Dose-Dependent Synergistic effect of Pomegranate Juice on the Bioavailability of Sildenafil in Rats by Using HPLC Method

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SUMMARY. World widely, the prevalence of erection dysfunction (ED) ranged from 2% in men younger than 40 years to 86% in men 80 years or older. Sildenafil citrate, a potent and selective inhibitor of cyclic guanosine monophosphate (cGMP), is widely prescribed for ED treatment. Pomegranate (Punica granatum) juice has a high content of polyphenolic flavonoids with antioxidant and anti-atherosclerotic properties. The food-drug interaction was assessed in this study to elucidate the role of pomegranate uptake on the pharmacokinetics of sildenafil citrate in a dose-dependent manner. A set of 30 rats was divided into 5 groups (group A to group E). In the experiment, group (A) had received sildenafil aqueous solution alone, while groups from (B) to (E) had received sildenafil solution with 2, 4, 6, and 8 mL of pomegranate juice, respectively. There was a significant increase in AUC for sildenafil in a proportion of 6% and 9% in groups of D and E, respectively. The maximum serum concentration (Cmax) of Sildenafil in the control group and the time required to reach maximum concentration (Tmax) were 131.12 ± 33.82 ng/mL and 0.5 h, respectively. The area under the serum curve (AUC) was 520.96 ± 64.04 (ng/mL*hr). The Cmax of Sildenafil with pomegranate in group D (129.11 ± 30.12) and group E (127.35 ± 27.9689) ng/mL were lower than Sildenafil alone, whilst Tmax was longer 1 and 1.5 h and AUC bigger (564.19 ± 54.46 and 547.78 ± 39.12) (ng/ml.h, P < 0.05). There was a sustained release for sildenafil when administered with pomegranate as the elimination rate has decreased and the values of T1/2 have increased. Pomegranate has increased the bioavailability of sildenafil by affecting both absorbance and concentrations of sildenafil in bloodstream in a dose-dependent manner; therefore, food-drug interactions should be taken in consideration when treating ED patients.

RESUMEN. La prevalencia de la disfunción eréctil (ED) en el mundo osciló entre el 2% en hombres menores de 40 años y 86% en los hombres de 80 años o más. El citrato de sildenafil, un inhibidor potente y selectivo de guanosina monofosfato cíclico (GMPc), es ampliamente prescrito para el tratamiento de la disfunción eréctil. Por su parte el jugo de granada (Punica granatum) tiene un alto contenido de flavonoides polifenólicos con propiedades antioxidantes y anti-ateroscleróticas. La interacción de alimentos con drogas fue evaluada en este estudio para dilucidar el papel de la absorción de la granada en la farmacocinética de citrato de sildenafil en forma dosis-dependiente. Un conjunto de 30 ratas se dividió en 5 grupos (grupos A al E). En el experimento, el grupo (A) había recibido sólo solución acuosa de sildenafil, mientras que los grupos (B) a (E) habían recibido solución de sildenafil con 2, 4, 6 y 8 mL de zumo de granada, respectivamente. Hubo un aumento significativo en el AUC de sildenafil en una proporción de 6% y el 9% en los grupos D y E, respectivamente. La concentración máxima (Cmax) en suero de sildenafil en el grupo control y el tiempo necesario para alcanzar la concentración máxima (Tmax) fueron 131.12 ± 33.82 ng/mL y 0.5 h, respectivamente, mientras que el área bajo la curva (AUC) de suero fue de 520,96 ± 64,04 (ng/mL*hr). Considerando que la Cmax de sildenafil con el jugo de granada en el grupo D (129,11 ± 30,12) y el grupo E (127,35 ± 27,9689) ng/mL fueron más bajos que el sildenafil solo, mientras que Tmax fue mayor de 1 y 1,5 h y AUC más grande (564,19 ± 54,46 y 547,78 ± 39,12) (ng/ml.h, P < 0.05). Hubo una liberación sostenida para sildenafil cuando se administra con el zumo de granada, ya que la velocidad de eliminación ha disminuido y los valores de T1/2 han aumentado. El zumo de granada ha incrementado la biodisponibilidad de sildenafil al afectar tanto la absorbancia como a la concentración de sildenafil en el flujo de sangre en una forma dependiente de la dosis; por lo tanto, las interacciones entre alimentos y medicamentos deben ser tomados en cuenta cuando se trate pacientes con ED.

KEY WORDS: drug-food interaction, pharmacokinetic, pomegranate, preclinical, sildenafil.

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INTRODUCTION

The National Institutes of Health consensus panel has defined the erection dysfunction (ED) as “the inability to achieve or maintain an erection sufficient for satisfactory sexual performance”. World widely, the prevalence of ED ranged from 2% in men younger than 40 years to 80% in men 80 years or older. In the United States of America, the prevalence of ED is estimated around 18 million in men aged over 20 years. Several factors such as obesity, stress, and cardiovascular events are contributing for the development of ED. As an efficient remedy for ED; Sildenafil citrate (Fig. 1) is a potent and selective inhibitor of cyclic guanosine monophosphate (cGMP) and widely prescribed.

