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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR ESTIMATION OF RELATED SUBSTANCES IN AVANAFIL

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ABSTRACT

The current proposal of the research is to estimation of related substances by using high-performance liquid chromatographic method has been developed and validated for the determination of Avanafil. Reversed-phase chromatography was performed on Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower^2 photodiode array detector using ACE C18 (250 mm × 4.6 mm, 5 µm particle size) column with pH 4.2 buffer: methanol in the ratio of 90:10 as mobile phase A and acetonitrile as mobile phase B at a flow rate of 1.0 mL/min. by gradient elution with UV detection at 245 nm. Recovery and Linearity was observed well within the limits (R^2 = more than 0.99 for concentration range of LOQ to 150% level for linearity and the % recovery was within the ICH acceptance limits of 85-115%) for all the impurities. The limit of quantitation (LOQ) and limit of detection (LOD) were found to be less than 0.05%. The method was validated as per ICH guidelines. The RSD for intra-day and inter-day (<3.0% RSD) precision were found to be less than 1 %. The percentage recovery was in good agreement with the labeled amount in the pharmaceutical formulations. from the method validation data, it can be concluded that the method is simple, specific, precise and accurate for the determination of Avanafil in pharmaceutical formulations.

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INTRODUCTION

Avanafil (Figure-1) as (S) -4-[(3-Chloro-4 methoxybenzyl) amino] - 2- [2- (hydroxymethyl)- 1 – pyrrolidinyl] – N -(2pyrimidinylmethyl) – 5 -pyrimidinecarboxamide. It is a practically White crystalline powder. It is freely soluble in Methanol & in Acetonitrile, Practically insoluble in water, soluble in 0.1 mol/L hydrochloric acid. Its molecular formula C23H26ClN7O3 and calculated molecular weight of 483.95 gm/mol. The physiologic mechanism of erection of the penis involves release of nitric oxide (NO) in the corpus cavernosum during sexual stimulation. NO then activates the enzyme guanylate cyclase, which results in increased levels of cGMP, producing smooth muscle relaxation in the corpus cavernosum and allowing inflow of blood. Avanafil has no direct relaxant effect on isolated human corpus cavernosum, but enhances the effect of NO by inhibiting PDE5, which is responsible for degradation of cGMP in the corpus cavernosum. Because sexual stimulation is required to initiate the local release of nitric oxide, the inhibition of PDE5 has no effect in the absence of sexual stimulation. Studies in vitro have shown that avanafil is selective for PDE5. Its effect is more potent on PDE5 than on other known phosphodiesterases (greater than 100-fold for PDE6; greater than 1,000-fold for PDE4, PDE8 and PDE10; greater than 5,000-fold for PDE2 and PDE7; greater than 10,000-fold for PDE1, PDE3, PDE9, and PDE11). Avanafil is greater than 100- fold more potent for PDE5 than PDE6, which is found in the retina and is responsible for phototransduction. In addition to human corpus cavernosum smooth muscle, PDE5 is also found in other tissues including platelets, vascular and visceral smooth muscle, and skeletal muscle, brain, heart, liver, kidney, lung, pancreas, prostate, bladder, testis, and seminal vesicle. Erectile dysfunction (ED) is sexual dysfunction characterized by the inability to develop or maintain an erection of the penis during sexual performance. A penile erection is the hydraulic effect of blood entering and being retained in sponge-like bodies within the penis. The process is often initiated as a result of sexual arousal, when signals are transmitted from the brain to nerves in the penis. Erectile dysfunction is indicated when an erection is difficult to produce**.** Avanafil is an effective treatment option for males suffering from ED. Its main advantage over the other available PDE5 inhibitors is its faster onset of action.[1-3] Till now only some methods published on Avanafil [4-7] but no stability indicating method published till now.

However no method has been published for the estimation of impurities in Avanafil tablets. the author has tried to develop a simple robust stability indicating analytical method by using HPLC for the estimation of impurities in Avanafil pharmaceutical substance. In the present work a simple estimation of impurities in Avanafil reverse phase liquid chromatographic method has been developed for the determination of impurities and validated as per ICH guidelines[11]. In the present work we developed simple, rapid and accurate reverse phase liquid chromatographic method for the determination of Avanafil and its impurities.

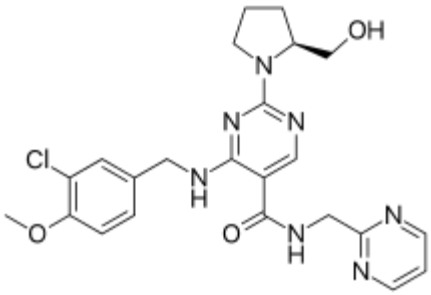


Figure: 1.1 Chemical Structure of Avanafil.

