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Development and Validation of Stability Indicating RP-HPLC Method For Estimation of Sildenafil Citrate and Estradiol Valerate In Tablet

Vijay P. Jodhani[1*], Ankit B. Chaudhary, Pooja J. Parmar, Dhaval B. Patel
1. Saraswati Institute of Pharmaceutical Sciences, Dhanap, Gandhinagar, Gujarat, India.

ABSTRACT

A simple, precise and accurate stability indicating RP-HPLC method has been developed and subsequently validated for the simultaneous estimation of Sildenafil citrate and Estradiol valerate in bulk and pharmaceutical formulation. The separation was carried out using C_{18} column (150mm x 4.6mm, 5µm), mixture of acetonitrile and water 80:20 % v/v as a mobile phase with a flow rate of 1 ml/min and the effluent was monitored at 290 nm using PDA detector. The retention time of Sildenafil citrate and Estradiol valerate were 2.55 min and 5.56 min respectively. The method is linear over the range of 125 - 750 µg/ml and 5 - 30 µg/ml for Sildenafil citrate and Estradiol valerate respectively. The method was found to be precise, accurate and specific during the study. The percentage assay was found to be 100.68 % and 99.58 % for Sildenafil citrate and Estradiol valerate respectively from the tablet formulation. Sildenafil citrate and Estradiol valerate were subjected to stress condition to check the degradation behaviour of them. The drugs undergo degradation under acidic, basic, oxidative and thermal condition. The proposed method enables rapid quantification and simultaneous analysis of both drugs from commercial formulations without any interference of excipients. So, the method can be used for routine analysis of Sildenafil citrate and Estradiol valerate in combined tablet formulation.

Keywords: Sildenafil citrate, Estradiol valerate, RP-HPLC, Simultaneous estimation, Stability indicating

*Corresponding Author Email: jodhani.vijay@gmail.com
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INTRODUCTION

Sildenafil citrate (SC) is designated chemically as 5-[2-ethoxy-5-(4-methylpiperazin-1-yl) sulfonylphenyl]-1-methyl-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-7-one;2-hydroxypropane- 1,2,3-tricarboxylic acid (Figure 1) is a compound of the pyrazolo-pyrimidinyl-methyl piperazine class, and is used to treat male erectile dysfunction and pulmonary arterial hypertension (PAH)[1]. It acts by inhibiting phosphodiesterase type5 (PDE5) enzyme which promotes degradation of cGMP and regulates blood flow in the penis. Increased cGMP level thus leads to smooth muscle relaxation and increased blood flow into the corpus cavernosum[2]. Various analytical methods have been reported for the estimation of Sildenafil citrate as alone as well as in combination with other drugs. They include spectrophotometric methods[3-4], HPLC[5-9], LC/MS/MS[10], stability indicating HPLC methods with Dapoxetine[11], Duloxetine[12] and Tadalafil[13].

Estradiol valerate (EV) is designated chemically as [(8*R*,9*S*,13*S*,14*S*,17*S*)-3-Hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]-phenanthren-17-yl]-pentanoate (Figure 2) is a Synthetic ester of Estradiol, a steroidal sex hormone[14]. It is generally used as a source of Estradiol in for the treatment of Estradiol deficiency. It act by binding to the estrogen receptors in the cell and leading to modulation of gene transcription and expression in the respective cells[15]. Various analytical methods have been reported for the estimation of Estradiol valerate as alone in formulation, as well as in combination with other drugs. They include simple spectrophotometric methods[16], derivative spectrophotometric method[17], HPLC methods[14], simultaneous estimation with Cyproterone acetate[18] and Dienogest[19].

