EXPERIMENTAL
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Part A: Method Development and Validation.

Though many methods are reported for the estimation of sildenafil, they are either tedious involving expensive instruments\textsuperscript{26, 28, 30 and 37} or not suitable for its determination in biological fluids\textsuperscript{29, 135}. So we decided to develop our own method in the laboratory, which is simple, precise and yet sensitive. As HPLC is the most commonly available instrument giving high precision and accuracy and is comparatively less expensive, we developed the method on HPLC with solvent extraction procedure.

EXPERIMENTAL CONDITIONS:

1. HPLC system: Merck Hitachi
2. Detector: Merck Hitachi L - 7400 Dual Wavelength absorbance detector.
3. Integrator: HSM Software
4. Analytical Column: LiChrosphere RP 18\(_e\), 5\(\mu\), 250X4.0 mm, Merck Germany.
5. Guard Column: LiChrosphere C18, 5\(\mu\), 30X4.0 mm, Merck Germany.

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.
7. Flow rate: 1.0ml per minute.
8. Sample Compartment Temperature: 15\(^\circ\)C.
9. Injection Volume: 100μl
11. Column Temperature: 35°C.
12. HPLC rinse solution: Methanol.

At the above conditions sildenafil was eluted at 11.0 min and diazepam at 16.0 minutes.

Plasma Extraction Procedure: 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 pH 9.0 were added and vortexed for 30 seconds. 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°C under nitrogen flushing. The residue was reconstituted in 150μl of mobile phase and 100μl from this was injected into HPLC system.

A set of seven calibration standards, a zero, a blank and three sets of Quality Control standards (QCS) were analysed with every series of standard curves. For each calibration standard, the peak height ratio of sildenafil to IS was calculated. A straight-line equation describing the relationship between this ratio and concentration, with a weighting factor of 1/X was arrived at.

Method validation:

Specificity: Blank plasma samples of six different sources were screened to check for any interference due to endogenous components. Same samples were used for all further analytical developmental work and QCs.

Linearity: The linearity was demonstrated over the concentration range if the coefficient of variation \( r^2 \) of the calibration curve is more than 0.98.

Precision:

a. Within day precision: Three sets of calibrators and six replicates of QCS by level were assayed the same day, the concentrations of all the QCS were determined with the first calibration curve. The within day precision was expressed as the coefficient of variation (CV) of the six determinations at each level of QCS and must not exceed 15% except at lowest level where 20% is acceptable.

b. Interday Precision: It was performed on three days with one set of calibrators and six replicates of QCS by level per day. The interday precision was expressed as the coefficient of variation (CV) of the eighteen determinations.
a. A single dose, 50mg single period study to determine the pharmacokinetics of sildenafil under fasting conditions in healthy human subjects.

b. A single dose, 50mg single period study to determine the pharmacokinetics of sildenafil under fed conditions in healthy human subjects, after ingestion of high fat meal.

c. A single dose, 50mg single period study to determine the pharmacokinetics of sildenafil under fasting conditions in healthy human subjects administered at 8.00PM.

Pharmacokinetic studies were performed on healthy human volunteers. These studies were conducted in the laboratories of M/S Synchron Research Services Pvt. Ltd., Ahmedabad. The particulars of the volunteers included in the studies, the reports on their biochemical and hematological parameters and the details about the withdrawal of blood samples, timings and analytical reports and raw data is available in the records of this CRO. The physicians of the company supervised all the studies conducted on volunteers. A protocol of the study was prepared and submitted to the institutional ethics committee for approval. Ethics committee approved the protocol without any amendments. Volunteers were given an informed consent form, which they read and signed. A copy of the protocol and informed consent form are enclosed as Annexure I and II at the end of this thesis.

Volunteers were selected on the basis of following inclusion and exclusion criteria.

Inclusion Criteria:

    Healthy male volunteers between 18-35 years.
Body mass within ±15% of ideal mass as per height weight chart of Life Insurance Corporation of India and not less than 50kg.

Normal health as determined by medical history and physical examinations and laboratory examinations within the normal range.

Normal ECG and vital signs.

Non or ex smokers.

Ability to comprehend and willingness to adhere to the protocol requirements as evidenced by written informed consent.

