Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i10.2426

ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND STABILITY INDICATING STUDIES OF AVANAFIL BY USING RP-HPLC TECHNIQUE

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Abstract:
Objective: The project was aimed at developing an analytical method to quantify Avanafil drug either alone or in tablet formulation, including stability studies by practicing RP-HPLC technique.
Methods: The RP-HPLC technique was carried to work out an analytical method and inspecting consistency of the method by performing the different validation parameters. The degradation studies were performed under different physical and chemical conditions following the ICH guidelines. The column used was Inertsil ODS Column C_{18} (4.6×250mm)5µm.
Findings: In the course of stream lining the analytical method, we have clinched on to use the mobile phase with the combination of Methanol: 0.1% OPA (75:25v/v). The drug was detected at 246 nm in UV. The retention time was at 3.14 min and the linearity range of was from 0.5 µg/ml to 10µg/ml with the Regression coefficient calculated to be (R^2) 0.9978. The corresponding recognition limits (LOD and LOQ) was 0.02µg/ml and 0.08µg/ml respectively. Precision studies were carried out and the RSD values were found to be less than two. The degradation studies were successfully conducted.
Novelty: The significant advantages were reduction of retention time almost one minute less, the lower limit in linearity being at least 10 times less and the mobile phase used was quite cheaper than the reported methods. The other part was that, the usability of the method to quantify even though the drug was degraded nearly to 10 % in presence of the unknown degradants. The method is also sensitive, reproducible, quick and economical.

Keywords: Avanafil, Methanol, HPLC, Degradation studies, ICH Guidelines.

1. Introduction

The main objective of this project is to develop and validate a simple, precise and accurate method by using RP-HPLC method. Avanafil is chemically spelled as 4-{[(3-chloro-4-methoxyphenyl) methyl]amino}-2-[(2S)-2(hydroxymethylpyrrolidin-1-yl]-N-(pyrimidin-2-ylmethyl) pyrimidine-5

carboxamide. The empirical formula is C23H26ClN7O and molecular weight is 483.95 g/mol. The drug is slightly soluble in Methanol, sparingly soluble in DMSO and practically soluble in water.. It is a phosphodiesterase type 5 (PDE5) inhibitor exhibiting vasodilatory effects and oral route is permitted. The breakdown of cyclic guanosine monophosphate (cGMP), present in the smooth muscle of the corpus cavernosa of the penis, is prevented by avanafil's selective inhibition of PDE5. Avanafil drug binds 99% of the administered drug to plasma proteins and is independent of total drug concentration. It is excreted in the urine mainly as unchanged drug (approximately 21% of the administered dose). And approximately 62% of the drug is excreted in the feces[1]. An analytical method was reported by Bhatt bhumik, Raval Kashyap for the estimation of Avanafil in the pharmaceutical dosage form and they have used acetonitrile, water, triethylamine and acetic acid as mobile phase(65:35:0.1:0.1) with the C_{18} stationary phase of length 250mm, resulting in the retention time of 4.7 minutes[2]. P. Pavani, T. Raja Rajeswari and G. Ramana Reddy have used HPLC to establish an analytical method for the quantification of Avanafil. They worked with the C_{18} column(250mm length, 4.6mm i.d, 5µm particle size), flow rate of 1 ml/minute, mobile phase of buffer(pH-4.2) and methanol in the combination of 90:10[3]. Nitin kumar, D. Sangeetha, L. Kalyanraman and K.Sainath have thoroughly investigated for developing an analytical for the drug Avanafil take the help of Inertsil ODS 3 column (4.6 mm × 250 mm, 3 µm); mobile phase consisting of mobile phase A(0.1% trifluoro acetic acid and triethylamine in water) and mobile phase B (water and acetonitrile) in the ratio of 20:80 (v/v). The flow rate was maintained at 1.2 ml/minute[4]. M. V. Jadhav, A. Akash, V. Munipalli, R. M. Singh, S. Nayak and B. Vaidhun disclosed an analytical method for the assessment of Avanafil with the retention time of 10.7 minutes maintaining the conditions- mobile phase composition of Water, Acetonitrile and Trifluroacetic acid(65: 35: 0.1%v/v); flow rate at 1.0 ml/min and detection was at 238 nm[5]. Mital Patel and Charmy Kothari followed quality by design approach for method development and validation of Avanafil drug[6]. J.Peris-Vicente, J.Esteve-Romero and S. Carda-Broch have written a book on chromatographic separation techniques[7].