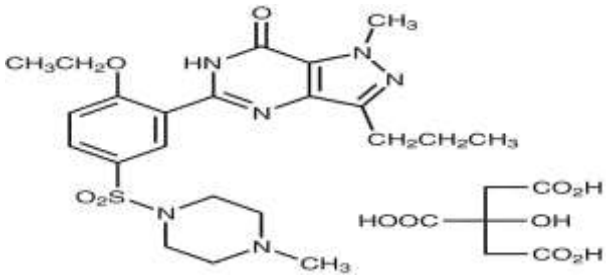

Figure 1: Chemical structure of Sildenafil citrate

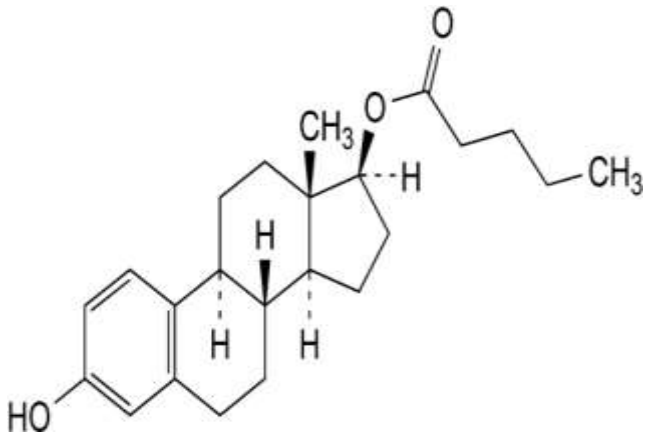

Figure 2: Chemical structure of Estradiol valerate

However an extensive literature search didn't reveal any estimation method for both the drugs in their combined dosage form. Therefore, attempt was made to develop and validate simple, precise, and accurate, stability indicating RP-HPLC method for simultaneous determination of both the drugs in their combined dosage form. The parent guideline on drug stability testing Q1A (R2) issued by international conference on harmonization stipulates stress studies be carried out on a drug in order to establish the drug's inherent stability characteristics[20].

MATERIALS AND METHOD

Reagents and Chemicals:

Sildenafil citrate and Estradiol valerate were obtained as gift samples from West coast pharmaceuticals, Ahmedabad. Sildenafil citrate and Estradiol valerate combined dosage form tablets were purchased from local market. HPLC grade Acetonitrile, Water and Hydrochloric acid, Sodium hydroxide and Hydrogen peroxide of analytical grade were obtained from SD Fine Chem Ltd.

Instruments and Chromatographic Conditions:

Young lin HPLC system was used for method development, degradation studies and validation. Data acquisition was performed on YL 9100 HPLC software. The separation were achieved on Inertsil ODS C_{18} (150 × 4.6 mm, 5µm) column. The column was maintained at room temperature and the eluent was monitored at 290nm using PDA detector. The mixture of Acetonitrile and Water in proportion of 80:20 %v/v at a flow rate of 1.0 ml/min was used as a mobile phase. The injection volume was 20µl.

Preparation of Standard Solutions of SC and EV:

Accurately weighed and transferred 100mg of SC and 100mg of EV into two different 100ml volumetric flasks and dissolved using mobile phase as a diluent, then volume was made up to mark

with mobile phase and used as standard stock solution (1000µg/ml). From the prepared stock solutions 12.5ml of standard stock solution of SC and 0.5ml of standard stock solution of EV were transferred in to 50ml volumetric flask, volume was made up to the mark with mobile phase to get working standard solution comprising 250 µg/ml SC and 10 µg/ml EV.

Preparation of Sample Solutions of SC and EV:

20 tablets were weighed accurately and powdered; a quantity of tablet powder equivalent to 25mg SC and 1mg of EV was weighed accurately and transferred to a 100ml volumetric flask. The tablet powder was dissolved in diluent with the aid of ultra-sonication, and then diluted up to mark with diluent and filtered through a whatman filter paper to produce a test solution having 250µg/ml SC and 10µg/ml EV. The resulted test solution was then analyzed for assay determination.

System suitability parameters:

System suitability tests were performed to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters monitored for system suitability includes retention time, theoretical plate number, peak area, tailing factor and resolution. The repeatability of these parameters was checked by injecting three times the test solution of 250µg/ml of SC and 10µg/ml of EV. The results shown in Table 1 were within acceptable limits.

