Open Access Contents
Int. J. Drug Dev. & Res.
| April - June 2014 | Vol. 6 | Issue 2 | ISSN 0975-9344 |
www.ijddr.in

Full Length Original Research Paper

Pharmacokinetics studies of Sildenafil and its Metabolite Piperazine N-Desmethyl Sildenafil by using LC-MS/MS in Human Plasma

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Abstract:

To assess the pharmacokinetics parameters of test drug (sildenafil citrate, 100 mg) with referenceViagra®(sildenafil citrate, 100 mg tablet) in healthy male volunteers. An open label, balanced, randomized, two-treatment, two-sequence, two-period, crossover, single-dose bioequivalence study was conducted in healthy adult male subjects, under fasting conditions.Twelve healthy subjects were enrolled in a single-centre, randomized, single-dose, open-label, 2-way crossover study, with a minimum washout period of 7 days. Plasma samples were collected up to 24.0 h postdosing. Concentrations of drugs weredetermined by using a validated LC-MS/MS method.

The pharmacokinetics parameter for the reference R and test T for sildenafil was as follow: $t_{1/2}$ was 2.77 (±1.14) and 2.69 (±0.41) h; Tmax was 0.94 (±0.0.48)h and 0.94 (±0.56) h; Cmax was 607.02 (±156.36) ng/ml and 614.74 (±197.42)ng/ml; AUC_{0-t} was 1988.80 (±676.68) ng.hr/mland 1648.02 (±378.68) ng.hr/ml. Elimination rate constant (K_{el}) for these products were calculated as 0.28 (±0.08) and 0.26 (±0.04) hrs^{-1} respectively(for R and T). For piperazine n-desmethyl sildenafilthe pharmacokinetics parameter for the reference R and test T was as follow: t1/2 was 3.74 (±1.47) and 3.99 (±1.59) hrs; Tmax was 1.25 (±0.58) hand 01.35 (±0.94) h; Cmax was 61.76 (±29.93) ng/ml and 69.00 (±29.31) ng/ml; AUC_{0-t} was 304.06 (±179.34) ng.hr/ml and 280.66 (±175.71) ng.hr/ml. Elimination rate constant (K_{el})for these products were calculated as 0.22 (±0.08) and 0.20 (±0.08) hrs^{-1} respectively for (R and T).

Keywords: Pharmacokinetics, Sildenafil citrate, LC-MS/MS, Plasma

Introduction

Sildenafil citrate, 1-(4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo(4,3-d)pyrimidin-5-yl)phenylsulfonyl)-4methylpiperazine, is primarily indicated in the treatment oferectile dysfunction (1). It acts by inhibitingc GMP - specific phosphodiesterase type 5, an enzyme that promotes degradation of cGMP, which regulates the blood flow in the penis. The chemical structure of Sildenafil is shown in fig. 1.

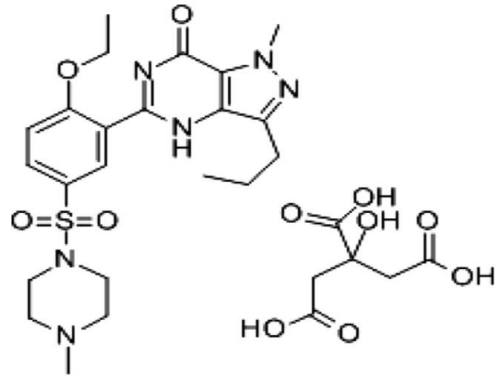

Fig. 1: Sildenafil structure

Sildenafil, when used properly, is relatively safe. There are, however, certain side effects that could create potential hazards. Sildenafil has been shown to potentiate the hypotensive effects of nitrates commonly employed in the treatment of certain heart conditions (2). Moreover, while sildenafil inhibitsPDE5, it also has a high affinity for phosphodiesterase type 6 (PDE6), which is a retinal enzyme involved in photo transduction (3, 4).

A literature survey reveals that only very few methods were reported for the estimation of sildenafil and its main metabolite piperazine n-desmethyl sildenafil from biological samples by LC-MS/MS (5-9), micellar electrokinetic capillary chromatography (10), capillary zone electrophoresis-ion trap mass spectrometry(11). Most of the reported methods show that

extraction of sildenafil and its main metabolites are made using solid phase extraction (5-9).

Many analytical methods, using high performance liquid chromatography (HPLC), have been published for quantification of the parent drug sildenafil in plasma and not for its active metabolite, using ultraviolet visible (UV–Vis) detector (12, 13).The development of ultra-HPLC coupled with MS/MS (UPLC-MS/MS) provided more sensitive and selective methods that have allowed for simultaneous quantitation of sildenafil and its major metabolite (12-15). Most of the methods were developed for estimation of sildenafil alone from pharmaceuticals(16, 17).Many physicians consider sildenafil a good first-line therapy for erectile dysfunction and an alternative to the use of oral androgens, injection of intracavemosal or transurethral prostaglandins, or vacuum devices(18-20).After oral administration, sildenafil is rapidly absorbed, reaching peak plasma concentrations in 30-120 min (21).