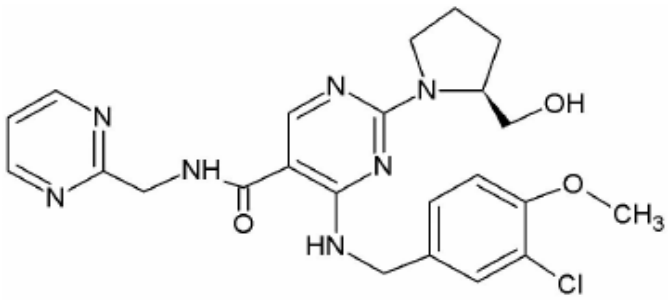


Figure 1: Chemical structure of Avanafil

The objective of the present work was to develop a chromatographic method for determination of Avanafil and to validate the method by using various parameters.

2. Methodology

2.1 Materials

HPLC (Shimadzu LC-20AD), UV Visible Spectroscopy (Shimadzu UV-1800 ENG240V), Electronic balance (Shimadzu ATY224), Digital ultrasonic cleaner (SONICA2200MH), hot air oven (INFRA DIGI ISO 9001-2015), UV Cabinet (MONOQUARTZ).

2.1.2 Chemicals

Avanafil (Carbanio), Methanol (HPLC grade, Merck), Water for HPLC(Merck), Ortho Phosphoric acid (HPLC grade, Merck).

2.1.3 Preparation of standard stock solution:

Standard stock solution was prepared by dissolving, accurately weighed 10mg of Avanafil Active Pharmaceutical Ingredient(API) in Methanol: 0.1%OPA(75:25) and the mobile was slowly added to reach the mark on the 10 ml volumetric flask (1^0 stock solution 1000µg/ml).

2.1.4 Preparation of secondary standard stock solution:

Secondary standard stock solution was prepared by pipetting 1ml of stock solution and volume of mobile phase added to make up to the mark on the 10ml volumetric flask (2^0 stock solution 100µg/ml).

2.1.5 Chromatographic condition in RP-HPLC

HPLC analysis was performed using Shimadzu Corporation, equipped with reservoir tray, column oven, detector(PDA). Reverse phase column of C_{18} packed with particle size of 5µm diameter with internal diameter of 4.6 mm, 250 mm length of the column. The mobile phase and the drug solution were filtered using filter of 0.45µm pore size. The various dilutions of Avanafil in the concentration of 0.5-10µg/ml was prepared. The solutions were injected using a 20 µl fixed loop into the chromatographic system at the flow rate of 1ml/min, the effluents were monitored at 246 nm and chromatograms were recorded.

The peak was eluted at 3.14 min. The method was extended for determination of Avanafil in pharmaceutical dosage form. The tablet dosage form containing 100 mg strength was taken. 10 tablets of Avanafil (containing 100 mg) were weighed, powdered in glass mortar, ground using the pestle and the powder equivalent to 100mg of Avanafil was transferred into 100ml volumetric flask. The diluents (mobile phase) was used to make up the volume to 100ml. Further dilutions were made with mobile phase to obtain working standard of 10µg/ml. The solution was filtered using micropore filter paper of 0.45µ pore size. The concentration of the drug in tablet sample solution was calculated by comparing with peak area of standard. The parameters such as system suitability, linearity, LOD and LOQ are done by API whereas precision, accuracy, robustness, and degradation studies are done by the tablet powder. The proposed method was validated as per the ICH guidelines[8-9].

3. Results and Discussion

3.1 System suitability:

Standard solution of 5µg/ml was prepared and six injections are injected into the chromatographic system. From the system suitability studies, it was observed that all the parameters were within limits viz Retention time (3.14min), theoretical plates (3018), tailing factor(1.300), peak area (659586). Table 1 contains the values for the system suitability parameter and Figure 2 showing the chromatogram for optimized conditions.

Figure 2: Chromatogram of optimised conditions Avanafil in the mobile phase of Methanol:0.1% OPA; 75:25.