Related Substance Structures:

Figure: 1.2 Chemical Structure of related compound-1
4-(3-Chloro-4-methoxybenzylamo)-2-((S)-2-(hydroxymethyl)pyrrolidin-1-yl)pyrimidine-5-carboxylicacid
Figure: 1.3 Chemical Structure of related compound-2
Ethyl-4-(3-Chloro-4-methoxybenzlamino)-2-((S)-(hydroxymethyl)pyrrolidin-1-yl)-pyrimidine-5-carboxylate
Figure: 1.4 Chemical Structure of related compound-3
Ethyl-4-(3-Chloro-4methoxybenzylamino)-2-(methylsulfinyl)pyrimidine-5-carboxylate
Figure: 1.5 Chemical Structure of related compound-4
Ethyl 7-((3-Chloro-4-methoxybenzyl)amino)-2-hydroxypyrimidine-5-carboxylate
Figure: 1.6 Chemical Structure of related compound-5
(S)-2-(2-(Hydroxymethyl)pyrrolidin-1-yl)4-((4+-methoxybenzyl)amino)-N-(oyrimidin-2-ylmethyl)pyrimidine-5-carboxamide
Figure: 1.7 Chemical Structure of related compound-6
(S)-(1-(4-((3-Chloro-4-methoxybenzyl)amino)-5-((Pyrimidin-2-ylmethyl)Carbomoyl) pyrimidin-2-yl)Pyrrolidin-2-yl)methyl acetate

Experimental

Reagents& Chemicals:

Potassium dihydrogen Phosphate, Methanol, Acetonitrile (HPLC grade), Potassium hydroxide, were obtained from Merck (India). All chemicals were of an analytical grade and used as received.

Chromatographic conditions:

Chromatographic separation was achieved by using a Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower[2] photodiode array detector using ACE C18 (250 mm × 4.6 mm, 5 µm particle size) column with pH 4.2 buffer: methanol (95:5) as mobile phase A and Acetonitrile as mobile phase B by using gradient mode of elution at a flow rate of 1.0 mL/min. with UV detection at 245 nm. Column maintained at temprature 35 ºC, sample temprature 5ºC. The overall run time was 60 min. and the flow rate was 1.0 mL/min. 10 µl of sample was injected into the HPLC system. Retention times of impurities were 11.1 for RC-1, 14.1 for RC-2,21.4min. for RC-3,27.55 for RC-4, 29.64 for RC-5, 42.21 for RC-6 and about 19 min for avanafil.

Method Validation

System Suitability

Perform the system suitability by analyzing the standard solution six times and sensitivity solution once as per recommendations from US pharmacopeia. Calculate the theoretical plates and tailing for main peak and S/N ratio for sensitivity solution.

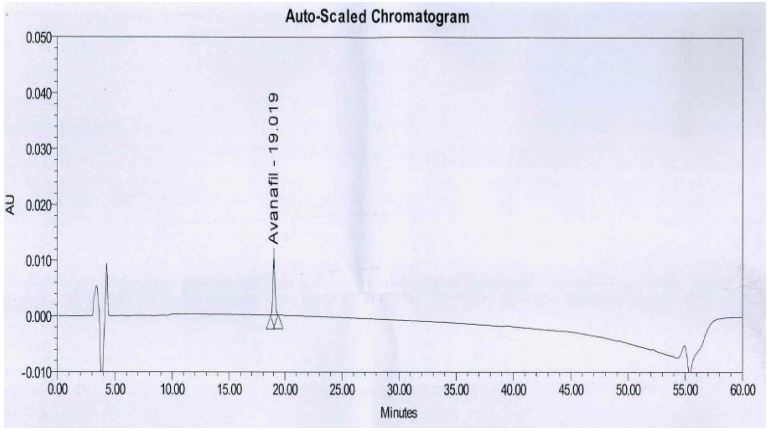


Figure: 1.5 Standard chromatogram of Avanafil by proposed method.

Table: 1.1 Summary of system suitability.

