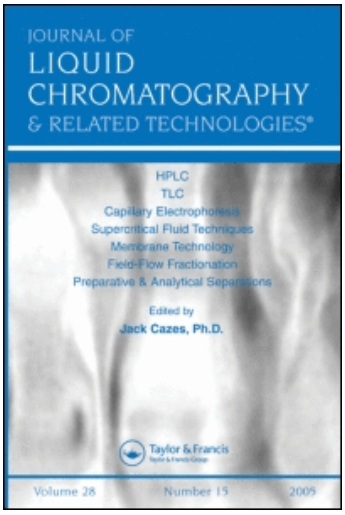

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Validation of a Reversed-Phase HPLC Method for the Analysis of Sildenafil Citrate in Pharmaceutical Preparations and in Spiked Human Plasma

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Abstract: A simple, precise, rapid, and accurate reversed-phase high performance liquid chromatographic (RP-HPLC) method has been developed for the determination of sildenafil citrate (SLD) in pharmaceutical dosage forms and spiked human plasma. Chromatography was carried out on a C_{18} reversed-phase column, using a mixture of acetonitrile:water(45:55, v/v) as a mobile phase at a flow rate of 1 $mL \cdot min^{-1}$. Phenobarbital sodium was used as an internal standard (IS) and detected using a diode array detector at 220 nm. Retention times of SLD and IS were 7.2 and 3.2 min, respectively. The linear range of SLD was found to be 5×10^{-8}–1×10^{-5} $mol \cdot L^{-1}$. Limit of quantitation (LOQ) and limit of detection (LOD) were calculated to be 7.5×10^{-8} and 2.2×10^{-8} $mol \cdot L^{-1}$, respectively. The method was validated for its linearity, precision, accuracy, stability, robustness, and ruggedness. The proposed method was applied to sildenafil tablets and spiked human plasma. In addition, the results were compared with those obtained from UV-spectrophotometry.

Keywords: Sildenafil citrate, RP-HPLC, Pharmaceutical analysis

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INTRODUCTION

Sildenafil citrate (SLD) 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d] pyrimidin-5-yl) –4-ethoxyphenyl] sulfonyl]-4-methyl piperazine citrate, is a novel orally active selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), and is used for increasing penile erectile activity in patients with male erectile dysfunction. Sildenafil causes cGMP to accumulate in corpus cavernosum[1–3] resulting in smooth muscle relaxation and increaser blood flow. Its chemical structure is shown in Fig. 1.

No pharmacopoeial method has been found for the assay of SLD formulations. However, various analytical methods have been reported for the determination of SLD including HPLC,[4–10] micellar electrokinetic capillary chromatography,[11,12] square-wave adsorptive stripping voltammetry,[13,14] flow injection analysis,[15] and electrochemical study.[16]

The aim of this study was to investigate and develop the method for determination of SLD by a simple, rapid, precise, and accurate reversed-phase HPLC combined with UV detection, and apply the method to the pharmaceutical dosage forms, as well as spiked human plasma. In addition, the results were compared with those obtained from UV-spectrophotometry.

EXPERIMENTAL

Apparatus

The HPLC system consisting of a Model Spectra System SCM 1000 degasser, Spectra System P1000 isocratic pump, Spectra System SN4000 connector, Spectra System UV6000LP diode array detector (Thermo Finnigan, USA)

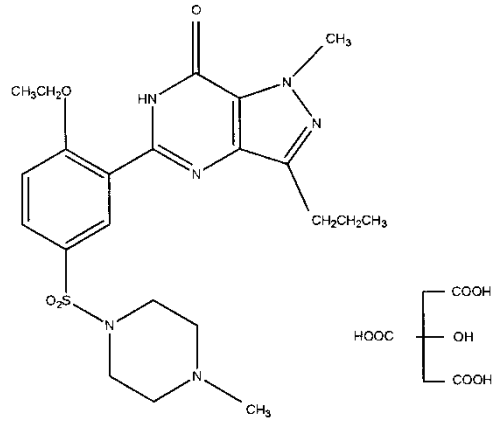

Figure 1. The chemical structure of sildenafil citrate.

was used. The analyte peaks were resolved at ambient temperature on a Phenomenex Luna C18 (150 × 4.6 mm I.D.; particle size 5 μm) column. The volume of the injection loop was 20 μL. The data were collected and analyzed with Chrom Quest™ 4.0 HPLC database system on a IBM Pentium IV computer. A Shimadzu Spectrophotometer Model UV 2401 PC (Japan) and quartz cells were used in the measurement of the absorbance by UV-spectrophotometry.