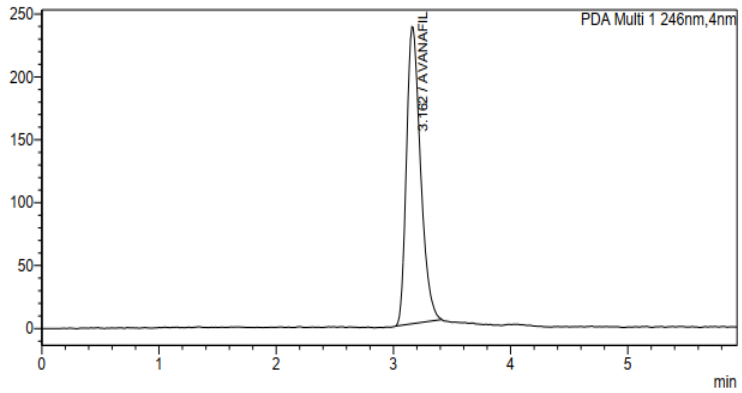
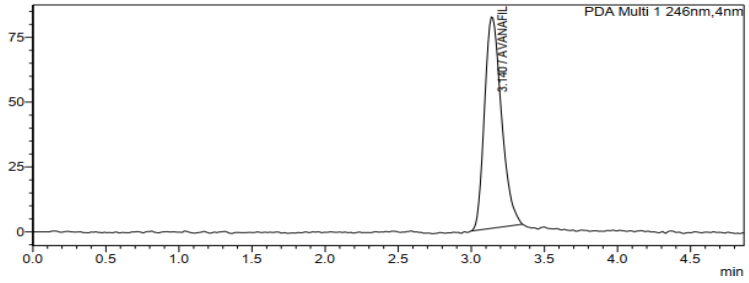


Table 1: System suitability parameters for proposed HPLC method

S. No	Peak Area	Ret Time	Plate Count	Peak Height	Tailing factor
1	652436	3.11	2936	81297	1.312
2	667490	3.14	3057	83320	1.306
3	659586	3.14	3018	81487	1.300
4	658723	3.14	2978	80948	1.325
5	666844	3.14	3028	82608	1.272
6	659109	3.138	3014	82427	1.249
Average	660698.00	3.13	3005.17	82014.50	1.26
STDEV	5653.80	0.01	42.36	911.83	0.03
% RSD	0.86	0.39	1.41	1.11	2.00
Limits	_	_	>2000	_	<2.0
% RSD	<2.0	<2.0	<2.0	<2.0	<2.0

Figure 3 : Representative chromatogram of System suitability.



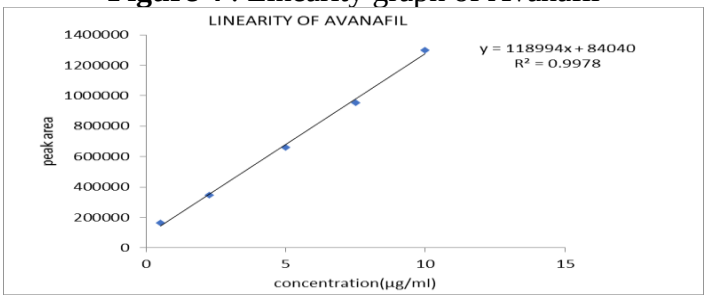
3.2 Linearity:

Linearity range was found to be 0.5 to 10µg/ml for Avanafil. The correlation coefficient was found to be 0.997. A calibration curve was prepared by plotting peak area as a function of concentration of drug solution. The values for calibration curve are presented in table 2 and in the Figure 4 graph is accomplished for the same.

Table 2: Linearity for proposed HPLC method

S. No	Conc. (µg/ml)	Peak area
1	0.50	162471
2	2.25	348122
3	5.00	659278
4	7.50	955335
5	10.00	1299588

Figure 4 : Linearity graph of Avanafil



3.3 Precision:

Concentration of 100% was prepared and six injections were injected into the chromatographic system and %RSD was found to be within the limits(limit <2). The values being presented in table 3, table 4

for inter day precision and intraday precision respectively. The chromatograms for the same are presented in figure no 5 and 6.

