DISCUSSION
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A simple, reverse phase, high performance liquid chromatographic method using C18 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°C 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 Validation153.

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 (Figure 3). During all the runs r² was found to be more than 0.98 (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 coefficient of variation of the five determinations was between 5 and 15%, the value
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 studies180.

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.

**Pharmacokinetic studies:**

Pharmacokinetics of sildenafil citrate tablets were studied in Indian, healthy, human, male volunteers. Volunteers were enrolled on the preset inclusion, exclusion criteria. Prescribed hematological tests and urine analysis was performed on the volunteers well before the study to ensure their health status. The tablets were administered to the volunteers, blood samples withdrawn at specified time intervals, plasma was separated and analysed for the concentration of sildenafil by the above method. Using the plasma concentration time curve, pharmacokinetic parameters were calculated.

To study the effect of high fat diet on pharmacokinetics of sildenafil, sildenafil citrate 50mg tablets were administered to the volunteers after 15 minutes of high fat breakfast. Pharmacokinetic parameters were calculated.

Pharmacokinetic parameters of sildenafil under fasting conditions were not significantly different from reported values. Intra individual variations observed amongst the parameters were calculated as percentage coefficient of variation. Maximum concentration, \( C_{\text{max}} \), varied by around 2.3 times between the volunteers (839.051 and 361.498 ng ml\(^{-1}\)). Elimination rate constant, \( K_{\text{el}} \), showed a variation of about 2.9 times (0.3930 to 0.1344). A variation of about 2.9 times was observed in half-life \( t_{1/2} \) (5.1545 to 1.7638 hours). Time to reach maximum concentration \( t_{\text{max}} \) varied by 3 times (1.5 to 0.5 hours). A 1.7 fold variation was observed with area under the curve AUC\(_{0-12}\) (2310.06 to 1333.02 ng. h. ml\(^{-1}\)). Individual pharmacokinetic parameters along with the error values are shown in table 9. Thus extent of sildenafil absorption as determined by AUC\(_{\text{inf}}\)
showed less intra subject variation (25.81%CV) than the rate of absorption as characterized by t\textsubscript{max} (37.26%CV) but the metabolism shows higher degree of intra subject variation as elimination rate constant shows a high coefficient of variation (46.32). There are reports, which confirm that sildenafil is metabolized through four cytochrome P450 enzymes: CYP3A4, CYP2C9, CYP2C19, and CYP2D6 to N-desmethyl sildenafil (UK-103, 320)\textsuperscript{110}. The degree of hepatic clearance of orally dosed drugs depends on hepatic extraction ratio\textsuperscript{154}. There are large individual differences in hepatic extraction efficiency and for drugs with high extraction ratios fraction of the absorbed dose available for general circulation will be flow rate dependent\textsuperscript{155}. This may be further complicated by changes in hepatic extraction efficiency to saturation of metabolizing enzymes when drug is carried to that organ at faster rates. Thus the metabolism of sildenafil in any individual will depend on hepatic extraction efficiency and saturation of metabolizing enzymes. This may be an important reason for individual variation observed in the metabolism of sildenafil. However this needs to be studied further.