Chemicals

Standard SLD (99.9%) and Viagra® tablets containing 50 mg active material were kindly supplied from Fako İlaçları A.S. (İstanbul, Turkey) and Pfizer İlaç San. Tic. A.S. (İstanbul, Turkey), respectively. SLD was used without further purification. Acetonitrile, LiChrosolv® for chromatography, and phenobarbital sodium (as internal standard (IS) 99.8%) were purchased from Merck (Germany). The water used in the experiment was double distilled.

Chromatographic Conditions

The proposed method was conducted using a reverse-phase technique, UV monitoring at 220 nm, and phenobarbital sodium as an IS. A simple mobile phase without the need of buffer was chosen, and did not involve a complex procedure to prepare sample solutions. The mobile phase consisting of acetonitrile:water (45:55, v/v) was filtered through a 0.45 μm membrane filter and degassed for 20 min before use, and pumped from the reservoir to the column at the rate of $1\,mL \cdot min^{-1}$.

Procedure

Stock and Standard Calibration Solutions

Stock solutions of SLD ($1.035 \times 10^{-3}\,mol \cdot L^{-1}$) and phenobarbital sodium (IS) ($1.32 \times 10^{-3}\,mol \cdot L^{-1}$) were prepared in water. Standard solutions of SLD were prepared with mobile phase in the range of 2.07×10^{-7}–$1.035 \times 10^{-6}\,mol \cdot L^{-1}$, maintaining the concentration of IS at a constant level of $1.32 \times 10^{-5}\,mol \cdot L^{-1}$. Twenty microliters of each solution was injected into the column and chromatograms were recorded. The calibration curve for the HPLC analysis was constructed by plotting the ratio of peak normalization of the SLD to internal standard against concentration.

Analysis of Tablets by HPLC

Ten tablets were weighed to get the average weight and then powdered. The fine powder, equivalent to 50 mg of SLD, was weighed and transferred into a

100 mL calibrated flask and dissolved using mobile phase. This mixture was sonicated for 20 min and then filtered through a 0.45 μm membrane filter. After filtration, the appropriate volume (0.1 mL) was taken into a 100 mL flask, added to 1.0 mL IS (final concentration is $1.32 \times 10^{-5}\,\mathrm{mol \cdot 1^{-1}}$), and made up to volume with mobile phase, to give an expected concentration in the calibration range (equivalent to $7.5 \times 10^{-7}\,\mathrm{mol \cdot L^{-1}}$ of sildenafil citrate). All determinations were conducted in triplicate. The amount of SLD was calculated from the related linear regression equations.

Recovery Studies in Spiked Human Plasma Samples

Plasma samples obtained from healthy volunteers were stored frozen until assayed. The frozen plasma samples to be used were thawed at room temperature and were centrifuged for 15 min at 3000 rpm. Separate aliquots of 1 mL plasma were spiked with SLD standard solution in water to achieve the final concentration of $1.035 \times 10^{-6}\,\mathrm{mol \cdot L^{-1}}$, and IS of $1.32 \times 10^{-5}\,\mathrm{mol \cdot L^{-1}}$, and was diluted to 10 mL with the same serum sample. Then, 1 mL acetonitrile was added and mixed well using a vortex. The precipitated proteins were separated by a centrifuge for 15 min at 3000 rpm. The supernatant was carefully taken, and filtered through a 0.45 μm membrane filter. After filtration, samples were injected to the HPLC apparatus under the same conditions as mentioned above.

UV-Spectrophotometric Studies

A series of standard SLD dilutions in the concentration range 1×10^{-5} and $5 \times 10^{-5}\,\mathrm{mol \cdot L^{-1}}$ was prepared using $1 \times 10^{-3}\,\mathrm{mol \cdot L^{-1}}$ stock solutions using bidistilled water. The powdered tablets mentioned above were used for the spectrophotometric assay. The fine powder equivalent to 50 mg of SLD was weighed and transferred into a 100 mL calibrated flask, and dissolved in bidistilled water. This mixture was sonicated for 20 min and then filtered through a 0.45 μm membrane filter. After filtration, an appropriate volume (5 mL) was taken into a 100 mL flask, and made up to volume with bidistilled water, to give an expected concentration in the calibration range (equivalent to $3.7 \times 10^{-5}\,\mathrm{mol \cdot L^{-1}}$ of SLD). The absorbances of the solutions were measured at 292 nm using quartz cells.