Retention time of Avanafil	Tailing factor for Avanafil peak	Theoretical plates for Avanafil peak	S/N Ratio Avanafil sensitivity solution
28.18	1.1	47868	24.26

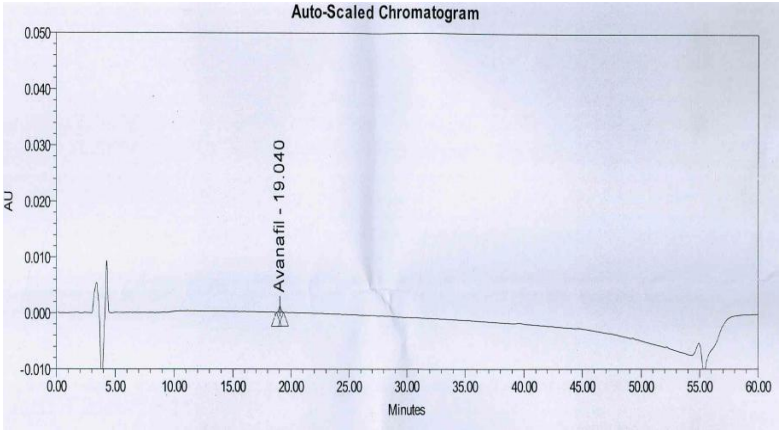
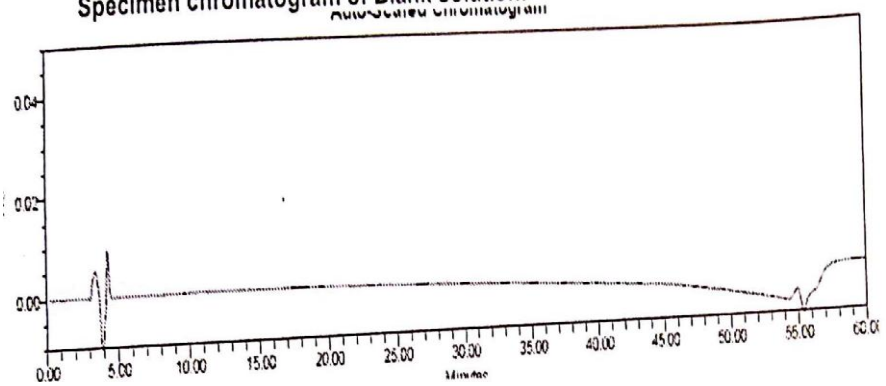


Figure: 1.6 Sensitivity Solution chromatogram of Avanafil.

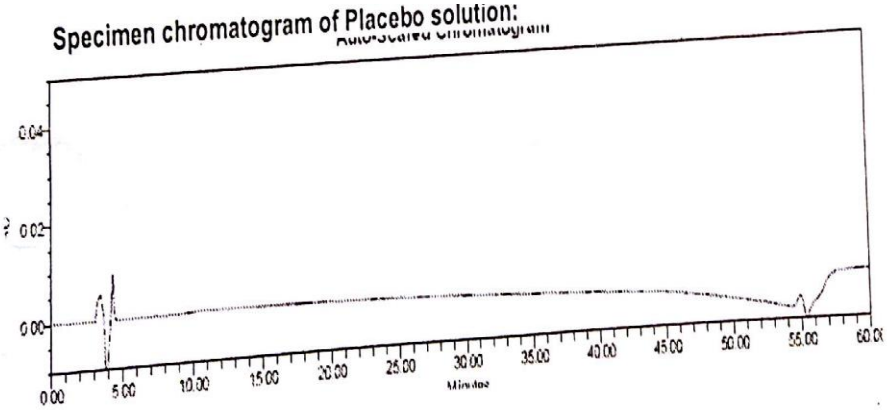
Specificity[8-14]

Prepare and analyze the solutions of monohydroxy impurity, BND-VI, Isopropyl ester impurity and Avanafil each individually. Prepare a spiked solution of each potential impurity to the Avanafil drug substance and analyze. Perform the analysis using PDA detector and determine the peak purity.

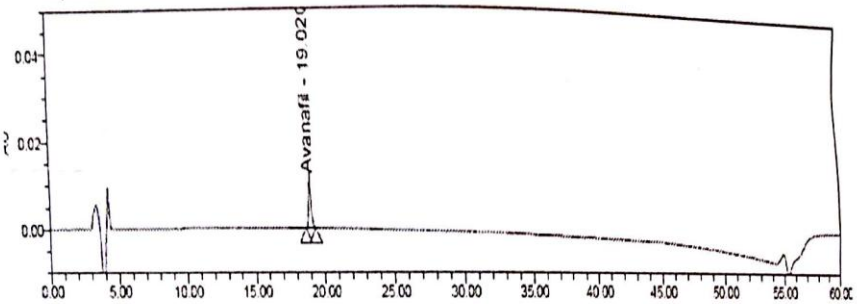
Specimen chromatogram of Blank solution:



Specimen chromatogram of Placebo solution:

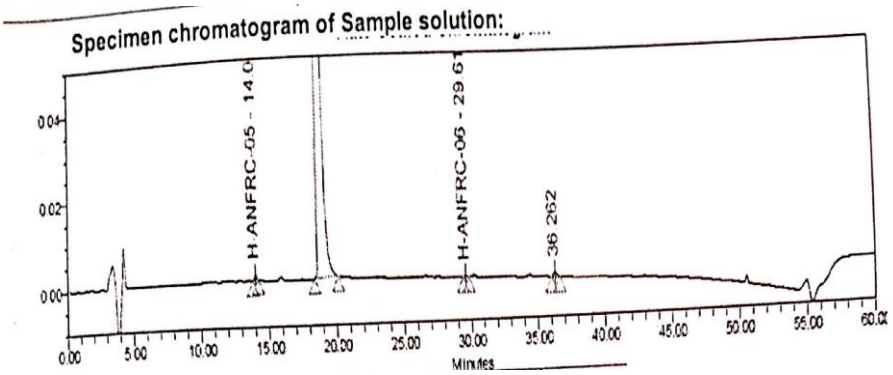


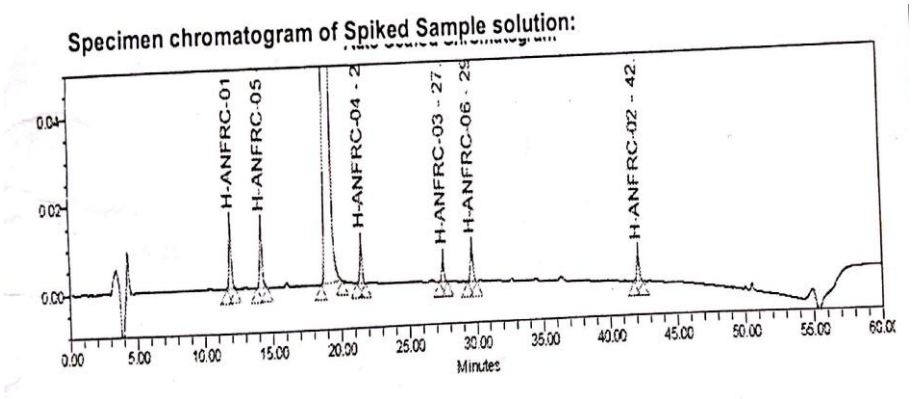
Specimen chromatogram of Standard solution:



	Sample Name	Vial	Inj	Name	RT	Area	USP Plate Count	USP Tailing
1	Standard solution	29	1	Avanafil	19.019	134651	47868	1.1

Specimen chromatogram of Sample solution:





S.No.	Condition	Purity angle	Purity threshold	Purity flag
1	Control sample	0.103	0.295	No
2	Spiked sample	0.054	0.255	No
3	5N Hydrochloric acid-7Hrs at 90°C	0.092	0.270	No
4	5N Sodium hydroxide-7Hrs at 90°C	0.087	0.252	No
5	5% Hydrogen peroxide-7Hrs at 90°C	0.087	0.249	No

Figure: 2.0 Specificity chromatogram of Spiked Solution and Forced Degradation summary.

Table: 1.3 summary of retention time, and relative retention time for known impurities.

Peak Name	Retention Time	Relative retention time(RRT)
RC-1	11.13	0.63
RC-2	14.11	0.75
Avanafil	19.00	1.00
RC-3	21.44	1.13
RC-4	27.54	1.45
RC-5	29.64	1.56
RC-6	42.18	2.22

This study showed that all the known impurities of Avanafil are adequately resolved. Therefore the method is selective for the determination of related substances in Avanafil.

Limit of detection[8-14]

Table: 1.4 Limit of detection (LOD) for Avanafil and impurities.

Component	Concentration (mg/ml)	Signal to noise	LOD (%)
RC-1	0.01485	3.9:1	0.07485
RC-2	0.01867	3.9:1	0.09400
Avanafil	0.01455	3.9:1	0.07273
RC-3	0.01543	3.2:1	0.07714
RC-4	0.01400	3.3:1	0.07029
RC-5	0.01742	3.4:1	0.08677
RC-6	0.01441	3.7:1	0.07206

The limit of detection values obtained for each impurity and Avanafil are within the acceptance criteria.

Limit of Quantitation

Table: 1.5 Limit of Quantitation for Avanafil and impurities.