In the present study, we have reported a highly sensitive, selective and reproducible analytical method for the determination of sildenafil, and its metabolite piperazine n-desmethyl sildenafil inplasma samples utilizing liquid chromatography coupled to electrospray (ES) tandem mass spectrometry by using liquid–liquid extraction methods. Clarithromycin was used as an internal standard. We have developed and validated the method as per the FDA guidelines over a concentration range of 1.0–1000.0 ng/ml forsildenafil and 1.0–200.0 ng/ml for piperazine n-desmethyl sildenafil using 0.3 mL plasma sample followed by a simple liquid–liquid extraction technique for extraction of drugs and internal standards. The retention times of sildenafil, piperazine n-desmethyl sildenafil and clarithromycin were0.24-0.30 min. The method validated as per FDA guidelines and was employed in the analysis of plasma samplesafter oral administration of sildenafil tablets (100 mg) in healthy human volunteers (22, 23).

MATERIALS AND METHODS

Reagents and Chemicals

Methanol and Acetonitrile were obtained from S. D. Fine Chem (HPLC grade) Ltd. Water was purified by Direct-Q water purification system from Millipore. Formic acid was of analytical grade and were obtained also from Merck. Sildenafil citrate and piperazine n-desmethylsildenafil were provided by Pfizer. Clarithromycin is used as an internal standard. 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 Bio systems Sciex, Ontario, Canada) equipped with a Turbo Ion Spray source for ion production. 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) 5µm at 35 °C using a mobile phase of acetonitrile: 0.02% formic acid (70:30, v/v). Sonication was done for 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 IonSpray Voltage (IS) was set at –4500V for ionization. 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°C until use.

Preparation of Samples

Sample preparation consisted of the addition of 0.3 ml of plasma sample in a 10 ml tube, then add 50 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°C 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 µ was injected into LC-MS/MS system.

Analysis of Patient Samples

The bioanalytical method developed previously was applied to quantify sildenafil and piperazine n-desmethyl sildenafil concentrations in plasma following oral administration of healthy human volunteers. These volunteers were contracted in Vertex Laboratory Research Ltd., New Delhi, India. Each one of the 12 healthy volunteers were administered a 100 mg dose (one 100 mg tablet) orally with 240 ml of drinking water. The product Viagra tablets R (Pfizer) 100 mg and the test viagra tablets T (formulation) were used. Blood samples were collected aspre-dose(0) hr 5 min prior to dosing followed by further samples at 0.25, 0.5, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 5.00, 6.00, 8.00,10.00, 12.00 and 24.0 hr. After dosing, 4 ml blood sample was collected each time in vaccutainers containing CPDA. Then they were keptfrozen at -25°Cuntil sample analysis. Test and reference were administered to the same human volunteers under fasting conditions separately with proper washing periods as perprotocol.

Pharmacokinetics and Statistical Analysis

Pharmacokinetics parameters from human plasma samples were calculated by using Win-Non-Lin 5.0 software. Plasma sildenafil, piperazine n-desmethyl sildenafil concentration-time profiles were visually inspected and Cmax and Tmax values were determined. The AUC_{0-t} was obtained by trapezoidal method. $AUC_{0-\infty}$was calculated up to the last measureable concentration and extrapolations were obtained using the last

measureable concentration and the terminal elimination rateconstant (Kel). The terminal elimination rate constant (Ke), was estimated from the slope of the terminal exponential phase of the plasma of sildenafil, piperazine n-desmethyl sildenafil concentration-time curve (by means of the linear regression method). The terminal elimination half-life t1/2 was then calculated as 0.693/Ke.

RESULTS AND DISCUSSION

Validation of LC-MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity andre producibility. The goal of this work is to quantify the drug sildenafil and its metabolite piperazine n-desmethyl sildenafil in human plasma. This procedure incorporates LLE and LC-MS/MS with APCI ion trap MS. The use of LLE provided acleaner extract and less expensive than did in the alternative SPE. Chromatographic conditions, especially the composition and nature of the mobile phase were optimized through several trials to achieve best resolution and increase the signal of analytes and internal standard.