Table 3: Inter day precision peak areas for the Day 1, Day 2 and Day 3

S. No	Day 1	Day 2	Day 3
1	665764	651820	658709
2	667490	652436	664855
3	659586	652235	666979
4	658723	653648	657640
5	666844	657661	659900
6	659109	652501	665763
Average	662919.33	653383.50	662307.67
STDEV	4186.29	2182.07	4019.55
% RSD	0.63	0.33	0.61
Limits	**% RSD: <2.0**	**% RSD: <2.0**	**% RSD: <2.0**

Table 4: Intraday precision peak areas within the day

S. No	9:00 AM	1:00 PM	5:00 PM
1	659149	656393	658709
2	656689	655290	664855
3	653741	653564	666979
4	655051	660455	657640
5	654142	651701	659900
6	652907	655594	665763
Average	655279.83	655499.50	662307.67
STDEV	2293.54	2952.39	4019.55
% RSD	0.35	0.45	0.61
Limits	**% RSD: <2.0**	**% RSD: <2.0**	**% RSD: <2.0**

Figure 5: Representative chromatogram of Inter day Precision

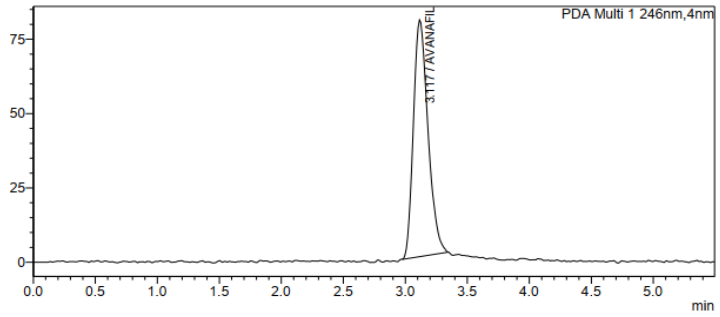
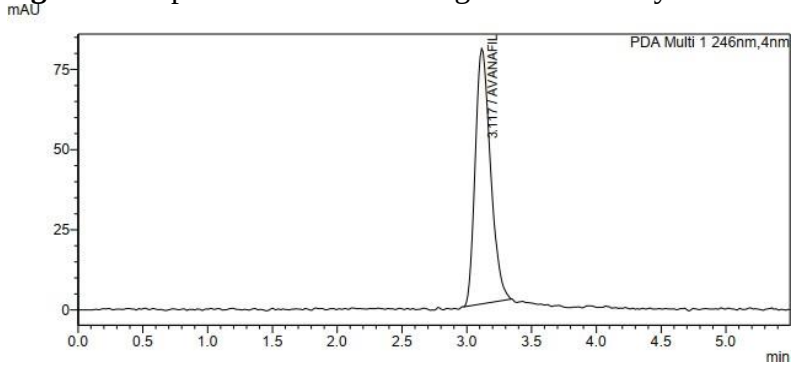


Figure 6: Representative chromatogram of Intraday Precision



3.4 Accuracy:

Series of 50%, 100% and 150% solutions were prepared by taking the tablet powder equivalent to Avanafil drug and by using standard solutions. Resulting chromatograms for the above concentrations were analyzed and percentage recovery was found to be within limits i.e., 98-102%. The values being presented in figure in table 4 and the resulted chromatograms are presented in figure 7.

Figure 7 : Representative chromatogram of Accuracy : (100%)

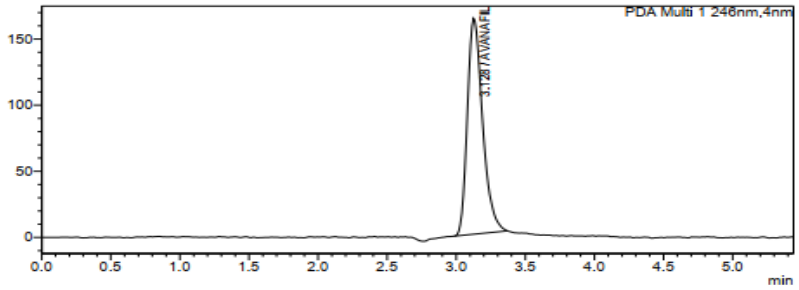


Table 5 : Results for Accuracy at 50%, 100 % and 150 %

Accuracy level	Peak area	Amount taken	Amount added	Amount found	% Recovery	Mean % Recovery
50%	972654	5	2.5	7.4677	98.7088	100.49
	980132	5	2.5	7.5306	101.2226	
	981032	5	2.5	7.5381	101.5251	
100%	1253688	5	5	9.8295	96.5894	97..96
	1262759	5	5	9.9057	98.1140	
	1269165	5	5	9.9595	99.1907	
150%	1604559	5	7.5	12.7781	103.7082	101.20
	1618549	5	7.5	12.8957	105.2758	
	1523378	5	7.5	12.9363	94.6118	

