Quantitation of Sildenafil in Human Plasma by Reverse Phase High Performance Liquid Chromatography

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Summary

A rapid, sensitive and specific method to quantify sildenafil in human plasma using diazepam as the internal standard (ISTD) was developed and validated. The drug was extracted from the matrix by liquid-liquid extraction. The analyte and the IS were separated on a reverse phase C_{18} analytical column, (LiChrosphere RP 18_e 250mm × 4.0 mm i.d. 5 μm) by isocratic separation mode and analyzed by HPLC using LiChrosphere C18, 5μ, 30X4.0mm as guard column. The run time was 20 min and a linear calibration curve over the range 10-500 ng ml^{-1} ($r^2 > 0.9822$). The method was shown to be accurate, with intra-day accuracy (92.30% to 98.83%) and inter-day (94.13% to 98.43%); and precise, with intra-day precision (6.38% to 14.22%) and inter-day precision (5.68% to 11.74%). The method is suitable for use in pharmacokinetic studies and routine plasma monitoring of sildenafil.

Keywords: Sildenafil; Diazepam; reverse phase high performance liquid chromatography; liquid-liquid extraction.

Introduction

Erectile dysfunction (ED) of varying degrees occurs in an estimated 20 to 30 million American men and is associated with adverse effects on quality of life; particularly personal well-being, family and social interrelationships[1]. It is a common condition in middle-aged and older men and frequently occurs in association with various chronic illnesses[2].

The two connotations, which mean sexual dysfunction, viz., impotence and erectile dysfunction (ED), express two different concepts. Impotence is a general male sexual dysfunction that includes libidinal, orgasmic, and ejaculatory dysfunction. ED is the inability to achieve or maintain an erection sufficient to allow satisfactory sexual intercourse and is part of the general male sexual dysfunction[3]. The incidence of erectile dysfunction increases with age and with risk factors for vascular disease, including smoking, diabetes and hypertension[4].

The introduction of sildenafil (UK-92, 480) has revolutionized the treatment of erectile dysfunction (ED). The availability of sildenafil, the first effective oral agent for ED, has also expanded the field of sexual healthcare to include general and primary care practitioners and other non-urology specialists[5]. Sildenafil is the most successful new drug launched in the recent times and has an annual sale of around $800 million[6]. Chemically sildenafil is (1-[4 ethoxy-3- (6,7 dilhydro-1-methyl-7-oxo-3-propyl-H-pyrazolo- {4,3} pyrimidin-5-yl) phenyl sulfonyl]-4-methyl pyperazine) [7]. Sildenafil is an innovative molecule, effective in most cases of erectile dysfunction, acting by inhibition of type 5 phosphodiesterase in the corpus cavernosum[8].

To date, many analytical methods are reported for the estimation of sildebafil, they are either tedious involving expensive instruments[9-12] or not suitable for its determination in biological fluids[13 and 14].

Methods

Chemicals and Reagents

Sildenafil and diazepam (internal standard) were procured from Sigma-Aldrich (Steinheim, Germany). Super gradient grade methanol and HPLC grade tertiary butyl methyl ether were purchase from Lab Scan Asia Limited (Samutsakorn, Thailand). Borate buffer was purchased from Merck limited (Mumbai, India). Purified water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Purified compressed nitrogen gas used was generated using a nitrogen generator Peak (Renfrewshire, UK). Blank human drug free plasma was procured from blood bank Synchron India Ltd., Ahemdabad. Pooled plasma was prepared and stored at -70ºC±5ºC until needed.

Instrumentation

The HPLC system consisted of a Merck Hitachi and Merck Hitachi L – 7400 Dual Wavelength absorbance detector with HSM software (for data acquisition). Chromatography was performed using a LiChrosphere RP 18_e (250mm x 4.0mm i.d, 5µ) column(Merck, Germany).

