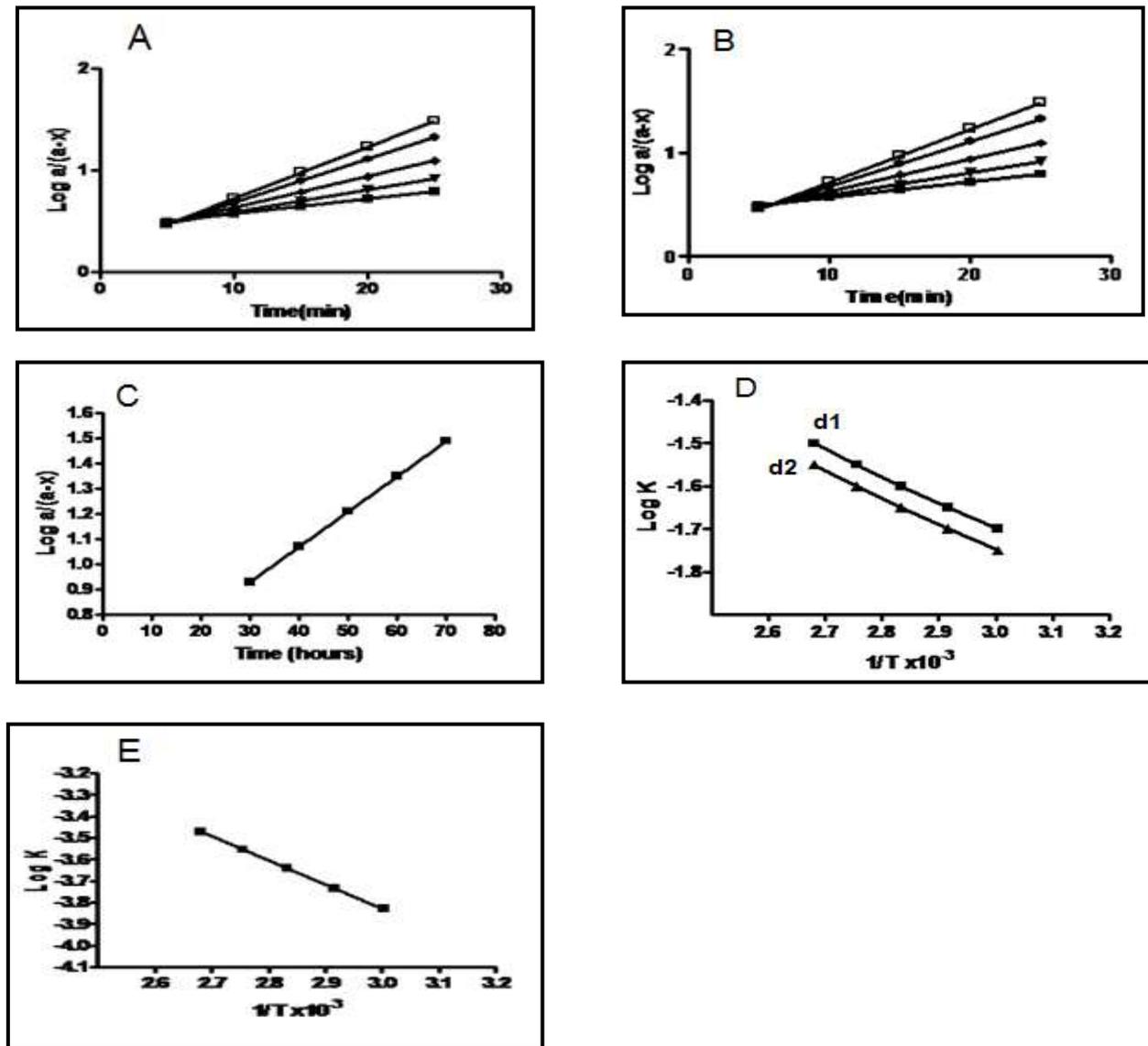


Fig 4: Kinetic parameters of sorafenib degradation reactions: (A),(B),(C) Semilogarithmic plot of the amount remaining of sorafenib 1.0 $\mu\text{g}/\text{mL}$ after acidic degradation,oxidative and photolytic degradation versus different heating times respectively.(D, d1,d2),(E) : Arrhenius plot for the degradation of sorafenib in acid and alkaline media and oxidative degradation respectively.