3.5 LOD & LOQ:

The limit of detection (LOD) was found to be 0.02 µg/mL and the limit of quantification (LOQ) was found to be 0.08 µg/mL, these parameters are summarized in Table 6 and the chromatograms are summarized in figure 8.

Table 6 : Values for LOD and LOQ for Avanafil

Parameters	Slope from Linearity	SD of peak from system suitability
	11899	106.332
LOD = 3 x SD/Slope	0.02 µg/ml	
LOQ = 10 x SD/Slope	0.08µg/ml	

Figure 8: Representative Chromatograms for LOD and LOQ

Figure 8(a) LOD

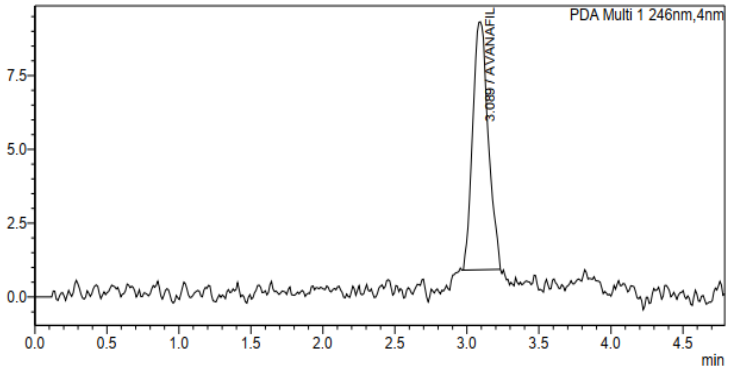
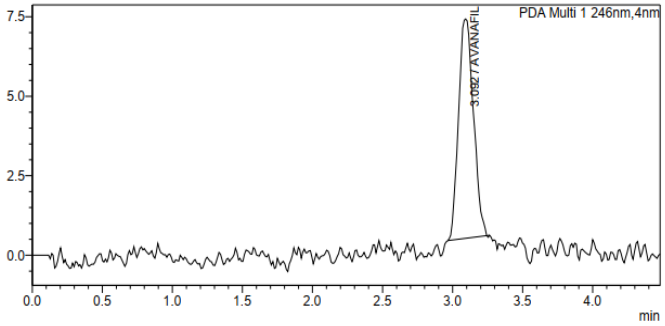


Figure 8(b) LOQ



3.6 Robustness:

A sample solution of 100% concentration was prepared and injected into the chromatographic system by following the below chromatographic conditions.

The observed values are within the acceptance limits (Limits: %RSD<2.0)

Table 7 : Robustness

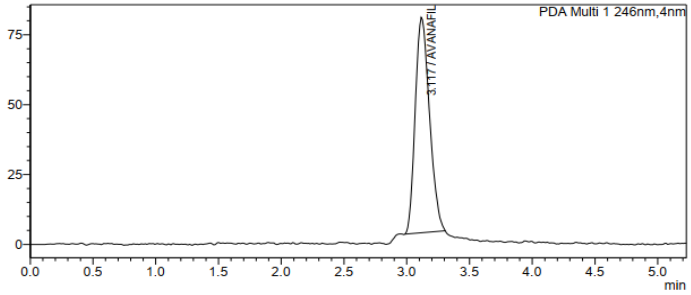
Parameter	Condition	Condition	RT	Peak area	Theoretical plates	% RSD
Flow (ml/min) min±0.2 ml	-0.2ml/min	0.8ml/min	3.831	824629	3642	2.04
	+0.2ml/min	1.2ml/min	2.609	623879	2894	1.92
Temp (°C) min±5°C	-5^{0}C	25°C	3.131	667476	3750	2.07
	+5^{0}C	35°C	3.110	654814	3748	0.99
Wave length (nm) min±5 nm	-2nm	244 nm	3.099	670582	3208	0.836
	+2nm	248 nm	3.098	663292	3220	0.482