Chromatographic conditions

The chromatographic analysis was performed at 35ºC (column oven temperature) at isocratic elution. The mobile phase consisted of 24: 240: 170: 90 v/v of ion pair solution: water: acetonitrile: methanol with flow rate of 1.0ml/min. The auto-sampler tray temperature was maintained at 15ºC and a sample volume of 100µl was injected into the HPLC system with detection wavelength 230nm.

Ion pair solution was prepared by dissolving 5.5g of heptane sulphonic acid in 50ml of acetic acid and making up the volume to 100ml with distilled water.

Preparation of standard stock solutions

Standard stock solutions of sildenafil and internal standard diazepam were prepared by dissolving appropriate amounts of each drug in methanol to obtain final drug concentrations of 1mg/ml respectively. Intermediate and working solutions were prepared by further diluting these stock solutions with methanol-water (50:50, %v/v). The stock solutions were prepared extemporaneously.

Calibration standards and quality control

Calibration curves of sildenafil were prepared by spiking drug-free plasma at concentration of 10, 20, 100, 200, 300, 400 and 500 ng/ml and the analysis was carried out in duplicate for each concentration. The quality control samples were prepared in drug-free plasma at concentrations of 15, 300 and 450 ng/mL low, mid and high quality control (LQC, MQC and HQC respectively). Calibration curves were constructed using ratios of the observed analyte peak area to internal standard area versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficient data which were then used to calculate analyte concentration in each sample.

Sample preparation procedure

All frozen blank human plasma samples were thawed to ambient temperature and centrifuged at 3500 *g* for 5 min at 4ºC to precipitate solids. One ml of the plasma was transferred to a clean 15ml glass tube. To this 100µl of 3µg ml^{-1} internal standard.solution, 200µl of borate buffer pH9.0 were added and vortexed for 30seconds. To this mixture 5ml of tertiary butyl methyl ether was added and vortexed for 5 minutes and centrifuged for 5 minutes. The organic layer was removed and the procedure repeated. The combined organic layers were evaporated at 50^0C under nitrogen flushing. The residue was reconstituted in 150µl of mobile phase and 100µl from this was injected in to HPLC system.

Results

Specificity

Representative chromatogram of blank extracted human plasma is shown in Fig. 2. The retention time of sildenafil and diazepam were 11.0 and 16.0 min respectively. No endogenous peak interfered with analyte and ISTD in blank plasma extracts were observed as evidenced in fig.1.

Linearity

Linearity was established over 00-500 ng/ml to assess the performance of the method. A linear least-squares regression with a weighting factor of $1/x^2$ was carried out on the peak area ratios of sildenafil and ISTD versus sildenafil concentration of 9 plasma calibration standards (in duplicate) to generate a calibration curve. The coefficient of determination of sildenafil was ≥0.9929±0.0081. The results are shown in Table 1.

Limit of quantification

The lowest limit of quantification (LLOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 10ng/ml.

Accuracy and precision

The results from the validation of this method at each quality control levels (mean±SD, n=5) in human plasma are shown in Table 2 and Table 3. The within batch accuracy ranged from 92.30% to 98.83% and between batch accuracy was from 94.13% to

98.43%. The within batch precision was ≤14.22% and between batch precision was ≤11.74.

Recovery

The absolute recovery of sildenafil from plasma extracted with liquid-liquid extraction was calculated by comparing the peak areas. The ratio of two concentrations of sildenafil in human plasma quality control samples that underwent liquid-liquid extraction to that of identical concentrations of sildenafil prepared in methanol without extraction. The recoveries observed (n=5) were 79.196and 80.910 (15 and 450 ng/ml) for sildenafil. The results are shown in Table 4.

Stability

The results of stability of sildenafil under various conditions are shown in Table 5, 6 and 7. Under all these conditions sildenafil proved to be stable.