Exclusion Criteria:

- History or presence of significant cardiovascular, pulmonary, hepatic, renal, hematological, gastrointestinal, endocrinological, immunological, dermatological, neurological or psychiatric disease.
- History or presence of alcoholism,
- History or presence of drug abuse,
- History or presence of ophthalmological disturbances (except for the need for corrective lenses),
- History or presence of thyroid disease,
- History or presence of adrenalin dysfunction,
- History or presence of organic intracranial lesion,
- History or presence of cancer,
- Use of any medication during two weeks before the first administration of study drugs,
- Use of enzyme modifying drugs during 30 days prior to the screening period.
Major illness/hospitalization during 3 months before the screening period.

History of hypersensitivity to sildenafil or related drugs.

Subjects who have been on an abnormal diet (for whatever reason) during the four weeks preceding the study.

Subjects who through completion of this study, would have donated in excess of 500ml of blood in 14 days, 750ml in 3 months, 1000ml in 6 months, 1500ml in 9 months or 2000ml in one year.

Subjects who have participated in another clinical trial within 8 weeks of study start.

Subjects were evaluated for the physical examinations and laboratory tests. Physical examinations include medical history and vital signs like blood pressure, ECG, pulse rate and body temperature.

Laboratory Tests included

- Hematology: Hemoglobin, Hematocrit, Total and differential leukocyte count, red blood cell count, platelets count.
- Serum Chemistry and Serology: Sodium, Chloride, Glucose, Creatinine, Urea, Bilirubin (total), Sodium, Calcium, Cholesterol, Alkaline Phosphate, Potassium.
- Phosphorous, SGOT, Total Protein, Albumin, SGPT, Globulin, Uric acid, BUN.
- Urine Analysis: pH, Colour, Blood, Protein, Glucose, Microscopic examinations.

Volunteers were abstained from any xanthine containing food or beverages or alcoholic products 48 hours prior to dosing and during the study.
**STUDY FLOW CHART**

- Screening
  - Eligibility Criteria
  - Subject Selection
  - Check in, Informed Consent Form and Safety Assessment
  - Catheter, Zero hour Sampling and Safety Assessment
  - Dosing
  - Blood Draw and Safety Assessment at appropriate time intervals
  - Safety Assessment and Check out

*High fat breakfast in phase II

The volunteers were admitted to the study unit on the evening of day one. They received a standard vegetarian diet of about 2800 calories at night and were rested till next morning. Water intake was restricted in the morning. After performing their morning ablutions, oral temperature, blood pressure and pulse rate were recorded. An indwelling catheter of viggo 18 gauge was inserted, and the zero hour sample was collected. Then the volunteers were administered sildenafil tablet 50mg with 240ml of water at ambient temperature. After drug administration 7ml of blood samples were collected at 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 hours post dose. The catheter was kept patent with
0.5ml of heparinised saline 20 units/ml. Before collecting each sample, 1.0ml of blood was withdrawn and discarded. Samples were collected in to a tube containing heparin and were centrifuged within 60 minutes of collection at laboratory temperature at 4000RPM for 4 minutes. The serum obtained was transferred to a vial, labeled indicating the volunteer number and sampling time and stored -20°C pending analysis. The samples were analysed by the above method and analysis was completed within 30 days of sample collection. During the study period volunteers were given uniform vegetarian diet at 4.0 and 8.0 hours post dose and at appropriate times thereafter. Water was restricted two hours pre dose and two hours post dose and allowed ad libitum at all other times. The oral temperature, blood pressure and pulse were also checked at 2.0, 6.0 and 10.0 hours post dose. When the sampling time and vital sign measurement were coincided, sample took the precedence over vital sign measurement. The volunteers were dosed in sitting position and remained seated for first 2 hours. For rest of the period they were allowed to engage in normal activities avoiding any severe physical exertion.
### SCHEMATIC REPRESENTATION OF STUDY DESIGN

<table>
<thead>
<tr>
<th>Time relative to dose administration in hours</th>
<th>Clock time in hours (Days)</th>
<th>Vital Signs</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.0 to -11.5</td>
<td>04.00 to 08.30 pm D₀</td>
<td>Yes</td>
<td>Check in, ICF, SF and CE</td>
</tr>
<tr>
<td>-11.0</td>
<td>09.00 pm D₀</td>
<td></td>
<td>Dinner</td>
</tr>
<tr>
<td>-1.0</td>
<td>07.00 am D₁</td>
<td>Yes</td>
<td>Pre dose blood and SF</td>
</tr>
<tr>
<td>*0.0</td>
<td>08.00 am D₁</td>
<td></td>
<td>Dosing</td>
</tr>
<tr>
<td>0.5</td>
<td>08.30 am D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>0.75</td>
<td>08.45 am D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>1.0</td>
<td>09.00 am D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>1.5</td>
<td>09.30 am D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>2.0</td>
<td>10.00 am D₁</td>
<td>Yes</td>
<td>SF and Blood draw</td>
</tr>
<tr>
<td>3.0</td>
<td>11.00 am D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>4.0</td>
<td>12.00 noon D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>6.0</td>
<td>02.00 pm D₁</td>
<td>Yes</td>
<td>SF and Blood draw</td>
</tr>
<tr>
<td>8.0</td>
<td>04.00 pm D₁</td>
<td></td>
<td>Blood draw</td>
</tr>
<tr>
<td>10.0</td>
<td>06.00 pm D₁</td>
<td>Yes</td>
<td>SF and Blood draw</td>
</tr>
<tr>
<td>12.0</td>
<td>08.00 pm D₁</td>
<td></td>
<td>SF, Blood draw and Check out</td>
</tr>
</tbody>
</table>