Forced Degradation Studies:

Forced Degradation Studies of the drugs, in combination, were performed under different stress conditions as mentioned in ICH guideline Q1A (R2). The standard solution containing 250µg/ml Sildenafil citrate and 10µg/ml Estradiol valerate was subjected to acidic, alkaline, oxidative, thermal and photolytic stress condition. Acidic and alkaline degradation were performed using up to 2N strength of acid/base at different temperature. Oxidative stress studies were carried out for using 3-20% H_2O_2. The sample solution containing Sildenafil citrate and Estradiol valerate was subjected to valerate were subjected to thermal and photo degradation as explained in detail below.

A) Acid catalyzed degradation

The solution containing each of SC (250µg/ml) and EV (10µg/ml) was subjected to different strengths of Hydrochloric acid (HCl) like 0.1N, 1N and 2N HCl at 40°C for 60min. and 50°C for 90min. for each strength of HCl. The solution was neutralized and volume was made up using the mobile phase. The maximum degradation was obtained with 2N HCl at 50°C for 90min. The representative results are shown in Table 2.

B) Alkali catalyzed degradation

The solution containing each of SC (250µg/ml) and EV (10µg/ml) was subjected to different strengths of Sodium hydroxide (NaOH) like 0.1N and 1N and at 40°C for 60min. and 50°C for

90min. for each strength of NaOH. The solution was neutralized and volume was made up using the mobile phase. The maximum degradation was obtained with 1N NaOH at 50°C for 90min. The representative results are shown in Table 2.

C) Peroxide degradation

The solution containing each of SC (250µg/ml) and EV (10µg/ml) was subjected to different strengths of Hydrogen peroxide (H_2O_2) like 3%, 10% and 20% and at 40°C for 60min. and 50°C for 90min. for each strength of H_2O_2. Final volume was made up using the mobile phase. The maximum degradation was obtained with 20% H_2O_2 at 40°C for 60min. The representative results are shown in Table 2.

D) Thermal Degradation

The sample solution was exposed to a different temperature for different time period. The solution containing SC (250µg/ml) and EV (10µg/ml) was prepared using the mobile phase for each set of thermal condition. The maximum degradation was obtained at 90°C for 150min. The representative results are shown in Table 2.

E) Photolytic Degradation

The sample solution was exposed to UV light at 254 nm for 3, 6, 12, 24, 36 and 48 hours. The solution containing SC (250µg/ml) and EV (10µg/ml) was prepared using the mobile phase for each set of photolytic condition. There was no much degradation found for any of the drug even after exposure of UV light up to 48 hour. The representative results are shown in Table 2.

METHOD VALIDATION:

1) Specificity:

Specificity of method can be termed as absence of any interference at retention times of samples. Specificity was performed by injecting blank and standard preparations. Chromatograms were recorded and retention times from sample and standard preparations were compared for identification of analytes.

2) Linearity and Range:

A series of standard solutions 125-750µg/ml of SC and 5-30µg/ml of EV were prepared. An aliquot of 20µl of each solution was injected 3 times for each standard solutions and peak area was observed. Plot of average peak area versus the concentration is plotted and from this the correlation coefficient and regression equation were generated. The calibration data of SC and EV is given in Table 3, while Figure 4A and Figure 4B represents linearity graphs of both drugs respectively.