RESULTS AND DISCUSSION

A reversed-phase isocratic procedure is proposed as a suitable method for the analysis of SLD in tablets. A mixture of acetonitrile:water (45:55, v/v) at a flow rate of $1\,\mathrm{mL \cdot min^{-1}}$ was found to be an appropriate mobile phase, allowing adequate and rapid separation of SLD and phenobarbital sodium

as IS. A typical chromatogram for analysis of SLD and IS is shown in Fig. 2. Under these conditions, the retention times obtained were 7.2 min for the SLD and 3.2 min for IS. The IS was clearly separated from the drug. The retention times for both drug and IS were highly precise. The calibration curve for SLD was calculated by plotting the ratio of peak normalization of drug to the IS against the concentration. The characteristics of regression equations are given in Table 1.

Application of the Method to Tablets and Spiked Human Serum

The methods used in the above study were applied to the tablets and spiked human serum. The results were tabulated for tablets as percent in Table 2. The percentage recovery results of SLD in spiked human serum are shown in Table 3.

Validation of the Method

The proposed HPLC method was validated in terms of linearity, precision, accuracy, stability, robustness, and ruggedness.

Linearity

The linearity of the method was determined in terms of the correlation coefficient between its SLD and the ratio of peak normalization of SLD to that of IS.

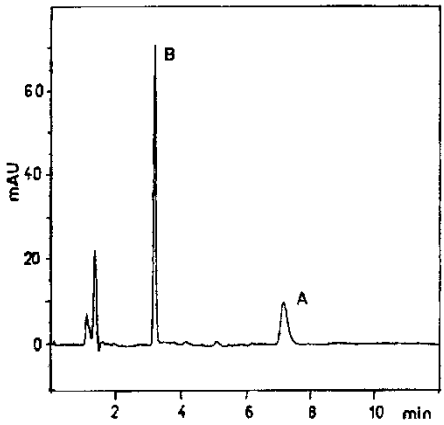

Figure 2. A typical chromatogram for sildenafil citrate (A) and phenobarbital sodium (B).

Table 1. Regression characteristics of the proposed method

Parameter	Value
Linearity range($mol \cdot l^{-1}$)	5×10^{-8}–1×10^{-5}
Slope (a)	217723.7
Intercept (b)	0.0017
Correlation coefficient (r)	0.9998
RSD of slope	1.16
RSD of intercept	0.20
LOQ ($mol \cdot l^{-1}$)	7.5×10^{-8}
LOD ($mol \cdot l^{-1}$)	2.2×10^{-8}

RSD, Relative standard deviation; LOD, Limit of detection; LOQ, Limit of quantitation.

The data were analyzed by linear regression least-squares method. The linear range of SLD was found to be 5×10^{-8}–1×10^{-5} $mol \cdot L^{-1}$. To calculate the limit of quantitation (LOQ) and limit of detection (LOD), signal to noise ratio 10 and 3, respectively, were used. Limit of quantitation (LOQ) and limit of detection (LOD) were calculated to be 7.5×10^{-8} and 2.2×10^{-8} $mol \cdot L^{-1}$, respectively.

Precision

The method was validated for its intra and inter day precision. As seen in Table 4, in the range of 2.07×10^{-7}–1.035×10^{-6} $mol \cdot L^{-1}$, RSD values were obtained between 0.35 and 0.70. These data indicate a considerable degree of precision and reproducibility for the proposed method, both during one day and between different days. Sample solutions, analyzed after one week, did not show any appreciable change in assay values. No interfering peaks were found in the chromatogram, indicating that the tablet

Table 2. Assay results of SLD as percent in tablets*

	HPLC	UV Spectrophotometry
Mean %	98.3	99.2
n	8	8
RSD	1.4	0.96
Confidence limit ($p = 0.05$)	± 1.18	± 1.83
t-test of significant	0.38	$t_{0.05} = 2.14$ (table)
F-test of significant	1.46	$F_{0.05} = 4.17$ (table)

*Each tablet contains 50 mg of SLD.