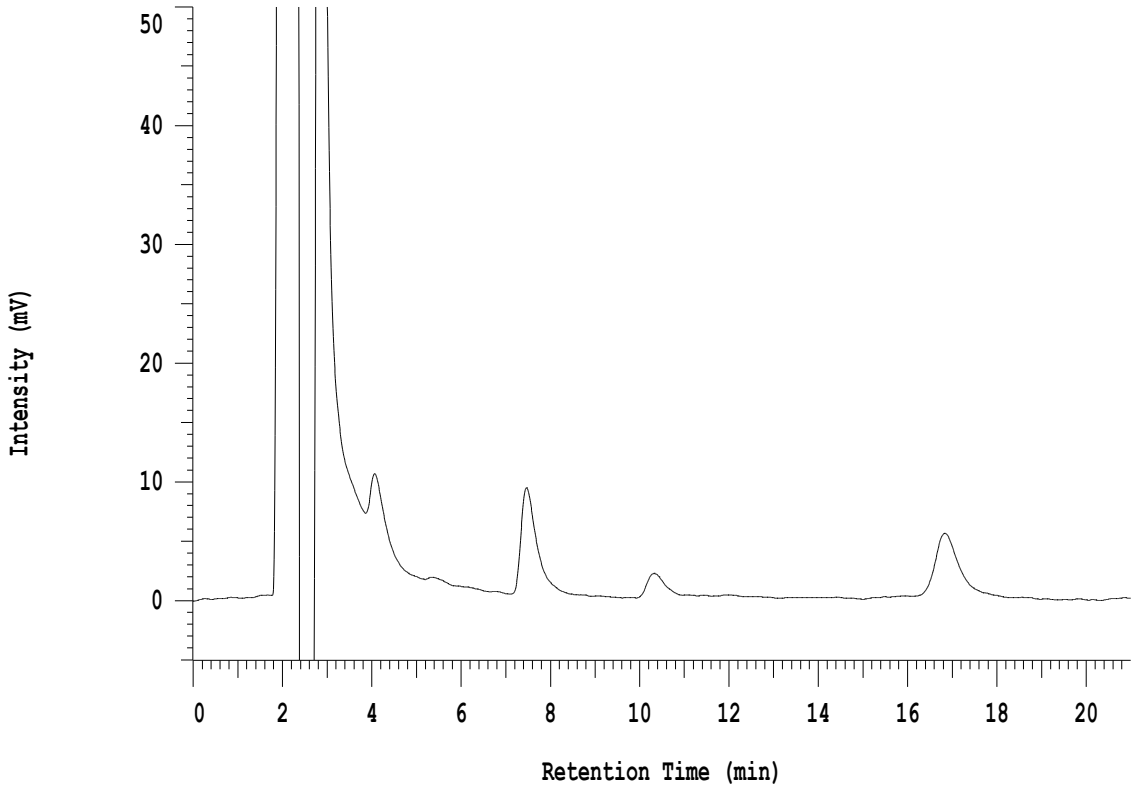

Figure 1. Representative blank chromatogram.