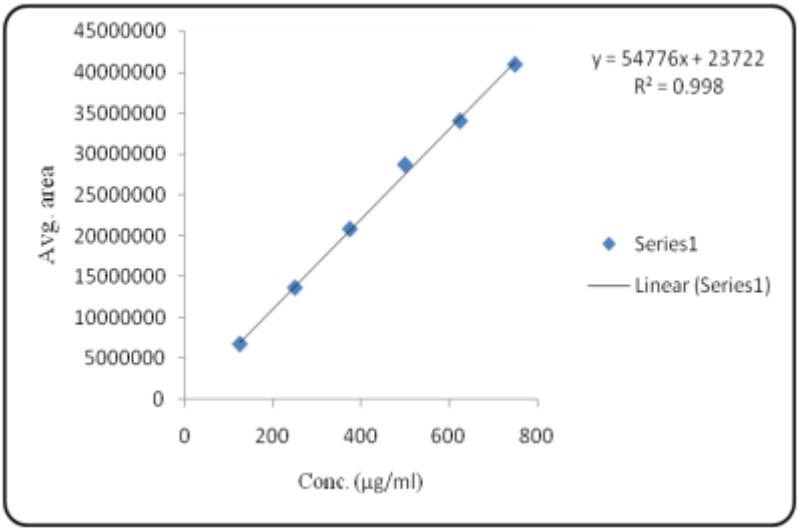

Figure 4A: Linearity graph for Sildenafil citrate

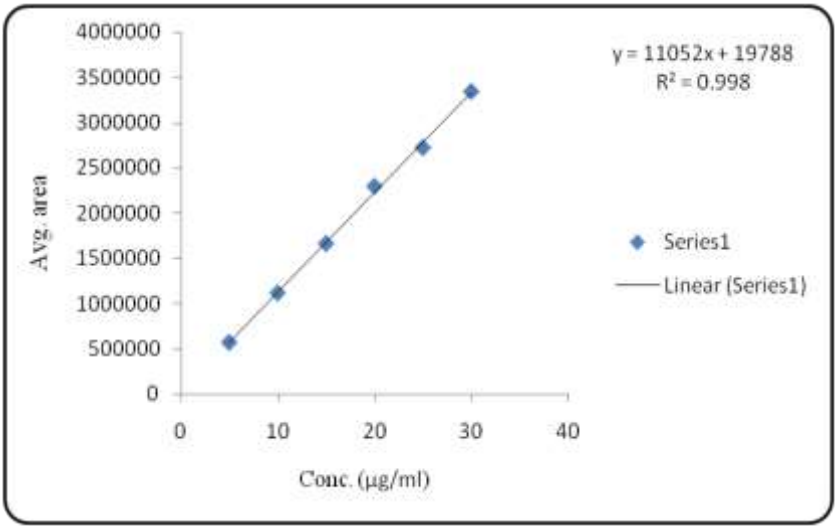

Figure 4B: Linearity graph for Estradiol valerate

3) Precision:

The method was validated in terms of intra-day and inter-day precision. The solution containing 250µg/ml of SC and 10µg/ml of EV was injected six times for repeatability study. Inter-day and Intra-day study was performed by injecting 250, 375 and 500µg/ml of SC and 10, 15 and 20µg/ml of EV solutions three times for each aliquots. The %RSD for precision study was found less than 2% as shown in Table 4.

4) Accuracy:

Accuracy was determined by calculating recovery of SC and EV by the standard addition method. Known amounts of standard solutions of SC (125, 250 and 375µg/ml) and EV (5, 10 and 15µg/mL) were added to a pre quantified test solutions of SC (250µg/ml) and EV (10µg/ml). Each

solution was injected in triplicate and the recovery was calculated by measuring peak areas. Results obtained are shown in Table 5.

5) LOD and LOQ:

LOD and LOQ for SC and EV were calculated as suggested by ICH guidelines using equations LOD = 3.3 σ/s and LOQ = 10 σ/s, respectively. Where, σ is the SD of the response and S is the slope of the calibration curve.

6) Robustness:

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by making two small changes. The mobile ration was changed by ± 0.2 ml and detection wavelength was changed by ± 2 nm. After each changes sample solution was injected and system suitability parameters were observed. The results were shown in Table 6.

RESULT AND DISCUSSION

System suitability study

The detection was carried out in the UV region at 290nm. The different composition of mobile phase was testing and the composition giving retention time of 2.55 min for SC and 5.56 min for SC with good resolution and theoretical plates was selected, that optimized mobile phase was acetonitrile: water (80:20 %v/v). A chromatogram of the mixture in optimized conditions is shown Figure 3 and the system suitability parameters are shown in Table 1.