There is considerable evidence that drug bioavailability is influenced by food. Food may alter the absorption of drug due to degrading of the drug, complexation of the drug with components of food, reduced dissolution due to the presence of food or altered physiology interfering active absorption process or altered gastrointestinal motility\textsuperscript{156}. It is reported that less than 10% of all the drugs are unaffected by food. In some cases drug availability is either reduced or delayed and in others increased. It is important to consider that if the bioavailability of the drug is impaired then any sophistication in formulation is a futile exercise\textsuperscript{143}.
In the present study of sildenafil bioavailability is both decreased and delayed. There is a statistically significant reduction at 95% C.I. in $C_{\text{max}}$, $t_{\text{max}}$ and AUC of sildenafil after high fat diet in comparison with fasting data, but there is no significant intrasubject variation observed under high fat diet as evidences by %CV of 10.98 to 26.84 observed for the pharmacokinetic parameters (Table 11). Maximum concentration, $C_{\text{max}}$ varied by around 1.3 times between the volunteers (291.66 to 218.02 ng ml$^{-1}$). Elimination rate constant, $K_{\text{el}}$ showed a variation of about 1.8 times (0.490 to 0.261). A variation of about 1.9 times was observed in half-life, $t_{1/2}$ (2.65 to 1.41 hours). Time to reach maximum concentration, $t_{\text{max}}$ varied by 1.3 times (4.0 to 3.0 hours). A 2.0 fold variation was observed with area under the curve $\text{AUC}_{0-12}$ (1655.38 to 793.62 ng h ml$^{-1}$). Detailed data and error values are shown in table 11. Thus the extent of absorption, the rate of absorption and the metabolism is fairly constant amongst the volunteers and there is no much intra subject variation.

Blood flow rate and the regulation of gastric motility and emptying are two important physiological factors that influences gastrointestinal physiology and in turn affect drug absorption. The most important absorption route is by direct transfer from the lumen of the GI tract, across the epithelial cell lining and into the adjacent capillary network leading to the portal circulation. The rate at which the compound diffuses across the capillary membrane is a function of concentration gradient across the membrane and hence of the rate of flow of blood through the capillary. Any changes in the rate of splanchnic blood flow, due to food ingestion might therefore be expected to have some influence on the absorption efficiency of drug. Studies in normal human beings have shown that changes in splanchnic blood flow may occur during food ingestion, but the
degree of change varies considerably depending on the type of food. After ingestion of a liquid protein meal, estimated splanchnic blood flow increased in the first hour and dropped slightly during the next half an hour. However with liquid glucose meal, estimated splanchnic blood flow decreased slightly during the first hour and returned to normal values during next half a hour. Increased splanchnic blood flow may also influence the absorption of the drugs that are extensively metabolized because of changes in the clearance during the first pass through the hepatoporal system.

In general, drugs are very well absorbed from the small intestine, and the absorption from stomach is relatively insignificant. It has been suggested that promoting gastric emptying will generally increase the bioavailability of the drug. Though it does not hold true for all cases, it does frequently happen. The most significant point is that gastro intestinal motility can be a variable in the absorption of orally administered products. Food, especially fatty food, closes the pyloric sphincter, delaying the stomach emptying. The simultaneous ingestion of food with drugs may influence gastro intestinal absorption by altering the dissolution rate, changing the gastric emptying time, altering the pH of stomach fluid or complexing drug to food or food components.

Weakly basic compounds, which tend to be un-ionised in relatively high intestinal pH, are optimally absorbed from the small intestine. So any factor, which has a potential of delaying the stomach emptying, can therefore delay the absorption of orally dosed drug. Gastric emptying may also be delayed by ingestion of solutions of high viscosity, by fats and to a lesser extent by protein and carbohydrates. Presence of solid food influences GI physiology in a very complicated manner and have been shown almost double stomach emptying time compared to liquid meal in rats. Absorption of
sildenafil, which is a weakly basic drug, may thus be delayed due to this reason. Although passage of a drug from stomach to the intestine is thus likely to be mechanically delayed by the presence of food, prolonged residence in the stomach may have varying effects on drug absorption depending on the drug's solubility and stability in the acidic gastric juices.

Apart from its influence on splanchnic blood flow and GI motility fat also stimulates the secretion of bile salts. These may either increase the dissolution of poorly soluble drugs, promoting absorption\(^{165}\) or impede the absorption of some compounds because they form insoluble complexes\(^{166}\). Solid or semisolid food may also act as a mechanical barrier preventing drug movement towards the mucosal surface of alimentary tract inhibiting the drug absorption.

In addition delayed and reduced absorption of some drugs may also occur due to adsorption of drug on to the food compound or chelation of drug by polyvalent metal ions such as calcium and magnesium\(^{167}\) or complexation with proteins\(^{168}\).