DEGRADATION STUDIES

1. Acid Degradation:

For acid degradation, 1ml of dosage form (5µg/ml) was taken and 1ml of 0.1 N HCl was added in a 10 ml volumetric flask and warmed at 60°C for 10min. The solution was cooled and 1ml of 0.1N Sodium hydroxide was added and make up to the mark using mobile phase and the solution was injected into the HPLC system and the peak responses were recorded and the chromatogram is shown in figure 9 (a) and in table 8 s.no 1.

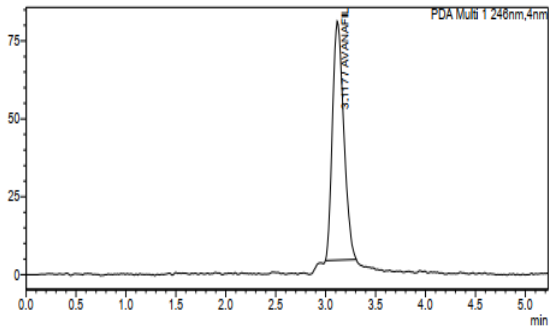
Figure 9 (a): Representative chromatogram of Acid Degradation study



2. Base Degradation:

For base degradation, 1ml of dosage form (5µg/ml) and 1ml of 0.1N sodium hydroxide was added in a 10 ml volumetric flask and warmed to 60°C for 10min. The solution was cooled and 1ml of 0.1N HCl was added and make up to the mark using mobile phase and the solution was injected into HPLC system and the peak responses were recorded and the chromatogram is shown in figure 9 (b) and in table 8 s.no 2.

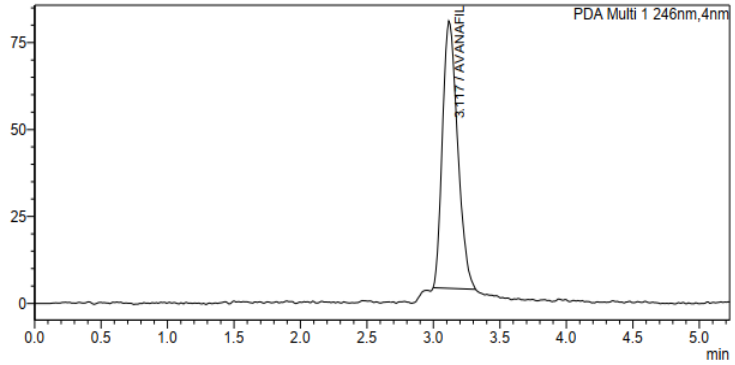
Figure 9 (b): Representative chromatogram of Base Degradation study



3. Peroxide Degradation:

For peroxide degradation, 1ml of dosage form (5µg/ml) and 1ml of 3% H_2O_2 was added in a 10ml volumetric flask and this volumetric flask was warmed to 60°C and make up to the mark using mobile phase. The solution was injected into HPLC system and the peak responses were recorded and the chromatogram is shown in figure 9 (c) and in table 8 s.no 3.

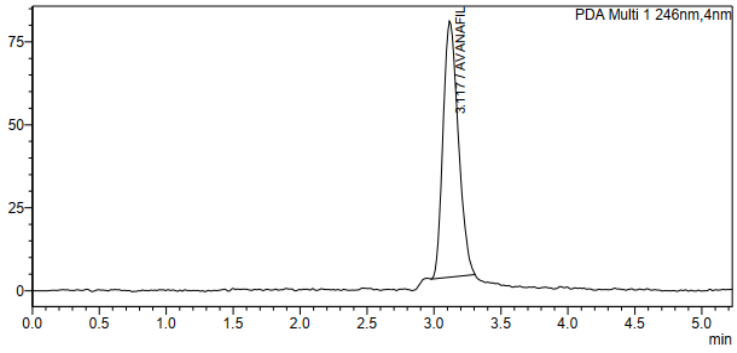
Figure 9 (c): Representative chromatogram of Peroxide Degradation study



4. Thermal Degradation:

For thermal degradation, 1ml of dosage form (5µg/ml) was transferred into 10 ml volumetric flask and was placed in hot air oven for 15min and make up to the mark using mobile phase. The solution was injected into HPLC system and the peak responses were recorded and the chromatogram is shown in figure 9 (d) and in table 8 s.no 4.