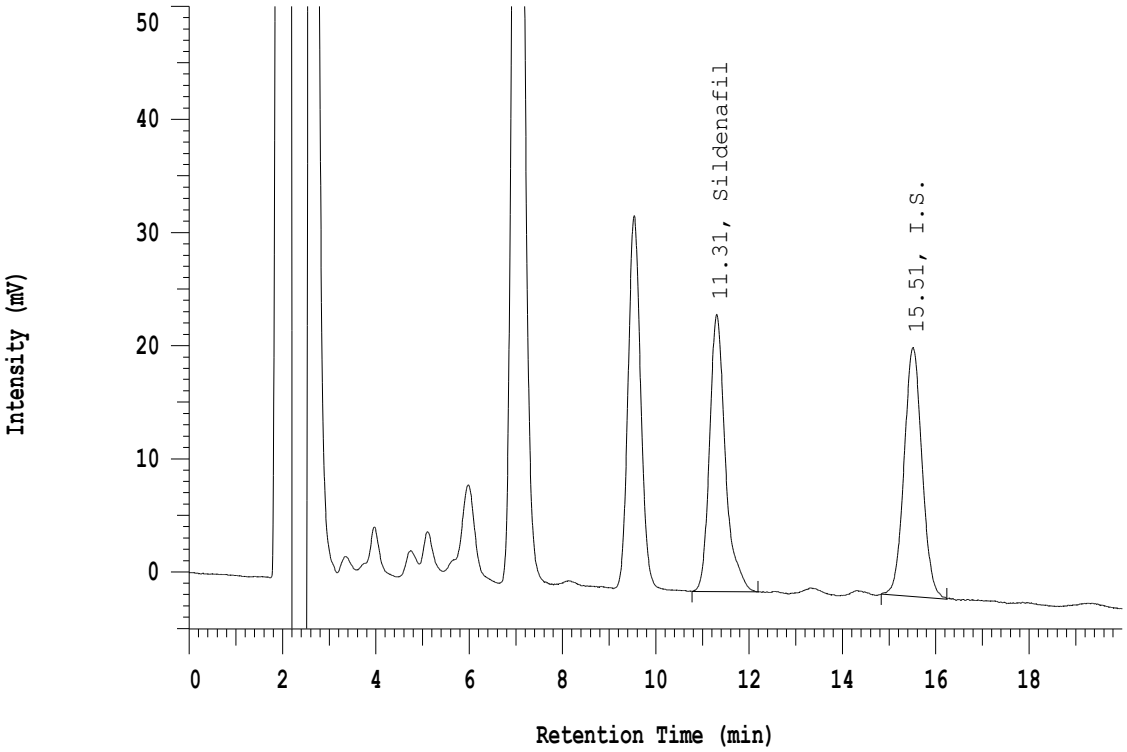

Figure 2. Representative chromatogram showing the elution of Sildenafil and Internal Standard.

Table 1. Data from calibration curves of sildenafil. (n=7)

Day	Slope	Intercept	r^2
1	0.00211649	0.03457964	0.9992
2	0.001950336	0.002303869	0.9993
3	0.001473829	0.020770370	0.9822
4	0.002168250	-0.0224517	0.9909
Mean	0.001927226	N.A.	0.9929
S.D.	0.0003	N.A.	0.0081
% RSD	16.4089	N.A.	0.8206

Table 2. Within day variability of quality control samples. (n=5)

Concentration added in ng ml-1	Concentration analysed ± S.D. in ng/ml	% C.V.	% Bias
15	14.6 ± 2.2	15.2228	-2.647
300	296.5 ± 16.8	5.6798	-1.169
450	415.4 ± 26.5	6.3843	-7.696

Table 3. Interday variability of quality control samples. (n=5)

Concentration added in ng ml^{-1}	Concentration analysed ± S.D. in ng/ml	% C.V.	% Bias
15	14.8 ± 1.7	11.7460	-1.575
300	282.4 ± 32.8	11.6033	-5.871
450	435.1 ± 24.7	5.6854	-3.315

Table 4. Percentage Extraction of sildenafil from plasma. (n=5)

	LQC		HQC	
	Peak height (Extracted)	Peak height (Non Extracted)	Peak height (Extracted)	Peak height (Non Extracted)
	907	1066	20426	25897
	917	1234	21235	26451
	926	978	19856	26874
	855	1066	23114	25321
	830	1256	20154	24965
Mean	887	1120	20957	25901.6
S.D.	42.113	119.883	1310.43	784.496
% Extracted	79.196		80.910	

Table 5. Bench top stability of sildenafil in human plasma. (n=3)

	0 hour		2 hours		4 hours	
Concentration added in ng ml^{-1}	Concentration analysed ± S.D. in ng/ml	%Bias	Concentration analysed ± S.D. in ng/ml	%Bias	Concentration analysed ± S.D. in ng/ml	%Bias
15	15.20 ± 1.710	-1.33	17.09 ± 3.232	-13.93	17.83 ± 0.699	-18.87
450	439.94 ± 16.168	2.24	471.62 ± 15.440	-4.80	433.55 ± 15.234	3.65

Table 6. Stability of freeze thaw cycle. (n=3)