Though most of the fat in present diet is from milk products, which are rich source of calcium and magnesium, chelation may not be responsible for the reduced rate and extent of sildenafil absorption. This is because the antacids containing aluminium or magnesium are reported to have no effect on pharmacokinetic parameters of sildenafil\(^{14}\). Hence calcium being responsible for such an effect is less probable. Complexation of sildenafil with fatty acids produced from fat or with the protein present in the diet may be one of the reasons for reduced bioavailability, but the structure of sildenafil (on page no.44) does not show any active group, which can make this possible. So the reason for delayed
absorption seems to be the reduced gastric motility and splanchnic blood flow. However further in depth investigations are necessary to support this hypothesis.

It is surprising to note that biological half-life and elimination rate constant of sildenafil after high fat diet are not significantly different from their fasting values. Thus though the rate of absorption is delayed and extent of absorption is reduced under high fat diet conditions, the elimination is unaffected. This may indicate that the metabolism of sildenafil, though involves enzyme P450, may not follow saturation kinetics.

In order to study the effect of circadian rhythm on pharmacokinetic parameters of sildenafil. Volunteers were administered 50mg tablets of sildenafil citrate after 7-8 hours of fasting, blood samples collected and analysed similarly. The chronopharmacokinetic data of sildenafil is identical to that under fasting condition. There are no statistically significant changes in the pharmacokinetic parameters due to circadian rhythm except for $t_{\text{max}}$.

The intrasubject variation observed amongst chronopharmacokinetic parameters is similar to that of fasting. Maximum concentration, $C_{\text{max}}$, varied by around 2.5 times between the volunteers (684.097 to 270.80 ng ml$^{-1}$). Elimination rate constant, $K_{el}$, showed a variation of about 1.6 times (0.1018 to 0.065). A variation of about 2.9 times was observed in half-life, $t_{1/2}$ (3.769 to 1.296 hours). Time to reach maximum concentration, $t_{\text{max}}$, varied by 3.0 times (3.0 to 1.0 hours). A 1.3 fold variation was observed with area under the curve AUC$_{0-12}$ (1758.10 to 1154.61 ng h ml$^{-1}$). The detailed data is given in table 13. Thus the extent of absorption and the rate of elimination are fairly constant amongst the volunteers, but other parameters showed a variation of about 2 to 3 times.
A set of environmental factors is able to influence within certain limits rhythm characteristics such as period, the amplitude and acrophase. For circadian rhythms, alternation of light and darkness cycle (LD Cycle) is a powerful synchronizer for a large number of plants and animals. For human beings alternation of activity and rest appears to be a powerful synchronizer of circadian rhythms. Drugs and potentially toxic agents and even physical agents do not have the same effect on the organism depending on the hour they are administered. The patient does not tolerate a surgical intervention in the same way at all hours in the 24h scale.

In the present study there were no statistical differences observed between fasting and Chronopharmacokinetic data except in $t_{\text{max}}$. This probably is due to a shorter duration of fasting (around 7 hours) than in fasted study where it was around 11 hours. The presence of small quantity of residual food might have delayed the absorption of the drug slightly. The extent of absorption and elimination do not change with the time of administration of the drug, proving its effectiveness during the actual use to the same extent as fasting.

In none of the studies any volunteer has to be withdrawn from the study, due to any adverse events. This shows that sildenafil is safe in subjects of the studied age group, the Indian male volunteers under all the studied conditions.

Thus sildenafil is equally effective irrespective of the time of administration. Hence it can be consumed at any time of the day, without any consideration of Light Dark Cycle. Nevertheless sildenafil after a high fat diet is severely contraindicated on pharmacokinetic terms due to severe reduction in extent and rate of absorption. This need not be cause of concern, as the dinner is usually light on fat, but the effect of other types
of food like carbohydrate and protein etc on pharmacokinetics of sildenafil needs to be studied further before concluding the effect of food.