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Original Article

METHOD DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE ESTIMATION OF SILDENAFIL AND ITS METABOLITE PIPERAZINE N-DESMETHYL SILDENAFIL IN HUMAN PLASMA

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ABSTRACT

Objective: To develop and validate a simple, sensitive and specific liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the quantification of sildenafil and its metabolite piperazine n-desmethyl sildenafil in human plasma using clarithromycin as internal standards (IS).

Methods: Chromatographic separation was performed on thermo hypersil gold column (50x2.1mm) 5µm with an isocratic mobile phase composed of 0.02% formic acid and acetonitrile (30:70 v/v), at a flow-rate of 0.6 ml/min.Sildenafil, piperazine n-desmethyl sildenafil and internal standard clarithromycin were detected at m/z 475.0→100.3, 461.2→310.8 and 748.5→158.3 respectively.Drug, metabolite and internal standards were extracted by liquid–liquid extraction.

Results: The method was validated over a linear concentration range of 5.0–1000.0 ng/ml for sildenafil and 1.00-200.0 ng/ml for N-desmethyl sildenafil. This method demonstrated with in batch and between batch precision within 0.24-11.36 % and 2.07-9.98 % for sildenafil and 1.56-11.66 % and 2.33- 9.20 % for piperazine n-desmethyl sildenafil.This method demonstrated within batch and between batch accuracy for sildenafil within 91.71-102.94 % and 95.42-101.54 % respectively and for piperazine n-desmethyl sildenafil within 91.71-109.66 and 97.70-106.12 % respectively.

Conclusion: Both analytes sildenafil and piperazine n-desmethyl sildenafil were found to be stable throughout three freeze/thaw cycles, bench top and long term studies.The method can be successfully applied to pharmacokinetic studies

Keywords: Sildenafil, Piperazine n-Desmethyl Sildenafil, LC-MS/MS, Pharmacokinetic.

INTRODUCTION

The impact of sildenafil citrate on medical practice and on society has been large and given its prescription drug status and the intimate nature of the condition for which it is indicated somewhat unexpected. Access to the drug has been fairly open provided patients could cover its cost, and marketing and media attention have kept sildenafil and erectile dysfunction in the public eye [1]. There have even been reports of sildenafil's use as a recreational drug [2]. Since sildenafil's March 1998 approval by the US Food and Drug Administration (FDA), approximately10 million prescriptions have been filled[3]. The use of sildenafil has altered the workup and management of erectile dysfunction in men, increasing these patients' access to health care and patient-support services [4]. Many physicians consider sildenafil a good first-line therapy for erectile dysfunctionand an alternative to the use of oral androgens, injection of intracavemosal or transurethral prostaglandins, or vacuum devices[5, 7-8].

Erectile dysfunction (ED) is a common medical condition that affects the sexual life of millions of men worldwide. Numerous physical and psychological factors are involved in normal erectile function, including neurological, vascular, hormonal and cavernous functions [9]. The physiological mechanism for penile erection during sexual stimulation involves release of nitric oxide (NO)from cavernous nerves and vascular endothelial cells in the corpus cavernosum, the activation of cyclic guanosine monophosphate (cGMP) and of cGMP-dependent protein kinases, responsible of free cytoplasmic calcium and smooth muscle relaxation lead to increased corporal blood flowand tumescence (erection) [10]. Phosphodiesterase type-5 (PDE-5) is responsible for degradation of cGMP [11]. Inhibition of PDE-5 slows cGMP degradation, leading to increased levels of cGMP and greater blood flow through the corpus cavernosum when NO is released during sexual stimulation [12].Oral PDE-5 inhibitors are the current first line treatment for ED [13].

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. Chromatographic methods are commonly used in regulatory laboratories for the qualitative and quantitative analysis of drug substances, drug products, raw materials and biological samples throughout all phases of drug development from research and quality control. Throughout the drug development process, method validation is carried out to ensure that an analytical method is accurate, specific. Reproducible and rugged over the specified range in which an analyte will be analysed [14, 15].