	Cycle 0		Cycle 1		Cycle 2		Cycle 3	
Concentration added in ng ml-1	Concentration analysed ± S.D. in ng/ml	% Bias	Concentration analysed ± S.D. in ng/ml	% Bias	Concentration analysed ± S.D. in ng/ml	% Bias	Concentration analysed ± S.D. in ng/ml	% Bias
15	15.01 ± 1.721	-0.067	15.41 ± 0.940	-2.73	15.44 ± 1.477	-2.93	17.89 ± 1.741	-19.27
450	467.17 ± 12.549	-3.82	469.61 ± 17.237	-4.36	464.35 ± 21.376	-3.19	441.82 ± 18.953	1.82

Table 7. Stability of the plasma samples in autosampler. (n=3)

	0 hour		4 hours		8 hours		12 hours	
Concentration added in ng/ml	Concentration analysed ± S.D. in ng/ml	% Bias	Concentration analysed ± S.D. in ng/ml	% Bias	Concentration analysed ± S.D. in ng/ml	% Bias	Concentration analysed ± S.D. in ng/ml	% Bias
15	14.23 ± 2.104	5.13	16.72 ± 0.992	-11.47	17.20 ± 1.993	-14.67	16.36 ± 1.537	-9.07
450	459.39 ± 3.710	-2.09	464.94 ± 14.261	-3.32	460.75 ± 1.487	-2.39	440.13 ± 15.334	2.19

Discussion

A simple, reverse phase, high performance liquid chromatographic method using C_{18} column, isocratic pump and the UV detection was developed for the quantification of sildenafil in human plasma. The drug was extracted into Tertiary Butyl Methyl Ether (TBME) after adding borate buffer of pH 9.0. The organic layer was evaporated under nitrogen at 50^0C and the residue was reconstituted in mobile phase and injected into the HPLC system. The method was validated as per USFDA guidelines for Bioanalytical Method Validation[15].

Blank samples of different sources were screened for any interference from the endogenous or exogenous components. Further all blanks that were used for method development were screened prior to use. There was no interference from any sources of blank plasma. Drug free plasma samples of all volunteer were also screened after extracting into TBME. There were no peaks at retention times corresponding to drug or internal standard (Figure 1 and 2). This shows that there were no interferences of any component of plasma.

The method was linear over a range of 10 to 500ng ml^{-1}. The linearity was demonstrated using the 7 calibration standards on different days. During all the runs r^2 was found to be more than 0.9822 (Table 1). This indicates the method was linear over the range and extrapolated concentration is reliable.

Three sets of QCs (15, 300 and 450ng ml^{-1}) were analysed at different times of the same day to assess the within the day precision and accuracy. The concentrations of all the QCs were determined by first calibration curve. The precisions expressed as percentage being higher at lower concentration and vice versa (Table 2). Similarly three sets of QCs of same concentration were analysed on different days to assess interday precision and accuracy. The concentrations of all the calibrators are calculated with the first calibration curve. The precisions expressed as percentage coefficient of variation of the five determinations was between 5 and 12%, the value being higher at lower concentration and vice versa (Table 3). Thus the method is accurate, precise and is suitable for all pharmacokinetic studies.

A recovery analysis was performed at two QC levels. The recovery is assessed comparing the concentration measured on a QC after extraction and on a solution of analyte. A mean recovery of 79-80% was obtained at both the levels (Table 4). Hence extraction with two quantities of 5 ml TBME was proved to be satisfactory. Though sildenafil is soluble in other solvents like ethanol, chloroform etc., ethanol is miscible with water and forms a homogenous layer with human plasma, hence is seldom used. Chloroform in our case, formed a stable gel with plasma, making the separation of organic layer difficult and unreliable. Because of these reasons, present solvent i.e. TBME was tried and found satisfactory.