Component	Concentration (mg/ml)	Signal to noise	LOQ (%)
RC-1	0.247	9.9:1	0.048
RC-2	0.256	9.9:1	0.049
Avanafil	0.240	9.5:1	0.048
RC-3	0.270	10.2:1	0.054
RC-4	0.246	10.3:1	0.049
RC-5	0.269	9.4:1	0.054
RC-6	0.245	9.7:1	0.049

Limit of quantitation values obtained for each impurity and Avanafil are within the acceptance criteria.

Precision at LOQ

The precision at LOQ is performed by analyzing six replicate injections of the standard solution containing all known impurities and Avanafil at LOQ level. . Determine the percentage relative standard deviation of peak areas of each impurity and Avanafil. Results of peak area of impurities and Avanafil are summarized in table 9.

Table: 1.6 Summary of peak areas for precision at LOQ.

Inj. No	RC-1	RC-2	Avanafil	RC-3	RC-4	RC-5	RC-6
1	15784	8051	11867	4872	8979	15526	8108
2	15689	8093	11200	4257	8413	15631	8109
3	15789	9150	12087	4751	8319	15743	8307
4	15827	8400	12500	4689	8719	16413	9710
5	15978	8800	11126	4891	8261	16428	8327
6	16100	8200	12047	4367	8971	15783	9157
Mean	15861	8449	11805	4638	8610	15921	8620
%RSD	0.95	5.20	4.57	5.73	3.76	2.50	7.67

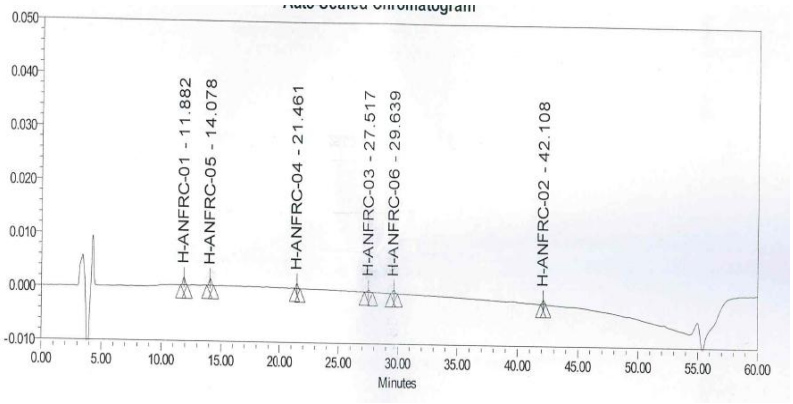


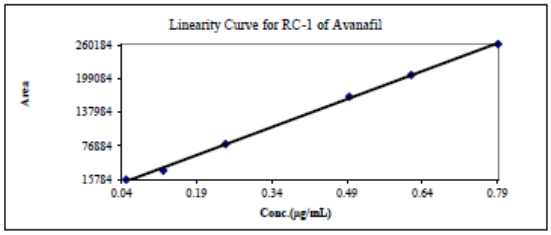
Fig. Typical Chromatogram at LOQ level for Avanafil and its impurities.

Linearty and Range[8-14]

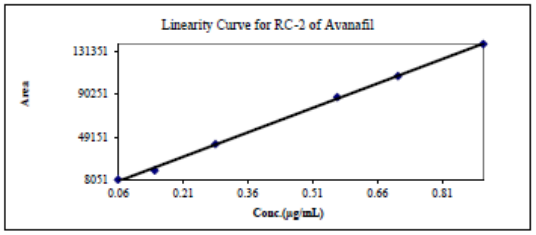
The linearty is determined by injecting the solutions in duplicate containing known impurities and Avanafil ranging from LOQ to 150% (LOQ, 20%, 40%, 80%, 100%, 120% and 150%) of the specified limit. Perform the regression analysis and determine the correlation coefficient and residual sum of squares. Determine the response factor for each impurity with respect to Avanafil. Report the linearty range as the range for determining the impurities.Results obtained are in the tables & figures show the line of best fit for peak area versus concentration for each impurity.

Table: 1.7 Linearity of Avanafil and Its Impurities.