A selective and sensitive method is required for quantitation of sildenafil in biological fluids. State-of-art LC-MS/MS technology based on assays provides exquisite analytical selectivity and is the method of choice for bioanalytical determination[16-20].The majority of the methods were developed for quantification of sildenafil alone from pharmaceutical, biological samplesand ayurvedic productsby using LC-MS/MS, capillary electrophoresis and ion mobility spectrophotometry [21-27].

A literature survey reveals that only a few methods were reported for quantification of sildenafil and its main metabolite N-desmethyl sildenafil from biological samples by LC-MS/MS,capillary zone electrophoresis ion trap mass spectrometry and micellar electrokinetic capillary chromatography [28-34]. Most of the reported methods show that extraction of sildenafil and its main metabolites are made using solid phase extraction.

Sildenafil citrate is designated chemically as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1 *H*–pyrazolo[4,3- *d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate.Sildenafil citrate is a white to off-white crystalline powder with a solubility of 3.5 mg/mL in water and a molecular weight of 666.7[35].

Scanning Optimization

Sildenafil and its metabolite piperazine N-desmethyl sildenafil are freely soluble in methanol. Stock solutions were prepared in HPLC

grade methanol. The required dilution of the stock was then made in methanol: water (50:50 v/v). The analyte were sildenafil using MRM mode sildenafil at m/z 475.0→100.3, piperazine n-desmethyl sildenafil at m/z 461.2→310.8 and internal standard clarithromycin at m/z 748.5→158.3, the lower limit of quantitation was 5.0 ng/ml.

MATERIALS AND METHODS

Reagents and Chemicals

Acetonitrile and methanol of HPLC grade were obtained from S. D. Fine Chem. Ltd. Water was purified by means of a Direct-Q water purification system from Millipore. Formic acid was of analytical grade and were obtained also from Merck.Sildenafil citrate and N-desmethylsildenafil were provided from Pfizer. Analytical columns Thermo Hypersil Gold Column (50x2.1mm) 5µm were purchased from Thermo Fischer Ltd. Extraction and purification

Instrumentation

The LC/MS/MS system consisted an LC-Shimadzu LC10 from Shimadzu coupled to an MS/MS (API 3000) from SCIEX Applied (Applied Biosystems Sciex, Ontario, Canada) equipped with a Turbo Ion Spray source for ion production. Data acquisition and integration were controlled by Applied Biosystems Analyst Software, version 1.4.1. Chromatography was performed with a HPLC system consisting of a LC-Schimadzu LC10. The pump was used under isocratic conditions on manual mode and detector was operated at 230 nm.

Chromatographic Conditions

The chromatographic separation was performed on Thermo Hypersil Gold Column (50x2.1mm) 5µmat 35 °C using a mobile phase of acetonitrile: 0.02% formic acid (70:30, v/v). Degassing was accomplished by sonicating the mobile phase prior to usage. The separation was under isocratic conditions with a flow rate of 0.500 mL/min. The injection volume was 2 µL.

Mass Spectrometry

The electrospray interface heater (IHE) was set to on mode and IonSpray Voltage (IS) was set at −4500V for ionization. The nitrogen curtain gas was adjusted to a constant value of 11 units and the source temperature (at set point) was 475°C. The mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution. The MRM mode for sildenafil, piperazine n-desmethyl sildenafil and internal standard clarithromycin were detected at m/z 475.0→100.3, 461.2→310.8 and 748.5→158.3 respectively.