It has been reported several adverse events associated with sildenafil usage as flushing, dizziness, headache, tachycardia, chest pain, drowsiness, hypotension, nausea, and syncope. Even though sildenafil has temporarily solved the problem of ED events but still there were no reports for priapism with therapeutic dose usage. One serious problem of sildenafil prescription is the drug-drug interaction, for instance, medications containing nitrates, alpha blockers, HIV protease inhibitors, and potent cytochrome P450 (CYP) 3A4 inhibitors namely macrolide and imidazole antimicrobials as well as nonspecific CYP inhibitor cimetidine are associated with increased plasma levels of sildenafil and may aggravate the actions of sildenafil. The extensive first-pass metabolism affects the low bioavailability of sildenafil, as only about 40% of the drug is absorbed after oral administration. Sildenafil is eliminated through hepatic metabolism pathway, namely cytochrome P450 3A4, and metabolized to an active metabolite with similar properties to the original drug having terminal half-lives of approximately 4-5 h. The maximum observed plasma concentration of sildenafil is reached within 30-120 (median 60) min of an oral dosing in a fasting state.

Pomegranate (Punica granatum) juice (POM) has a high content of polyphenolic flavonoids. The flavonoids have potent antioxidant and anti-atherosclerotic properties. Owing to these nutritional characteristics, HEART-UK, and the cholesterol charity encourages consumption of POM as part of a routine heart healthy diet. This endorsement motivates people to drink the juice for its potential health benefits. Pomegranate is commonly used as a juice or even an ingredient of food recipes in Jordan and some other middle-eastern countries. There is a concomitant confounder for the interaction of pomegranate with drugs, for instance, Nagata et al. showed an inhibitory activity for pomegranate on diclofenac metabolism through measuring the formation of 4-hydroxy-diclofenac in the presence or absence of pomegranate juice. Whereas, Voruganti et al. reported that pomegranate juice has improved the single pass of a solution of nitrendipine in the presence of pomegranate juice as both absorption rate constant and fraction of drug absorbed were greater in the pomegranate juice pre-treated group. On the other hand, priapism following the concurrent use of sildenafil and POM juice is reported for the first time in the literature to create awareness on this entity. In addition, Senthilkumaran et al. described a priapism effect of pomegranate ingestion with sildenafil relied on pharmacokinetic interactions with co-administered medications. However, pomegranate increased the bioavailability of the co-administered drugs as it increased warfarin levels in the bloodstream after the admission of pomegranate juice. Consistently, a massive increase was reported by Sheu et al. as there was an increase of 168% in the AUC and 35% in Cmax of sildenafil levels after grapefruit juice ingestion.

Currently, drug-juice interactions become in the center of our interest. Upon our knowledge, there is no study has been conducted on pomegranate-sildenafil interaction in a dose-dependent manner. However, the pomegranate was found to augment the bioavailability of sildenafil. In this study, we hypothesized that pomegranate juice co-administration would augment bioavailability of sildenafil administered orally in a dose-dependent manner.
MATERIAL AND METHODS

Chemicals and reagents
Distilled water, nano pure (Fischer Scientific), methanol and acetonitrile gradient grade were purchased from Fischer scientific, triethylamine from TEDiA, phosphoric acid from Medex, sildenafil and carbamazepine were obtained from Dar Al Dawa Pharma, Jordan. Rat plasma was obtained from Animal House of Applied Science University.

Instrumentation
The HPLC (Finnigan Surveyor) system was performed and composed of Chrom Quest software 4.2.34, solvent delivery systems pump (LC Pump Plus), auto-sampler plus, UV-VIS plus, Sepax GP-C18, (150 × 4.6 mm), 5 µm, computer system.

Preclinical study and protocol
Adult female Sprague-Dawley laboratory rats were generously donated by the animal house of the Applied Science University. The average weight of the rats was about 250 g, and they were healthy. They were placed in an air-conditioned environment (20-25 °C) and exposed to a photoperiod cycle (12 h light/12 h dark) daily.

Preclinical experiments were performed in compliance with FELASA (Federation of European Laboratory Animal Science Association) guidelines and the study protocol was approved (April 2014) by the ethical committee at the Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan.

A set of 30 rats was included in the drug interaction study in a crossover pattern. The rats were divided into 5 groups (group A to group E), 6 rats each. In the first period of the experiment, group (A) had received sildenafil aqueous solution alone, while groups from (B) to (E) had received sildenafil solution with pomegranate juice (purchased from local market), respectively and spontaneously.

The rats which were aimed to take pomegranate in each part of the experiment were supplied with 15 mL, 16 h before sildenafil solution administration. They received another 2 mL (group B), 4 mL (group C), 6 mL (group D), and 8 mL (group E) of pomegranate orally after the administration of sildenafil solution using a syringe with a specific needle for the oral administration of both sildenafil and pomegranate. Whereas, for the rats (group A) which were aimed to take sildenafil solution alone, drinking water was administrated in relevant amounts to pomegranate intake.