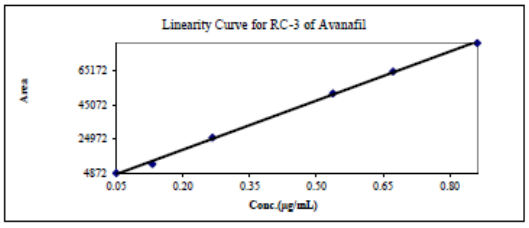
RC-1			
Name of the level	**% level**	**in %**	**area**
Level-1	loq	0.0494	15784
Level-2	25	0.124	32189
Level-3	50	0.2476	80537
Level-4	100	0.4944	166570
Level-5	125	0.618	205911
Level-6	150	0.791	262135
		Slope	338989
		Intercept	-4114
		Res sum of squ	3714
		CC (r)	0.9994
		RSQ (r2)	0.9989



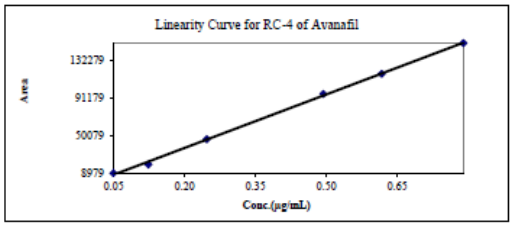
RC-2			
Name of the level	**% level**	**in %**	**area**
Level-1	loq	0.0564	8051
Level-2	25	0.141	16934
Level-3	50	0.2818	42305
Level-4	100	0.5636	87623
Level-5	125	0.7044	107909
Level-6	150	0.9016	138750
		Slope	157140
		Intercept	-2441
		Res sum of squ	1811
		CC (r)	0.9995
		RSQ (r2)	0.9991



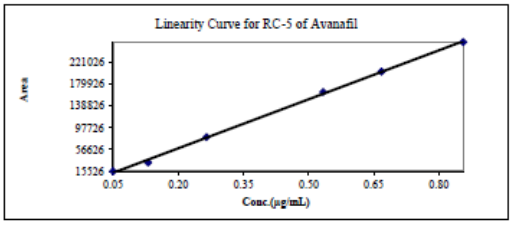
RC-3			
Name of the level	**% level**	**in %**	**area**
Level-1	loq	0.054	4872
Level-2	25	0.135	10298
Level-3	50	0.2704	25793
Level-4	100	0.541	51836
Level-5	125	0.6762	64764
Level-6	150	0.8656	81471
		Slope	96285
		Intercept	-961
		Res sum of squ	1198
		CC (r)	0.9994
		RSQ (r2)	0.9988



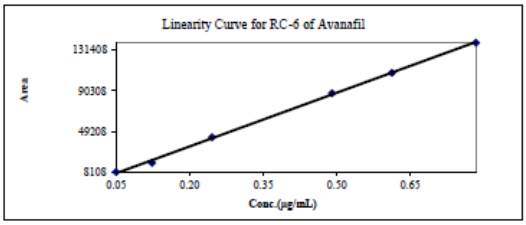
RC-4			
Name of the level	**% level**	**in %**	**area**
Level-1	loq	0.0492	8979
Level-2	25	0.123	18484
Level-3	50	0.2464	46304
Level-4	100	0.493	95960
Level-5	125	0.6162	117924
Level-6	150	0.7888	151711
		Slope	196235
		Intercept	-2548
		Res sum of squ	2080
		CC (r)	0.9995
		RSQ (r2)	0.9990



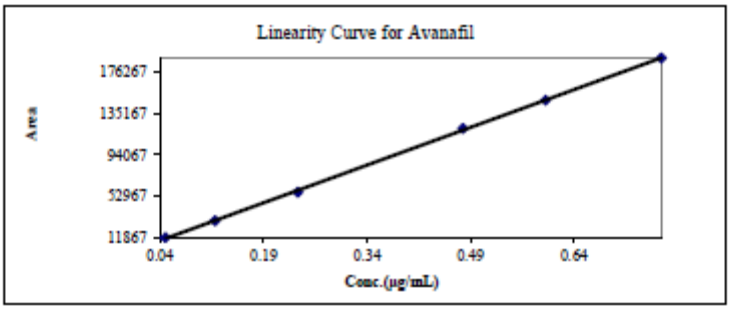
RC-5			
Name of the level	% level	in %	area
Level-1	loq	0.0538	15526
Level-2	25	0.134	32149
Level-3	50	0.2688	80312
Level-4	100	0.5376	165631
Level-5	125	0.672	204192
Level-6	150	0.8602	260225
		Slope	309273
		Intercept	-3906
		Res sum of squ	3666
		CC (r)	0.9995
		RSQ (r2)	0.9989



RC-6			
Name of the level	% level	in %	area
Level-1	loq	0.049	8108
Level-2	25	0.122	17355
Level-3	50	0.2448	43353
Level-4	100	0.4896	87797
Level-5	125	0.612	108677
Level-6	150	0.7834	139143
		Slope	181033
		Intercept	-2027
		Res sum of squ	1752
		CC (r)	0.9996
		RSQ (r2)	0.9991