Preparation of Calibration Standards and Quality Control Samples

A stock solution of sildenafil, piperazine n-desmethyl sildenafil were prepared in methanol at a concentration of 1.00 mg/ml and 500.00 µg/ml. Standard solutions for sildenafil (5.0, 10.0, 20.0, 100.0, 300.0, 600.0 and 1000.0 ng/ml)and for piperazine n-desmethyl sildenafil (1.0, 2.0, 4.0, 20.0, 60.0, 120.0 and 200.0 ng/ml) were prepared by serial dilution of the stock solution with methanol. Low, medium, and high concentration quality control for sildenafil (QC) solutions (15.0, 500, and 800 ng/ml) and for piperazine n-desmethyl sildenafil (3.0, 100.0 and 160.0 ng/ml) were prepared in the similar way. The stock solution of IS (1.0mg/ml) was also prepared in methanol and then diluted with methanol to a final concentration of 5.0 ng/ml. All solutions were stored at 2-8°Cuntil use.

Sample Preparation

Sample preparation consisted of the addition of 0.30 ml of plasma sample in a 10 ml tube, then add 50.0 µl of internal standard 50.0 ng/ml clarithromycin was added, then vortex for 30 seconds, 5ml solvent MTBE vortex 6 minutes, centrifugation for 6 minutes at 4400 r.p.m., transfer the organic layer to evaporation glass tube and evaporate under steam of nitrogen and 45 water bath, reconstitution with 200 µl of mobile phase and vortex for 1.5 minutes.Finally, the solution was transferred to the autosampler vials, and 2 µl was injected into LC-MS/MS system.

Method Validation

A full validation was performed in human plasma according to FDA guideline on Bioanalytical Method Validation [36] and EMEA [37].

Selectivity

Six lots of blank plasma were screened and interference at retention time of analyte and internal standard was evaluated. Only those lots which are under the acceptance criteria (<20 % in comparison to the spiked LOQ and <5 % in comparison to IS area) were selected. No significant interference was observed at the RT and m/z of sildenafil and Piperazine N-desmethyl sildenafil and clarithromycin (internal standard) in all the batches screened.

Matrix Effect

The absence of matrix interferences was determined by the analysis of 6 independent sources of control matrix. Each independent control matrix after being assessed and proved no interference was used to produce standard curves and quality control samples throughout the whole study.

Linearity

For the determination of linearity, standard calibration curves of seven points (nonzero standards) were used. Seven non-zero points 5.0, 10.0, 20.0, 100.0, 300.0, 600.0 and 1000.0 ng/ml for sildenafil and 1.0, 2.0, 4.0, 20.0, 60.0, 120.0 and 200.0 ng/ml for piperazine n-desmethyl sildenafil. In addition, a blank plasma sample were also analyzed to confirm absence of interferences, these sample was not used to construct the calibration function.

The data from three precision and accuracy batches were subjected to goodness of fit analysis using 1/x and 1/x2 weighing factor. Deviation from nominal concentration should be within +20% for LLOQ and within +15% for the other concentrations.

At least 75% of non-zero standards should meet the criteria, including LLOQ and ULOQ standard. Linear coefficient of correlation (r) should be ≥0.98.

Accuracy and Precision

The precision of the assay was measured by the intra-day and inter-day percent coefficient of variation over the concentration range of 5.0 to 1000.0 ng/ml for sildenafil and 1.0 to 200.0 ng/ml for piperazine n- desmethyl sildenafil during the course of the validation. The batches run on two different days to provide the intra and inter batch precision and accuracy.

The intraday precision and accuracy of the assay were measured by analyzing six spiked samples of sildenafil at each QC level (5.0, 15.0, 500.0 and 800 ng/mL) and 1.0, 3.0, 100.0 and 160.0 ng/ml) for sildenafil and piperazine n-desmethyl sildenafil respectively. Three batches run over two adjacent days to provide the inter batch estimate. The acceptance criteria for precision and accuracy deviation values should be within ±20% of LLOQQC and within ±15% the actual values for other concentrations.