Degradation Kinetics

The different forced degradations were achieved under different temperatures and over different time intervals. Figure 2 shows gradual decrease of the drug peak area with time and temperature which indicate that the degradations were time and temperature dependent. Different reaction kinetic parameters were calculated such as the reaction order, reaction rate constant, half life time and the activation energy. The degradation reaction were found to be pseudo-first order fig. 4A,B,C. The other kinetic parameters were summarized in Table 4,5. The activation energies were calculated from Arrhenius plot¹² (Fig.4 D,E).

Table 4: Degradation rate constant (K) and half life time ($t_{1/2}$) for idrocilamide

Medium	Temperature(°C)	K(min ⁻¹)	$t_{1/2}$ (min.)	Ea(K.Joule)
1 M HCl	60	0.02070	33.6	15.4
	70	0.0243	30.8	13.2
	80	0.0241	27.7	8.6
	90	0.0293	24.6	7.5
	100	0.0330	21.80	11.20
Mean				
2 M NaOH	60	0.0154	39.90	16.5
	70	0.0165	37.60	14.7
	80	0.011	32.70	10.50
	90	0.0230	29.10	8.80
	100	0.0256	25.40	8.80
Mean				12.60

Table 5: Rate constant (K) and half life time ($t_{1/2}$) for oxidative degradation sorafenib

Medium	Temperature(°C)	K(min ⁻¹)	$t_{1/2}$ (min.)	Ea(K.Joule)
H ₂ O ₂ degradation	60	0.017	45.5	35.70
	70	0.025	30.5	35.10
	80	0.051	22.10	34.90
	90	0.053	15.80	19.20
	100	0.071	13.40	31188.40
Mean				
Photolytic degradation		0.012(h ⁻¹)	60 h.	

Table 6: Application of the proposed method and comparison methods to determination of sorafenib in tablets:

Preparation	Proposed method			Comparison method(7)	
	Amount taken, $\mu\text{g/mL}$	Amount found, $\mu\text{g/mL}$	% Found*	Amount taken, $\mu\text{g/mL}$	% Found
1)Nexavar tablets (200mg sorafenib)	0.05	0.05021	100.43	0.50	99.50
	0.30	0.30060	100.20	5.00	99.83
	1.00	0.99600	99.60	10.00	100.33
	2.00	1.99640	99.82	20.00	100.60
Mean \pm SD					
t-test			100.01 \pm 0.43		100.10 \pm 0.56
F-test		0.51	(2.45)**		
		1.70	(9.277)**		

*The value of tabulated t and F, (at $p = 0.05$) ⁽¹¹⁾

Application to Nexavar Tablets

The present method was successfully utilized for the estimation of sorafenib in tablets. The obtained results were compared with those obtained by the comparison method ^[7] and there is no significance difference recorded Table 6 . The latter involved HPLC determination of sorafenib using of column (15 cm X 5 mm) of ODS using a mobile phase consists of 40% ammonium acetate (20mM) and 60% acetonitrile. The proposed method is fairly sensitive since it can measure down to 0.05 $\mu\text{g/mL}$. The comparison method is linear over the range 0.5-20 $\mu\text{g/mL}$.

Calibration of Sorafenib in Spiked Biological Fluids

The attained high sensitivity of the present method as it can measure down to 0.05 $\mu\text{g/mL}$ allows the analysis of sorafenib in biological fluids. Table 7 shows the results of recovery studies from the corresponding calibration curve for spiked plasma and urine. A corresponding regression equation was derived to calculate the drug level in real plasma.

Table 7: Determination of sorafenib in spiked biological fluids by the proposed method:

Sample	Proposed method		
	Amount added, $\mu\text{g mL}^{-1}$	Amount found, $\mu\text{g mL}^{-1}$	% Recovery
1)Spiked Plasma	0.05	0.0492	98.31
	0.30	0.09933	99.33
	2.00	2.022	101.10
Mean \pm SD			99.61 \pm 1.15
2)Spiked Urine	0.05	0.049	98.00
	0.30	0.0999	99.90
	2.00	2.020	101.00
Mean \pm SD			99.64 \pm 1.24

*Each result is the average of three separation determinations.