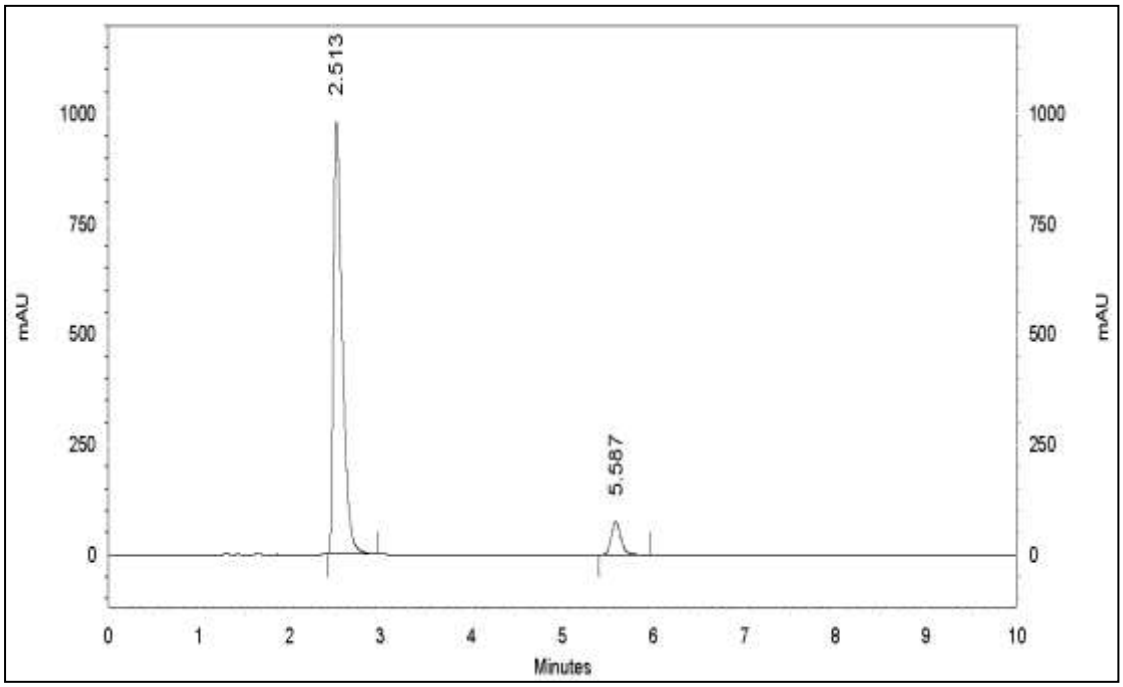

Figure 3: Optimized condition chromatogram of SC and EV

Table 1: Results for System suitability parameters

Parameters	Sildenafil citrate (mean ± SD)*	Estradiol valerate (mean ± SD)*
Retention time (min)	2.553 ± 0.04	5.561 ± 0.05
Theoretical plate	3532.67 ± 73.45	14244 ± 273.81
Avg. area ± SD	13717616.67 ± 28946.4	1123867.33 ± 2932.51
Tailing factor	1.787 ± 0.11	1.180 ± 0.06
Resolution	17.49 ± 0.18	
*** = average of three determinations, SD=Standard deviation**		

Forced degradation study

The mentioned percent degradation of both Sildenafil citrate and Estradiol valerate is with respect to their decrease in the areas. Peak purity of the drug is not affected. There are few impurities peaks have been observed for acidic, basic, oxidative and thermal degradation but no degradant observed for photolytic condition. Thus, the conditions subjected to the drugs make them undergo forced degradation thereby being able to detect any difference in the response in terms of their areas and impurities. The final results for the stress conditions are shown in Table 2.

Table 2: Forced degradation results for SC & EV

Degradation method	Optimized Condition	% Degradation	
		SC	EV
Acid	2 N HCl for (60 °C for 90 min.)	18.37%	12.43%
Base	1 N NaOH for (60 °C for 90 min.)	10.12%	17.75%
Oxidation	20% H_2O_2 (30 °C for 60 min.)	15.36%	18.45%
Thermal	90 °C for 150 min.	11.64%	19.46%
Photolytic	254nm UV light (48hour)	2.61%	2.38%

METHOD VALIDATION

A) Specificity

The method was found to be specific as there was no interference observed in any of the parameters under observation.

B) Linearity and Range

The linearity of SC and EV were found between 125-750 µg/ml and 5-30 µg/ml, respectively. The results are shown in Table 3.