Stability

Low quality control and high quality control samples (n=6) were retrieved from the deep freezer after three freeze-thaw cycles. Samples were stored at -30 °C in three cycles of 24, 48 and 72 hrs. In addition long-term stability of sildenafil and N-desmethyl sildenafil in quality control samples were also evaluated by analysis after 120 days of storage at -25 & -70°C. Bench top stability wasstudied for 7 hrs period with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within ±15% respectively of their nominal concentrations.

Autosampler stability

To assess the autosampler stability, six sets of QC samples (LQC and HQC) were processed and placed in the autosampler. These samples were injected after a period of around 24 hours and were quantified against freshly spiked calibration curve standards. The batch follows

the same acceptance criteria of as for the precision and accuracy batches.

Ruggedness

The ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Ideally a method will be able to tolerate small changes in the reagents, different batches of columns and different operators, etc. without compromising the analytical results. The batch follows the same acceptance criteria of as for the precision and accuracy batches.

Recovery

Recoveries were evaluated by comparing the mean peak area of six extracted low, medium and high quality control samples (QC Low; 15.0 ng/ml, QC Mid; 500.0 ng/ml and QC High; 800.0 ng/ml for sildenafil and QC Low; 3.0 ng/ml, QC Mid; 100.0 ng/ml and QC High; 160.0 ng/ml for Piperazine N- Desmethyl Sildenafil) to mean peak area of six extracted spiking drug free plasma samples with the same amount of low, medium and high sildenafil/ piperazine n-desmethyl sildenafil quality control samples. Similarly recovery of clarithromycin were evaluated by comparing the mean peak area of six extracted quality control samples to mean peak area of clarithromycin in samples prepared by spiking extracted drug free-plasma samples with the sameamount of clarithromycin.

Dilution Integrity (DI)

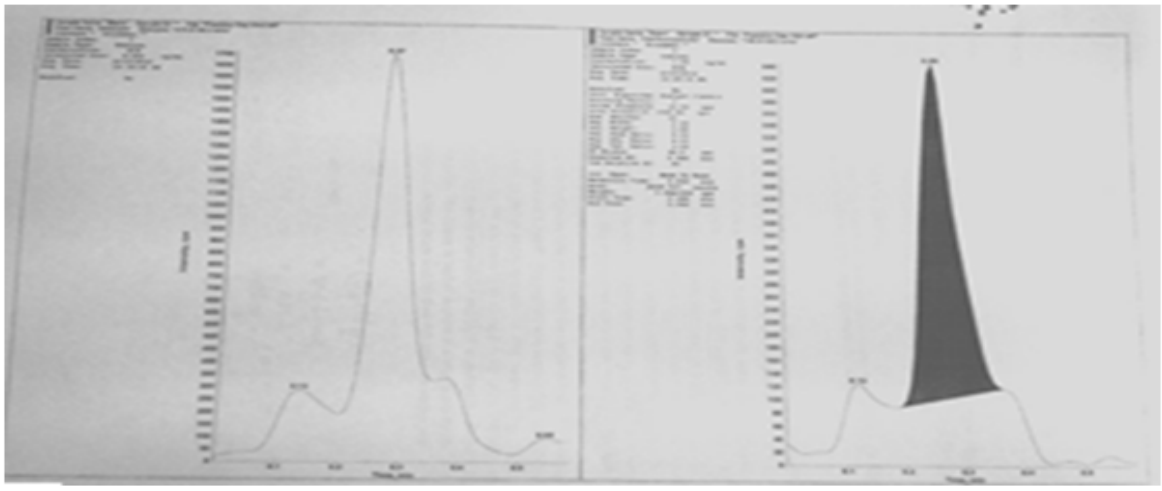
The ability to dilute samples originally above the upper limit of the standard curve is determined by accuracy and precision parameters.