Mean plasma concentration of sildenafil

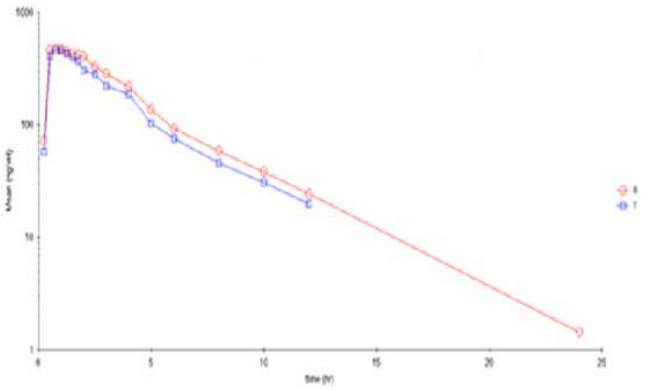

Fig. 2: Plasma concentrations versus time profiles for sildenafil

Mean plasma concentration of piperazine n-desmethyl sildenafil

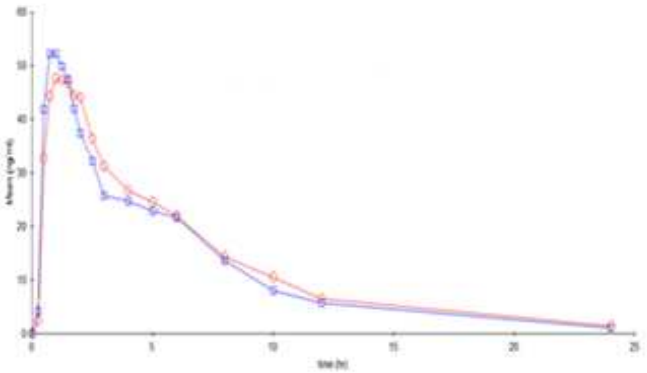

Fig. 3: Plasma concentrations versus time profiles for piperazine n-desmethyl sildenafil

Table 1: Pharmacokinetic parameters for sildenafil and piperazine n-desmethyl sildenafil

Pharmakokinetics Paramateres	Reference	Test
Pharmacokinetic details of Sildenafil		
C_{max} ng/ml	607.02 (±156.36)	614.74 (±197.42)
T_{max}hr	0.94 (±0.048)	0.94 (±0.056)
AUC_{0-t} ng.hr/ml	1988.80 (±676.68)	1648.02 (±378.68)
$AUC_{0-\infty}$ ng.hr/ml	2060.70 (±696.11)	1727.82 (±404.09)
$t_{1/2}$hr	2.77 (±1.14)	2.69 (±0.41)
Kel hr	0.28 (±0.08)	0.26 (±0.04)
Pharmacokinetic details of piperazine n-desmethyl Sildenafil		
C_{max} ng/ml	61.76 (±29.93)	69.00 (±29.31)
T_{max}hr	1.25 (±0.58)	1.35 (±0.94)
AUC_{0-t} ng.hr/ml	304.06 (±179.34)	280.66 (±175.71)
$AUC_{0-\infty}$ ng.hr/ml	318.44 (±188.38)	297.73(±185.37)
$t_{1/2}$hr	3.74 (±1.47)	3.99 (±1.59)
Kel hr	0.22 (±0.08)	0.20 (±0.08)

Table 2: Bioequivalence of analyzed formulations

Test/Reference	Sildenafil	Piperazine n-desmethyl Sildenafil
C_{max}(T/R)	99.56	114.56
AUC_{0-t}(T/R)	85.53	92.74
$AUC_{0-\infty}$ (T/R)	86.47	94.16

APPLICATION TO BIOLOGICAL SAMPLES

The validated method was used in the determination of sildenafil and piperazine n-desmethyl sildenafil in plasma samples for

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establishing the pharmacokinetics parameters of a single 100 mg dose (one 100 mg tablet) in 12 healthy volunteers. Plasma concentrations versus time profiles are shown in Fig. 3 and Fig. 4. All the plasma concentrations of sildenafil and piperazine n-desmethyl sildenafil were in the standard curve region and retained above LLOQ for the entire sampling period. The observed values were reported inTable 4 and Table 5 for sildenafil and for piperazine n-desmethyl sildenafil.Therefore, it can be concluded that the two analyzed formulations(reference and test) are bioequivalent.

Conclusions

In this paper we have reported the use of LC-MS/MS for theprecise and reliable quantification of sildenafil and piperazine n-desmethylsildenafil concentrations in human plasma after oraladministration of 100 mg to healthy volunteers. The methoddescribed here is accurate, robust and fast. Each sample requiresless than 1 min run time. The sensitivity of the assay is sufficient to followaccurately the pharmacokinetics of sildenafil and piperazine n-desmethylsildenafil 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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Article History: ----------

Date of Submission: 02-06-2014

Date of Acceptance: 06-06-2014

Conflict of Interest: NIL

Source of Support: NONE