Table 3: Linearity study data for SC and EV

Sr. No.	Sildenafil citrate			Estradiol valerate		
	Conc. (µg/ml)	Average area *	%RSD	Conc. (µg/ml)	Average area *	%RSD
1	125	6835213	0.458073	5	569619	0.206922
2	250	13713396	0.83852	10	1118249	0.304744
3	375	20873581	0.469674	15	1665046	0.360653

4	500	28738938	0.798329	20	2298643	0.152681
5	625	34075796	0.965848	25	2725399	0.191940
6	750	40973853	0.614986	30	3347031	0.125331

*= average of three determinations, RSD=Relative standard deviation

C) Precision

The %RSD for repeatability study for SC and EV was found to be 0.65 and 0.72 respectively. The Inter-day and Intra-day study also show %RSD value for SC and EV within the acceptable limit. Results for precision study are shown in Table 4.

Table 4: Precision study results for SC and EV

Parameters	**Conc.**		**% RSD**	
	SC (µg/ml)	**EV (µg/ml)**	**SC**	**EV**
Intra-day* precision	250	10	0.58	0.35
	375	15	0.77	0.47
	500	20	0.70	0.49
Inter-day* precision	250	10	0.28	0.69
	375	15	0.70	0.43
	500	20	0.73	0.78
Repeatability**	250	10	0.65	0.72

*= average of three determinations,
**= average of six determinations

D) Accuracy

Accuracy of the method was confirmed by recovery study at three levels (50%, 100 % and 150%) of standard addition. Percentage recovery for SC was found to be 98.90-101.43%**,** while for EV it was found to be 99.06-101.23% as shown in Table 5.

Table 5: The accuracy study results of SC and EV

Drug	**Accuracy Level %**	**Amount taken (µg/ml)**	**Amount added (µg/ml)**	**Total Amount found* (mg/ml) ± S.D.**	**% Recovery ± SD**
Sildenafil citrate	50%	250	125	370.86 ± 0.81	98.90 ± 0.22
	100%	250	250	507.16 ± 1.34	101.43 ± 0.27
	150%	250	375	627.68 ± 0.96	100.43 ± 0.15
Estradiol valerate	50%	10	5	14.86 ± 0.12	99.06 ± 0.77
	100%	10	10	20.24 ± 0.11	101.23 ± 0.54
	150%	10	15	25.04 ± 0.13	100.14 ± 0.52

*= average of three determinations

E) LOD and LOQ

The LOD was found to be 10.59 µg/ml for SC and 1.17 µg/ml for EV, while the LOQ was found to be 32.10 µg/ml for SC and 3.55 µg/ml for EV.

F) Robustness

The typical variations studied under this parameter were mobile phase composition and detection wavelength. Overall %RSD was found to be less than 2% for all the variations which indicates that the proposed method is robust. Robustness data are shown in Table 6.

Table 6: Robustness study results for SC and EV

Parameter	Change Level	Peak Area	
		SC	EV
Mobile phase composition (±2.0 ml)	78: 22	13405350	1091614
	80: 20#	13710639	1119103
	82: 18	13699761	1118367
	% RSD	1.27	1.41
Detection wavelength (±2.0 nm)	288	13496062	1153426
	290#	13742377	1122073
	292	13885468	1119124
	% RSD	1.44	1.68
#= actual parameter as control standard			

G) Analysis of marketed formulation by proposed method

Applicability of the proposed method was tested by analyzing the commercially available marketed formulation. The percentage of SC and EV was found to be 100.68% for SC and 99.58% for EV. Results as % Assay are shown in Table 7.

Table 7: Analysis of marketed formulation by proposed method

Sildenafil citrate			Estradiol valerate		
Labelled amount (mg)	Amount found (mg)	% Assay	Labelled amount (mg)	Amount found (mg)	% Assay
25 mg	25.115	100.46	1mg	0.999	99.94
	25.441	101.76		1.005	100.53
	24.952	99.81		0.982	98.25
Mean ± SD	25.169 ± 0.249	100.68 ± 0.99	Mean ± SD	0.996 ± 0.012	99.58 ± 1.19
%RSD	0.988	0.99	%RSD	1.191	1.19

CONCLUSION

From the above discussion it can be concluded that the proposed method is precise, accurate and stability indicating. Results are in good agreement with label claim which indicates there is no interference of exicipients. Therefore the proposed method can be used for routine analysis of Sildenafil citrate and Estradiol valerate in combined tablet formulation.

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