RESULTS AND DISCUSSION

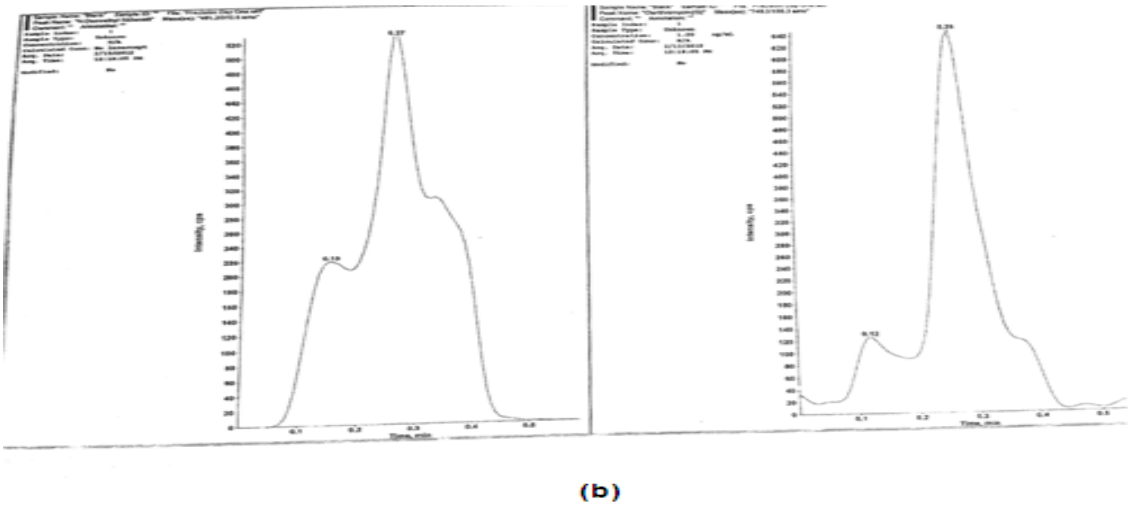
Method Development and Validation

LC-MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The goal of this work is to develop and validate a simple, rapid and sensitive method for the quantification of sildenafil and piperazine n-desmethyl sildenafil in human plasma. A simple extraction technique was utilized in theextraction of drug, metabolite and internal standards from plasmasamples. Chromatographic conditions, especially the compositionand nature of the mobile phase were optimized throughseveral trials to achieve best resolution and increase the signal ofanalytes and IS. The MS optimization was performedby direct infusion of solutions into ESI source of the mass spectrometer.

After the MRM channels were tuned, the mobile phasewas changed from an aqueous phase to more organic phase toobtain a fast and selective LC method. A good separation andelution were achieved using 0.02% formic acid and acetonitrile(30:70, v/v) as the mobile phase, at a flow-rate of 0.6 ml/min and injection volume of 2 µl.