* - High Fat breakfast in phase II.

**Legends:**
- ICF – Informed Consent Form.
- SF – Safety Assessment.
- CE – Clinical Examinations.

To study the effect of high fat diet on the pharmacokinetics of sildenafil a similar protocol was followed. The diet was designed as per USFDA requirements on a vegetarian basis.

Recommended calories for a 1000calorie high fat breakfast is 150-200 calories from protein, 500-600 calories from fat and 200-350 calories from carbohydrate. The diet followed was as follows:
<table>
<thead>
<tr>
<th>Food Items given for the breakfast</th>
<th>Calories from 100g</th>
<th>Average portion</th>
<th>Calories supplied</th>
<th>Protein in g</th>
<th>Fat in g</th>
<th>Carbohydrate in g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two slices of Toast</td>
<td>240</td>
<td>31 g</td>
<td>74</td>
<td>2.88</td>
<td>0.80</td>
<td>15.2</td>
</tr>
<tr>
<td>20g butter</td>
<td>714</td>
<td>20 g</td>
<td>143</td>
<td>0.06</td>
<td>8.1</td>
<td>0.04</td>
</tr>
<tr>
<td>3 slices of Cheese</td>
<td>370</td>
<td>84g</td>
<td>311</td>
<td>19.5</td>
<td>25.11</td>
<td>1.68</td>
</tr>
<tr>
<td>4 ounce of potato finger chips tossed in 10g of butter</td>
<td>98</td>
<td>113g</td>
<td>112</td>
<td>2.26</td>
<td>0.1</td>
<td>21.6</td>
</tr>
<tr>
<td></td>
<td>714</td>
<td>10g</td>
<td>71</td>
<td>0.06</td>
<td>8.1</td>
<td>0.04</td>
</tr>
<tr>
<td>226 ml of whole milk</td>
<td>68</td>
<td>226ml</td>
<td>154</td>
<td>7.9</td>
<td>8.8</td>
<td>11.1</td>
</tr>
<tr>
<td>A tin of Backed Beans</td>
<td>336</td>
<td>50g</td>
<td>168</td>
<td>11.5</td>
<td>0.85</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>1033</td>
<td></td>
<td>44.16</td>
<td>51.86</td>
<td></td>
<td>~9.66</td>
</tr>
</tbody>
</table>

1 ounce = 28.24g

1g protein = 4.27 calories

1g fat = 9.0 calories

1g carbohydrate = 4.0 calories.

The volunteers were admitted to the study unit on the evening of day one. They received a standard vegetarian diet of about 2800 calories at night and were rested till next morning. Water intake was restricted in the morning. After performing their morning ablutions, oral temperature, blood pressure and pulse rate were recorded. An indwelling catheter of viggo 18 gauge was inserted, and the zero hour sample was collected. All the volunteers compulsorily made to consume the above diet and after 15 minutes 50mg sildenafil tablets were administered with 240ml of water at ambient temperature. The catheter was kept patent with 0.5ml of 20units/ml heparin in saline. Blood samples were
collected at 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 hours post dose. 1.0ml of blood was discarded before each sample collection to avoid any dilution of the sample due to heparin. The blood was centrifuged at 4000rpm for 4 minutes as soon as possible, within 60 minutes of sample collection. Serum obtained was immediately stored at -20°C till the analysis. Analysis was completed within 30 days of sample collection. The volunteers were dosed in sitting position and remained seated for first 2 hours. For rest of the period they were allowed to engage in normal activities avoiding any severe physical exertion. Oral temperature, pulse and blood pressure were checked at 2.0, 6.0 and 10.0 hours post dose. When the sampling time and vital sign measurement were coincided, sample took the precedence over vital sign measurement.