Avanafil			
Name of the level	% level	in %	area
Level-1	loq	0.048	11867
Level-2	25	0.120	28489
Level-3	50	0.2396	56979
Level-4	100	0.479	120275
Level-5	125	0.5988	148422
Level-6	150	0.7664	190366
		Slope	250245
		Intercept	-1176
		Res sum of squ	1327
		CC (r)	0.9999
		RSQ (r2)	0.9997



The linearity results for Avanafil and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

Accuracy[8-14]

Prepare Avanafil solution spiked with a known amount of each impurity at five levels each in triplicate (in total 15 determinations) and analyze as per the method. The impurities are to be spiked at LOQ, 25%, 50%, 100% and 150% of the specified limit.

Table:2.0 Summary of % recoveries for RC-1 and RC-2.

Level	% of RC-1 (w/w)	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	0.0494	0.247	0.246	100.41	1.17
		0.243	0.247	98.38	
		0.245	0.244	100.41	
25%	0.1236	0.618	0.619	99.84	0.33
		0.616	0.621	99.19	
		0.615	0.617	99.68	
50%	0.2476	1.238	1.234	100.32	0.26
		1.234	1.235	99.92	
		1.239	1.241	99.84	
100%	0.4944	2.472	2.468	100.16	1.21
		2.471	2.423	101.98	
		2.476	2.484	99.68	
150%	0.791	3.955	3.958	99.92	0.08
		3.945	3.952	99.82	
		3.956	3.957	99.97	

Level	% of RC-2 (w/w)	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	0.0564	0.282	0.284	99.30	
		0.281	0.286	98.25	
		0.285	0.287	99.30	
25%	0.1409	0.7045	0.706	99.79	
		0.705	0.709	99.44	
		0.704	0.715	98.46	
50%	0.2818	1.409	1.411	99.86	
		1.408	1.412	99.72	
		1.404	1.409	99.65	
100%	0.5636	2.818	2.82	99.93	
		2.819	2.824	99.82	
		2.912	2.921	99.69	
150%	0.9016	4.508	4.518	99.78	
		4.507	4.52	99.71	
		4.509	4.523	99.69	

Table: 2.1 Summary of % recoveries for RC-3 and RC-4.

Level	% of RC-3 (w/w)	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	0.054	0.270	0.274	98.54	0.36
		0.271	0.276	98.19	
		0.268	0.271	98.89	
25%	0.1352	0.676	0.676	100.00	0.7
		0.675	0.673	100.30	
		0.671	0.678	98.97	
50%	0.2704	1.352	1.357	99.63	0.59
		1.358	1.351	100.52	
		1.351	1.359	99.41	
100%	0.541	2.705	2.712	99.74	0.17
		2.709	2.711	99.93	
		2.704	2.715	99.59	
150%	0.8656	4.328	4.335	99.84	0.06
		4.324	4.329	99.88	
		4.329	4.331	99.95	

Level	% of RC-4 (w/w)	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	0.0492	0.246	0.246	100.00	1.24
		0.248	0.244	101.64	
		0.247	0.249	99.20	
25%	0.1232	0.616	0.619	99.52	1.15
		0.618	0.612	100.98	
		0.621	0.629	98.73	
50%	0.2464	1.232	1.239	99.44	0.92
		1.237	1.224	101.06	
		1.239	1.245	99.52	
100%	0.493	2.465	2.461	100.16	0.2
		2.461	2.472	99.56	
		2.469	2.477	99.68	
150%	0.7888	3.944	3.951	99.82	0.09
		3.943	3.956	99.67	
		3.958	3.965	99.82	

Table: 2.2 Summary of % recoveries for RC-5 and RC-6.

Level	% of RC-5 (w/w)	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	0.0538	0.269	0.267	100.75	1.08
		0.268	0.271	98.89	
		0.266	0.269	98.88	
25%	0.134	0.672	0.679	98.97	0.00
		0.671	0.678	98.97	
		0.674	0.681	98.97	
50%	0.2688	1.344	1.349	99.63	0.08
		1.347	1.352	99.63	
		1.351	1.358	99.48	
100%	0.5376	2.688	2.695	99.74	0.58
		2.689	2.681	100.30	
		2.699	2.675	100.90	
150%	0.8602	4.301	4.309	99.81	0.18
		4.311	4.304	100.16	
		4.307	4.312	99.88	