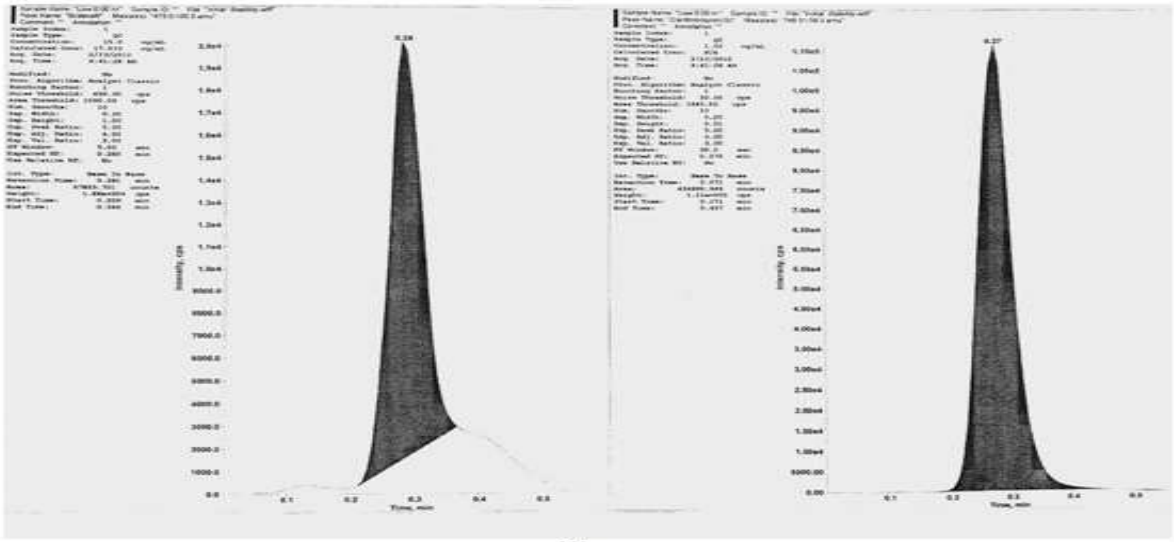


(a)



(b)

Fig. 1: Representation of blank sample chromatograms of Sildenafil (a) and piperazine n-desmethyl Sildenafil (b) respectively.



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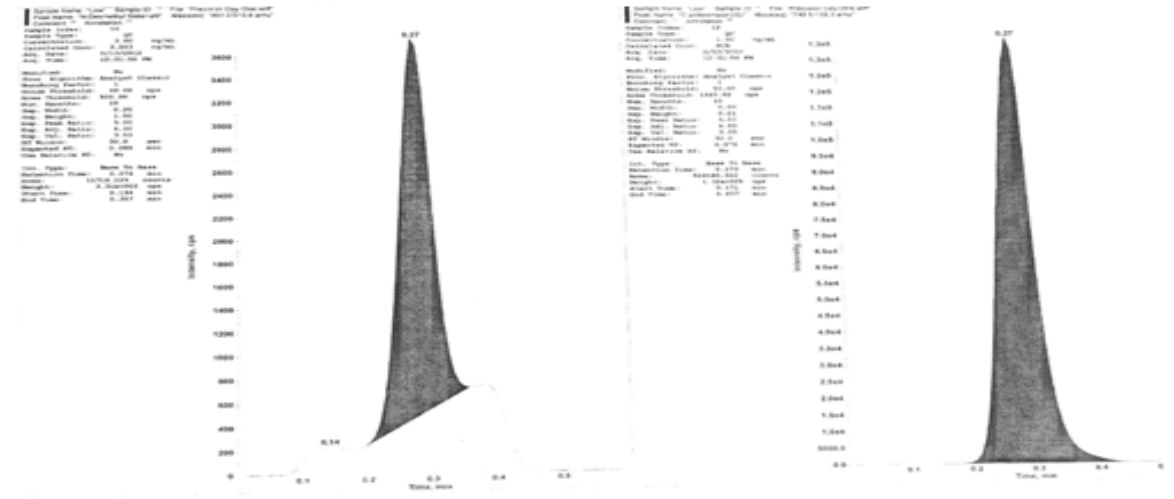

(d)

Fig. 2: Representation of LLOQ sample chromatograms of Sildenafil (c) and piperazine n-desmethyl Sildenafil (d) respectively.

Table 1:Spiked plasma concentration and RSD (%) for Sildenafil and piperazine n-desmethyl Sildenafil

Spiked plasma Concentration (ng/ml)	Concentration Measured (mean) (ng/ml) ± SD(n=6)	RSD (%)	Accuracy %
Sildenafil			
5.0	4.32 ± 0.159	3.71	86.29
10.0	9.62 ± 0.266	2.77	96.18
20.0	20.38 ± 0.978	4.80	104.92
100.0	108.41 ± 2.588	2.39	108.41
300.0	329.52 ± 7.255	2.20	109.84
600.0	608.42 ± 9.367	1.54	101.40
1000.0	954.23 ± 7.208	0.76	95.42
Piperazine n-desmethyl Sildenafil			
1.0	1.13 ± 0.051	4.48	113.42
2.0	1.98 ± 0.090	4.53	99.18
4.0	3.85 ± 0.190	4.94	96.24
20.0	17.97 ± 0.881	4.90	89.88
60.0	60.21 ± 1.037	1.72	100.34
120.0	120.04 ± 1.378	1.15	100.03
200.0	201.81 ± 0.950	0.47	100.90