Stability of the analyte in the biological matrix is important for the validity of method for pharmacokinetic and other biological work. It was tested at various stages of sample processing that is bench top, freeze thaw and auto sampler stability. The analysed concentration in most of the cases hovered around the spiked concentration as indicated by percentage bias. Bias observed in most cases was around 3-4% and was never above 20%, in any case. This is evident from tables 5, 6 and 7 respectively. Hence the drug was stable in the human plasma *in vitro* under the normal laboratory conditions for the time period tested. Thus the method developed is precise, accurate and is suitable for pharmacokinetic studies of sildenafil.

Conclusions

A sensitive and specific method was developed and validated for the estimation of sildenafil in human plasma. The method satisfies the requirements of high sensitivity, specificity, and rapid sample throughput required for pharmacokinetic studies. The method can be applied for bioequivalence studies and therapeutic drug monitoring of sildenafil in human plasma.

References

1. Nehra A, Colreavy F, Khandheria BK, Chandrasekaran K. Sildenafil citrate, a selective phosphodiesterase type 5 inhibitor: urologic and cardiovascular implications. World J Urol 2001; 19: 40-45.

2. Piccoli M, De Santis LM. Myocardial ischemia after administration de sildenafil: description of a case and review of the literature. Ital Heart J 2000; 1: 1196-98

3. Bellinghieri G, Santoro D, Lo Forti B, Mallamace A, De Santo RM, Savica V Erectile dysfunction in uremic dialysis patients: diagnostic evaluation in the sildenafil era. Am J Kidney Dis 2001; 38(Suppl 1): S115-17.

4. Stephen LA. Potassium channels and erectile dysfunction. Vascular Pharmacology 2002; 38: 61-71.

5. Padma-Nathan H. A new era in the treatment of erectile dysfunction. Am J Cardiol., 1999; 84: 18N-23N.

6. Foye's Principles of medicinal chemistry. 5th Edition, Lippincot, Williams and Wilkins; 2002.

7. Boolell M, Allen MJ, Ballard SA, Get Atlee S, Muirhead GJ, Naylor AM, Osterloh I Jignell C. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. Int. J. Impot Res. 1996; 8: 47-52.

8. Wespes E, Sternon J, Hirsch E, Schulman C. Sildenafil. Rev Med Brux 1998; 19: 437-41.

9. Berzas Nevado JJ, Rodríguez Flores J, Castañeda Peñalvo G, Rodríguez Fariñas N. Determination of sildenafil citrate and its main metabolite by sample stacking with polarity switching using micellar electrokinetic chromatography. J. Chromatogr. A 2002; 953: 279-86.with polarity switching using micellar electrokinetic chromatography. J. Chromatogr. A 2002; 953: 279-86.

10. Eerkes A, Addison T, Naidong W. Simultaneous assay of sildenafil and desmethylsildenafil in human plasma using liquid chromatography-tandem mass spectrometry on silica column with aqueous-organic mobile phase. J Chromatogr B Analyt Technol Biomed Life Sci. 2002; 768: 277-84.

11. Saisho K, Scott KS, Morimoto S, Nakahara Y. Hair analysis for pharmaceutical drugs. II. Effective extraction and determination of sildenafil (Viagra) and its N-desmethyl metabolite in rat and human hair by GC-MS. Biol Pharm Bull 2001; 24: 1384-88.

12. Altiokka G, Atkosar Z, Sener E, Tunçel M. FIA of sildenafil citrate using UV-detection. J Pharm Biomed Anal 2001; 25: 339-42.

13. Moriyasu T, Shigeoka S, Kishimoto K, Ishikawa F, Nakajima J, Kamimura H, Yasuda I. Identification system for Sildenafil in health foods. Yakugaku Zasshi 2001; 121: 765-69.

14. Dinesh ND, Vishukumar BK, Nagaraja P, Made Gowda NM, Rangappa KS. Stability indicating RP-LC determination of sildenafil citrate (Viagra) in pure form and in pharmaceutical samples. J Pharm Biomed Anal 2002; 29: 743-48.

15. Available from: http://www.fda.gov/cder/guidance/4252fnl.htm