Figure 9 (d): Representative chromatogram of Thermal Degradation study



5. Photolytic Degradation:

For photolytic degradation, 1ml of dosage form (5µg/ml) was transferred into 10ml volumetric flask and was placed in UV Cabinet for 1hour and make up to the mark using mobile phase. The solution was injected into HPLC system and the peak responses were recorded and the chromatogram is shown in figure 9 (e) and table no 8 S.No 5.

Figure 9 (e): Representative chromatogram of UV Degradation study

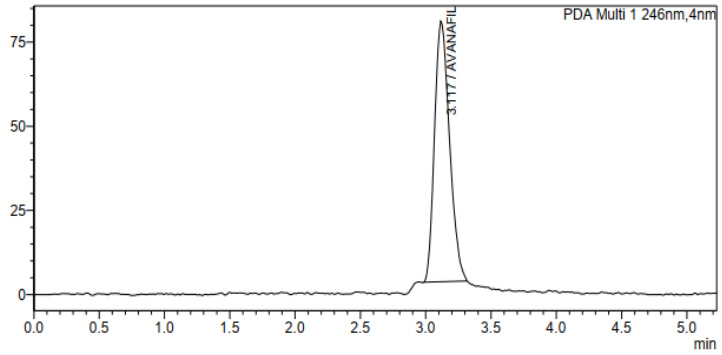


Table 8 : Degradation studies of Avanafil

S.No	Condition	Peak Area	% Assay	% Degradation
1	Acid Degradation	610595	92.5	7.5
2	Base Degradation	602420	91.3	8.7
3	Peroxide Degradation	610080	92.4	7.6
4	Photolytic Degradation	611767	92.7	7.3
5	UV Degradation	618800	93.8	6.2

Summary table of the parameters for the method development, validation and degradation studies

Table no.7

S. No	Parameters	HPLC Results
1	Mobile phase	Methanol : OPA (75:25v/v)
2	Wave length detection	246 nm
3	Calibration range (µg/ml)	0.5-10 µg/ml
4	Regression equation	y = 118994x + 84040
5	Correlation coefficient (r^2)	0.997
6	Retention time	3.162 min
7	Systems Suitability	0.86 %
8	LOD (µg/ml)	0.02 µg/ml
9	LOQ (µg/ml)	0.08 µg/ml
10	Accuracy	98-102 %
11	Inter-day Precision(%RSD)	0.33-0.63
12	Intraday Precision(%RSD)	0.35-0.61
13	Robustness Flow rate(%RSD)	1.92-2.00
14	Robustness Temperature(%RSD)	0.99-2.07
15	Robustness Wave length(%RSD)	0.48-0.91
16	Acid degradation	92.5 %
17	Base degradation	91.3 %
18	H_2O_2 degradation	92.4 %
19	Photolytic degradation	92.7 %
20	UV Degradation	93.8 %

4. Conclusion :

The estimation of Avanafil in API and its tablet dosage form was done by using RP-HPLC technique. The method was successfully validated and degradation studies were completed following ICH Q2R1and Q1A guidelines respectively. The linearity was in the range of 0.5 µg/ml to 10 µg/ml and the %RSD for accuracy, precision, and robustness were all within the limits of <2 %.

The advantages were reduction of retention time(to 3.14min) almost one minute less, thus reducing

the time of analysis, significant reduction in solvent usage, which in turn can be stated as economical. The lower limit(being o.5µg/ml in this method) in linearity being at least 10 times less than the reported methods in literature. The mobile phase/solvent used was Methanol and 0.1% OPA (75:25) which are comparatively cheaper than many solvents used in the literature. The other part was that, the usability of the method to quantify even though the drug was degraded nearly to 10 % in presence of the unknown degradants.

Thus, we can consider this method as sensitive, economical, reproducible and considerably rapid in the assay of Avanafil in API and dosage form.

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