All the mean accuracies were calculated against their nominal concentrations

Matrix effect

Sample Extraction and Optimization

For the extraction of the analytes, liquid–liquid extraction was selected comparing to solid phase extraction in order to allow the optimization of a simple, cost effective and rapid method that would be applicable in analysis. However the replacement ofsodium hydroxide with disodium hydrogen phosphate bufferappeared to facilitate the separation of the drug from theprotein complex providing a significant response. Theextraction were done in different solvents or solventmixtures [diethyl ether, ethyl acetate, chloroform/isopropanol/n-heptane (25:10:65, v/v/v)] and MTBE were further investigated. The first three solvents providedhigh matrix noise that overlapped with analytes's peaks. Theoptimum solvent was MTBE. Retention times of sildenafil and piperazine n-desmethylsildenafil were0.24-0.32 minutes**.**

Selectivity

Six lots of blank plasma were screened and interference at retention time of analyte and internal standard was evaluated. Only those lots which are under the acceptance criteria (<20 % in comparison to the spiked LOQ and <5 % in comparison to IS area) were selected. No significant interference was observed at the RT and m/z of sildenafil and Piperazine N-desmethyl sildenafil and clarithromycin (internal standard) in all the batches screened. Fig. 1 & 2, illustrates the representative chromatogram for blank and LLOQ.

The absence of matrix interferences was determined by the analysis of 6 independent sources of control matrix. Each independent control matrix after being assessed and proved no interference was used to produce standard curves and quality control samples throughout the whole study.

Linearity

A calibration curve was established on each validation day. The calibration curve was linear over the concentration range of 5.0–1000.0 ng/ml for sildenafil and 1.00–200.0 ng/ml for piperazine n-desmethyl sildenafil respectively in human plasma with a coefficient of relation (r) ≥0.99. The average slope and intercept of regression equations were 0.008573 and 0.008231 for sildenafil and 0.007432 and -0.001217 for piperazine n-desmethyl sildenafil. Linearity was found to be satisfactory and reproducible with time. The determination coefficients (r2) for sildenafil, and piperazine n-desmethyl sildenafil were greater than 0.9970 and 0.9990, respectively for all curves (Table 1).

Precision—accuracy

Precision and accuracy for this method was controlled by calculating the intra and inter-day batch variations of QC samples in six replicates at four concentrations (5.0, 15.0, 500.0 and 800.0 ng/ml) for sildenafil and (1.0, 3.0, 100.0 and 160.0 ng/ml) for piperazine n-desmethyl sildenafil as shown in Table 2.

Intraday precision and accuracy for sildenafil ranged from 0.24-11.36 % &91.71-102.94 % respectively and for piperazine n-desmethyl sildenafil ranged from 1.56-11.66 % & 91.71-109.07 % respectively.Intra-day batch precision and accuracy ranged from 1.85-10.47 % and 98.82-102.94 % respectively for sildenafil and 1.84–7.44 % and 98.01-109.06 % respectively for piperazine n- desmethyl sildenafil. These results indicated that the method was reliable, reproducible and accurate as all the parameters were within the acceptance limit of < 15 % and ± < 15 % for precision and accuracy respectively for LQC, MQC and HQC and ≤ 20 % for LLOQQC.