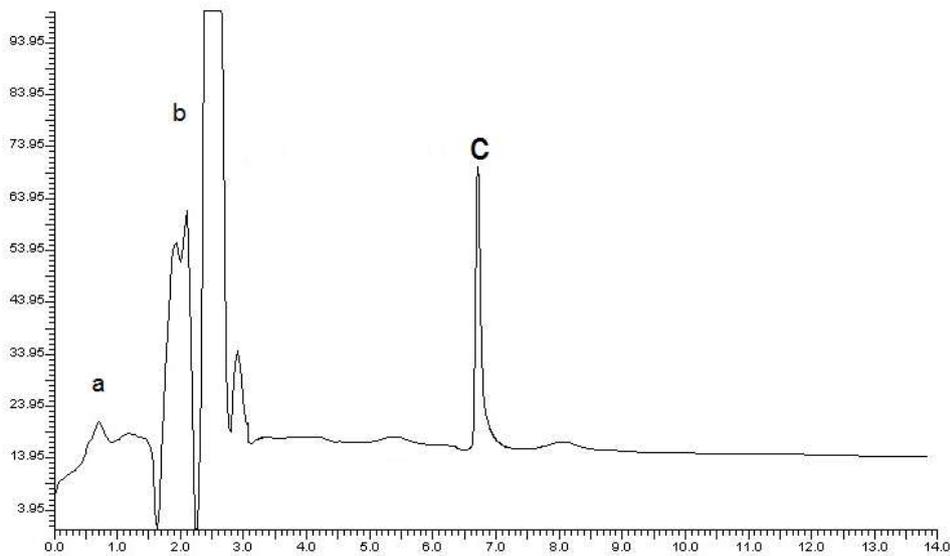
** The value of tabulated t and F, (at $p = 0.05$)

Table 8:Determination of the sorafenib in $\mu\text{g/mL}$ in patient plasma using:

Tablet/Time	0.5 hr	1 hr	2 hr	3 hr	4 hr
Nexavar [®] 200 mg tablets	0.28	0.80	1.50	2.1	1.20

Sorafenib Level in Patient Plasma

The volunteer plasma samples were analyzed to calculate the different sorafenib levels after different time intervals using the spiked plasma regression equation. The peak plasma level was 2.1 $\mu\text{g/mL}$ and it was reached after 3 hrs. This successful application allows the therapeutic dose monitoring of the drug. Fig 5. Table 8.



a:solvent front

b:plasma peak

c: sorafenib peak

Fig.5: Chromatogram of sorafenib in patient plasma, 3 hours after administration of Nexavar tablet.

Testing of Content Uniformity

The content uniformity testing was studied according to the United States Pharmacopoeia [13]. The method is able to analyze the drug in single tablet as it merits high accuracy and precision. Each table was subjected to analysis and the acceptance value AV was calculated for each of one. The results show smaller AV than the maximum allowed AV Table 9.

Table 9: Content uniformity testing of sorafenib in its dosage forms using the proposed methods

Parameter	Percentage of the lable claim
Data	Nexavar (using aqueous method)
	100.33
	100.41
	100.12
	99.74
	100.53
	99.93
	99.56
	99.28
	100.50
	99.90
Mean	100.03
SD	0.44
% Error	0.15
Acceptance value(AV)	1.10
Maximum allowed value (L1)	15

Stability

The stability of the standard solution was verified by reanalyzing the methanolic solutions at room temperature (25°C) for 24 hours. It was noticed no indication of any decomposition of sorafenib.

Chromatographic Performance

The present chromatographic method produces symmetrical well resolved peaks of sorafenib and the degradation products. The sorafenib peak was obtained at retention time of 6.3 min, the alkaline and acidic degradation products were obtained at 2.7, 4.0 min. The oxidative and photolytic degradation products peaks were obtained at 3.6 and 5.1 min. respectively.

Conclusion

The present investigation represents a stability indicating method for the analysis of sorafenib. Moreover the proposed work can be utilized for the bioanalysis of sorafenib in biological fluids and for routine analysis of the stability of the drug.

Disclosure

All authors read and approved the final manuscript. This paper is unique and is not under consideration by other publication and has not been published else where. The authors declare that they have no competing interest.

Conflict of Interest: Author declares that he has no conflict of interest.

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