Level	% of RC-6 (w/w)	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	0.049	0.245	0.241	101.66	2.01
		0.248	0.252	98.41	
		0.246	0.251	98.01	
25%	0.122	0.612	0.618	99.03	0.41
		0.612	0.621	98.55	
		0.615	0.619	99.35	
50%	0.2448	1.224	1.214	100.82	0.8
		1.249	1.241	100.64	
		1.243	1.251	99.36	
100%	0.4896	2.448	2.453	99.80	0.69
		2.443	2.459	99.35	
		2.418	2.456	98.45	
150%	0.7834	3.917	3.924	99.82	0.44
		3.919	3.921	99.95	
		3.317	3.296	100.64	

The percentage recovery values obtained for each impurity are in the range of about 91.2-108.5, which are within the specified criteria. The relative standard deviation values of recoveries obtained for all impurities are found less than 2%.

Precision[8-14]

System precision

Perform the analysis of reference solution six times and determine the percentage relative standard deviation of peak area of replicate injections of each impurity and Avanafil.

Table 2.2: Summary of peak areas of the Avanafil standard.

Injection No	Avanafil
1	122028
2	122448
3	122010
4	122781
5	121815
6	122293
Mean area	122229
%RSD	1.58

The relative standard deviation observed for Avanafil and impurities are less than 10%.The results comply with the acceptance criteria and indicate acceptable precision of the system.

Method precision

The precision of the method is determined by analyzing a sample of Avanafil solution spiked with impurities at 100% of the specification limit.

Table 2.3: Summary of results for precision of the method.

Inj. No	% of RC-1	% of RC-2	% of RC-3	% of RC-4	% of RC-5	% of RC-6	% of Any other individual impurity	% of total impurities
1	0.486	0.478	0.529	0.501	0.539	0.531	0.07	0.52
2	0.485	0.475	0.519	0.491	0.511	0.533	0.09	0.52
3	0.471	0.460	0.502	0.477	0.535	0.516	0.06	0.51
4	0.482	0.478	0.519	0.492	0.550	0.529	0.08	0.51
5	0.484	0.472	0.518	0.492	0.552	0.532	0.07	0.52
6	0.489	0.483	0.536	0.500	0.558	0.537	0.07	0.52
Mean (%)	0.483	0.474	0.520	0.492	0.548	0.530	0.07	0.52
% RSD	1.28	1.67	2.35	1.71	1.59	1.36	0.15	1.00

Similarly solution stability and robustness also established and found that the method is robust enough for the estimation of related substances in Avanafil.

CONCLUSION

A simple, economic, accurate and precise HPLC method was successfully developed. In this method it was carried out by using ACE C18, (250× 4.6mm) with 5µm particle size. Injection volume of 10µl is injected and eluted with the mobile phase A as Methanol and buffer of KH_2PO_4 pH 4.2 with potassium hydroxide and Acetonitrile as mobile phase B over gradient program, which is pumped at a flow rate of 1.0 ml/min. Detection, was carried out at 235 nm. all impurities are well resolved from the main peak and there is no interference from blank and placebo. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, robustness, and stability of solution.

For Selectivity, the chromatograms were recorded for standard and sample solutions of Avanafil and its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore the method is selective for the determination of related substances in Avanafil.

The limit of detection (LOD) and limit of quantitation (LOQ) was found to be for RC-1 0.0148 µg/ml,0.048 µg/ml, for RC-2 0.0186 µg/ml,0.049 µg/ml, for Avanafil n0.0148 µg/ml,0.048 µg/ml, for RC-3 0.0154 µg/ml,0.054 µg/ml, for RC-4 0.0140 µg/ml, 0.049 µg/ml, for RC-5 0.0174 µg/ml,0.054 µg/ml and for RC-6 0.0149 µg/ml, 0.049 µg/ml respectively. The linearity results for Avanafil and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.Calibration curve was plotted and correlation co-efficient for Avanafil and its impurities found to be more than 0.999.

The accuracy studies were shown as % recovery for Avanafil and its impurities at 25%, 50%, 100% and 150%.The limit of % recovered shown is in the range of 90 and 110% and the results obtained were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showed % recovery of the Avanafil and its related substances in the range 91.2-108.5 respectively.

For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Avanafil and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits. For intermediate precision the bias is not more than ± 0.03, the bias observed for individual impurities are within the acceptance criteria.
Hence, the chromatographic method developed for Avanafil and its related substances are rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its quality during its formulation.

Further as part of future course of extended research, the method can be further applied directly for estimating impurities in pharmaceutical substances and formulation in commercial labs as well as can be extended for identifying the impurities in the drug substances.

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