Table 2: Intra and inter-day accuracy and precision of the method in human plasma

Nominal (ng/ml)	Intraday (n=6)			Inter day (n = 18)		
	Mean observed (ng/ml)	Precision (%)	Mean accuracy (%)	Mean observed (ng/ml)	Precision (%)	Mean accuracy (%)
Sildenafil						
5.0	5.09	10.47	101.83	4.98	9.98	99.62
15.0	14.98	4.84	99.861	4.31	7.62	95.42
500.0	514.72	2.48	102.94	507.68	2.63	101.54
800.0	790.56	1.85	98.82	778.74	2.07	97.34
Piperazine n-desmethyl sildenafil						
1.0	1.09	7.44	109.06	1.06	9.20	106.12
3.0	3.23	4.54	107.82	2.93	8.84	97.70
100	98.01	1.84	98.01	97.88	2.33	97.88
160.0	157.60	1.87	98.50	59.31	2.95	99.57

All the mean accuracies were calculated against their nominal concentrations

Table 3: Stability data for Sildenafil and piperazine n-desmethyl Sildenafil

Stability	Analytes	Nominal concentration (ng/ml)	Mean concentration (ng/ml)	% Nominal	% CV
Freeze thaw	Sildenafil	5.000	15.048	100.32	8.15
(3-cycle, 72 h)		800.000	837.203	104.65	5.61
N-desmethyl	Sildenafil	3.000	2.925	97.51	5.78
		160.000	168.025	105.02	1.39
Bench top (7 h)	Sildenafil	15.000	15.235	101.57	7.49
		800.000	841.400	105.18	5.44
N-desmethyl	Sildenafil	3.000	2.813	93.76	5.45
		160.000	168.146	105.09	1.68
Long term	Sildenafil	15.000	15.247	101.65	7.13
(120 days)		800.000	835.015	104.38	6.92
N-desmethyl	Sildenafil	3.000	2.754	91.81	5.31
Auto sampler		160.000	166.560	104.10	1.67
(24 h)	Sildenafil	15.000	15.041	100.27	8.66
		800.000	829.962	103.75	6.18
N-desmethyl	Sildenafil	3.000	2.995	99.82	5.70
		160.000	170.326	106.45	2.15

Stability (Freeze–thaw, bench top, long term and Auto sampler)

The stabilities data of sildenafil and n-desmethyl sildenafil, which includes freeze thaw, bench top, long term and autosampler were within the acceptance limit. Results are demonstrated in Table 3.

Recovery

The percent recovery was determined by measuring the absolute peak area of Sildenafil and Piperazine N- desmethyl Sildenafil and the internal standard from a plasma sample prepared according to the method of analysis.The extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible. The mean overall recovery of sildenafil and clarithromycin was found to be 95.27 % and 97.13 % respectively. The mean overall recovery of metabolite piperazine N- desmethyl sildenafil and clarithromycin was found to be 85.54 % and 95.42 % respectively.

Dilution Integrity

Up to 20 fold of sildenafiland piperazine n-desmethyl sildenafil were diluted by blank human plasma and was tested with spiked samples exceeding the upper limit of the calibration curve and samples with the highest concentration. The % nominal was within ±15% and the observed precision was within ≤15 %. This demonstrates that the sample can be diluted up to 20 times and yet the results are predictable and reproducible.

Ruggedness

The ruggedness results suggest that the method was rugged under the change of solutions/columns and analyst.For sildenafil within batch precision and accuracy was found to be 0.35 to 10.99 % and 95.17 to 101.37 % respectively.For piperazine n-desmethyl sildenafil within batch precision and accuracy was found to be 2.06 to 8.47 % and 97.51 to 104.51 % respectively.

CONCLUSION

In this article the LC-MS/MS method for quantification of sildenafil and piperazine n-desmethyl sildenafil in human plasma has been successfully developed and validated. The samples for LC-MS/MS analysis were prepared by liquid-liquid extraction. Each sample requires less than 1 min run time. The validated method is also highly sensitive and specific due to the selection of mass spectrometry and advantages over other techniques like GC, GC mass and HPLC etc. The sensitivity of the assay is sufficient to follow accurately the pharmacokinetics of sildenafil and piperazine n-desmethyl sildenafil following oral administration.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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