For chrono pharmacokinetic study, the volunteers were checked in at least 4.0 hours before the study to acclimatize with the laboratory conditions. The diurnal cycle of the volunteers is 06.00 - 23.00. They were on normal diet before dosing avoiding excess of xanthine containing beverages and any other medication. Vital signs were measured and recorded. An indwelling catheter of viggo 18 gauge was inserted, and the zero hour sample was collected. A 50mg of sildenafil tablet was administered to the volunteers at 8.00pm clock time with 2 minutes interval between volunteers to facilitate sample collection. The catheter was kept patent with 0.5ml of 20units/ml heparin in saline. Blood samples were collected at 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 hours post dose. 1.0ml of blood was discarded before each sample collection to avoid any dilution of the sample due to heparin. The blood was centrifuged at 4000rpm for 4 minutes as soon as possible, within 60 minutes of sample collection. Serum obtained was immediately stored at -20°C till the analysis. Analysis was completed within 30 days of sample collection.
The volunteers were dosed in sitting position and remained seated for first 2 hours. Water intake was restricted for 2.0 hours before dosing and 2.0 hours after dosing. Rest of the time water was allowed ad libitum. Standard vegetarian meal of around 2800 calories was given at 4.0 hours post dose. Because volunteers slept after 6.0 hours sample till morning no food was given in between. Oral temperature, pulse and blood pressure were measured at 2.0, 6.0 and 12.0 hours post dose. When the sampling time and vital sign measurement were coincided, sample took the precedence over vital sign measurement. The following pharmacokinetic parameters were calculated from the plasma drug concentration, with the help of the software Kinetica 2000 Version 4.0.2® (Innaphase, USA)

- **AUC<sub>0-t</sub>:** The area under the plasma concentration versus time curve, from time 0 to the last measurable concentration, as calculated by the linear trapezoidal method.

- **AUC<sub>0-inf</sub>:** The area under the plasma concentration versus time curve from time 0 to Infinity. AUC<sub>0-inf</sub> was calculated as the sum of the AUC<sub>0-t</sub> plus the ratio of the last measurable plasma concentration to the elimination rate constant.

- **C<sub>max</sub>:** Maximum measured plasma concentration.

- **t<sub>max</sub>:** Time of the maximum measured plasma concentration. If the maximum value occurs at more than one time point, t<sub>max</sub> was defined as the first time point of this value.

- **K<sub>el</sub>:** Apparent first-order elimination or terminal rate constant calculated form a semi-log plot of the plasma concentration versus
time curve. The parameter was calculated by linear least-squares regression analysis using the last three (or more) non-zero plasma concentrations.

\[ t_{1/2} : \] 

The terminal-phase plasma half-life, obtained by dividing 0.693 by the elimination rate constant.

The variability of data may often be better described as a relative variation rather than as an absolute variation, such as represented by the standard deviation or range. One common way of expressing the variability, which takes into account its relative magnitude, is the ratio of standard deviation to the mean. This ratio often expressed as percentage is called the coefficient of variation (CV) or relative standard deviation (RSD). This way of expressing the variability puts the variability in perspective relative to the magnitude of the measurement and allows a comparison of the variability of different kinds of measurements. In biological data the coefficient of variation is often between 20 and 50%, and one would not be surprised to see an occasional CV as high as 100% or more. The relatively large CV observed in biological experiments is due mostly to "Biological Variation", the lack of reproducibility in living material\(^1\). So CV is used to express the intrasubject variation of pharmacokinetic parameters.

Analysis of variance (ANOVA) is perhaps the most powerful statistical tool. ANOVA is a general method of data from designed experiments whose objective is to compare two or more group means. The t test is a special case of ANOVA in which only two means are compared.

The statistical evaluation of treatment differences is based on the ratio of the observed treatment differences to the variability of difference. When this ratio is translated into
statistical statement results in “Statistically Significant” implying that the difference observed between treatments is real, not merely a result of random variation.

The ratio of the difference of averages of the two treatments to its experimental error (standard deviation) is referred to an approximate tabulated probability distribution. The treatment difference is statistically significant if the ratio is sufficiently large relative to the tabulated probability values. The testing procedure is based on the concept of a null hypothesis. The null hypothesis is a hypothetical statement about parameters, which will subsequently be compared to the sample estimate of the parameter, to test for treatment differences. Hence the test was used to compare the effects of experimental conditions on pharmacokinetics of sildenafil.