Table 3. The assay results of SLD in spiked plasma for recovery studies

Concentration added ($mol \cdot l^{-1}$)	Concentration $\pm$ SD* ($mol \cdot l^{-1}$) recovered	Percentage recovered
2.07×10^{-7}	$2.10 \times 10^{-7} \pm 0.45$	101.47
4.14×10^{-7}	$4.11 \times 10^{-7} \pm 0.85$	99.3
6.21×10^{-7}	$6.18 \times 10^{-7} \pm 0.55$	99.5
8.28×10^{-7}	$8.20 \times 10^{-7} \pm 0.67$	99.0
1.035×10^{-6}	$1.02 \times 10^{-6} \pm 0.37$	98.5

*Mean $\pm$ Standard deviation of five determinations.

excipients did not interfere. Known amounts of the analyte at 3, 5, and 10 μg concentration levels were added and assayed for the recovery of SLD from standard solutions and powdered tablets. The results obtained are given in Table 5. The lower RSD values of the assay indicate that the method is highly precise and accurate.

The precision of the assay was determined in terms of the intra-day and inter-day variation in the ratio of peak normalization using five replicate injections of five different concentrations, which were prepared and analyzed on the same day and on three different days, respectively.

Accuracy

To study the accuracy and to check the interference from the excipients used in the formulations, recovery studies were carried out by the standard addition method. The accuracy of the proposed method was assessed by adding known amounts (3, 5, 10 $\mu g \cdot mL^{-1}$) of the SLD to the solutions of known

Table 4. Intra- and inter-day precision for SLD assay

Concentration of SLD (10^{-7} $mol \cdot l^{-1}$)	Observed concentration of SLD (10^{-7} $mol \cdot l^{-1}$)			
	Intra-day		Inter-day*	
	Mean (n = 5)	RSD	Mean (n = 5)	RSD
2.07	2.08	0.35	2.06	0.47
4.14	4.16	0.45	4.12	0.53
6.21	6.20	0.51	6.18	0.60
8.28	8.31	0.58	8.25	0.65
10.35	10.38	0.40	10.31	0.70

*On three different days.

Table 5. Recovery and accuracy for the determination of SLD in tablets

	Recovery from standard solution		Recovery from tablet	
Amount added (μg)	Mean (±RSD) amount (μg) (n = 5)	Mean (±RSD) recovery (%) (n = 5)	Mean (±RSD) amount (μg) (n = 5)	Mean (±RSD) recovery (%) (n = 5)
3	3.13 ± 0.18	104.1 ± 0.11	2.95 ± 0.16	98.3 ± 1.51
5	5.08 ± 0.26	101.6 ± 0.52	4.92 ± 0.43	98.4 ± 0.96
10	9.95 ± 0.42	99.5 ± 0.82	9.90 ± 0.64	99.1 ± 0.83

concentrations along with IS, and then assayed using this method. Also, a known amount of SLD was added to the volumetric flask containing the powdered sample of the tablet formulation with a known amount of the SLD and IS. In both cases, the recovery studies were replicated five times. The accuracy was expressed in terms of recovery, and was calculated by the ratio of peak normalization of measured SLD concentration to the expected concentration with 100, so as to give the percent recovery.

Stability

The sample solutions of SLD in pure drugs and tablets were tested for stability over three days. The samples were analyzed by the optimized HPLC conditions in fresh and stored solutions at room temperature. The RSD values were obtained in the range of 0.25 to 0.62. This indicated that the SLD solutions were highly stable over a period of three days without degradation.

Robustness and Ruggedness

The optimum HPLC conditions (such as mobile phase ratio, flow-rate, detection wavelength, sonication time, column, and different analysts) have produced slight changes in samples of SLD. Considering the modifications in the system parameters, we did not observe significant changes. All these results indicate that the proposed method is robust and rugged.

There is generally no need for a comparison of the method for validation. However, the results of the above studies were supported by analysis of SLD by the UV-spectrophotometric studies. Calibrations were carried out by preparing standard solutions. The relationship between absorbance (A) and concentration of sildenafil citrate (C) was found to be $A = 11807.5\ C\ (mol \cdot L^{-1}) + 1.26 \times 10^{-3}$; $r = 0.9998$ at 292 nm.

The results were also evaluated statistically. No statistically significant difference was observed between the methods at the 95% probability level (F- and t-test). The results of the study indicated that the proposed HPLC method is suitable, simple, precise, rapid, and accurate for the determination of SLD in the pharmaceutical dosage form and human serum.

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