All blood samples were withdrawn from the optical vein of the rat’s into EDTA-containing micro-tubes at the following time intervals: (0.0, 0.5, 1, 1.5, 2.5, 4, and 6 h). The micro-tubes were immediately centrifuged for 15 min at 4,000 rpm and separated plasma were transferred into labeled Eppendorf tubes then stored at -30 °C until analysis.

Preparation of solutions
Preparation of sildenafil solution for oral use
An equivalent weight of 0.081 g of sildenafil citrate (equal to 0.057 g sildenafil) was dissolved in 100 mL of distilled water, to get a concentration of 0.57 mg/mL to be given to the rats.

Preparation of stock solution of sildenafil
Ten mg of sildenafil working standard was dissolved in 10 mL of methanol to get a concentration of 1000 µg/mL stock solution of sildenafil. From this stock solution, the standard concentrations of sildenafil were prepared to cover calibration range (20-500) ng/mL (low, medium and high ) quality control samples are 60, 250, and 425 ng/mL.

Preparation of stock solution of carbamazepine (internal standard)
An equivalent weight to 10 mg of carbamazepine working standard was dissolved in 100 mL of ACN to get a concentration of 100 µg/mL stock solution of carbamazepine.

Preparation of working solution of carbamazepine (I.S)
A volume of 20 µL were taken from carbamazepine stock solution (100µg/mL) and then diluted to 100 mL using acetonitrile which was considered to be (I.S) working solution that contains 20 ng/mL of carbamazepine.

Method of extraction
A labeled disposable Eppendorf tubes were placed in a rack, then 100.0 µL aliquots of each test samples (blank, zero, standards, QCL, QCM, QCH or rat samples) were drawn into the appropriate tubes by pipette, after that 150.0 µL of internal standard (20 ng/mL carbamazepine in ACN) was added, vortex each sample vigorously for 1.0 min. and then centrifugation at 14000 rpm for 15 min.

Chromatographic conditions
The chromatographic conditions of analyzing sildenafil in rat plasma are summarized in Table 1.
Validation

Accuracy and precision

The inter-day accuracy and precision were detected by analyzing three trials of QC samples and LLOQ samples on three different days. The accuracy percent was calculated by dividing the measured mean concentration over the nominal analyte concentration. Precision was expressed as CV%. According to EMA guideline, the accepted limits of accuracy and precision should be below 15%, except at the LLOQ which should be below 20% 20.

Linearity

The determination of linearity was evaluated by a series of six injections to a seven calibration concentration levels for the analyte. Peak areas of the calibration standards were plotted in the Y-axis against the nominal standard concentration. The linearity of the plotted curve was calculated through the value of the correlation coefficient (R²) which should be exceed 0.98 20.

Stability

Stability test of sildenafil in rat plasma was evaluated using low and high QC samples which were analyzed for auto-sampler stability and “3 cycles” freeze-thaw stability. The mean concentration should be within ± 15% of the nominal concentration 20.

RESULTS

Validation

Selected chromatograms for sildenafil samples (blank, LLOQ and QCM, sildenafil control and sildenafil with 6.0 mL pomegranate) were shown in Figs. 2-6.

Accuracy and precision

Table 2 represents inter-day accuracy and precision for quality control samples of sildenafil in three days of validation. All of the obtained accuracy and precision data were within the accepted range which is 85-115 % for all concentration except for LLOQ, which is 80-120 % for the accuracy, and for the precision (± 20 % for LLOQ and ± 15 % for other concentrations).

Table 1. Chromatographic conditions of the analytical method.
Linearity

$R^2$ which represents the strength of correlation coefficient for standard calibration curve was greater than 0.99 during validation course (Fig. 7). Data of standard curve with regards to correlation, slope, $R^2$, and intercept are shown in Table 3.

Stability

The stability tests of sildenafil were evaluated on low and high QC samples. The accuracy for the auto-sampler stability and freeze and thaw cycles were in the range of 85%-115%.

Effect of Pomegranate doses on sildenafil pharmacokinetic

As shown in Table 4 and Figs. 8 and 9, significant effect of pomegranate on the bioavailability of sildenafil was observed in 6.0 and 8.0 mL pomegranate doses, the maximum concentrations (Cmax) were 129.11 and 120.35 ng/mL, respectively and the area under the curve (AUC) were 564.19 and 547.78 ng/mL.hr. While the Cmax and AUC of sildenafil control was 131.12 ng/mL and 520.96 ng/mL.h, respectively.

DISCUSSION

The pharmacokinetic parameters of sildenafil in this study are consistent with previous studies. Statistical analyzes of the results using independent T-test has revealed a significant effect of pomegranate on the bioavailability of sildenafil hence sildenafil levels have increased in the blood of rats ingested 6 mL and 8 mL pomegranate in a ratio of 9% and 6%, respectively. There was a sustained release for sildenafil when administered with pomegranate as the elimination rate has decreased and the values of $T_{1/2}$ have increased (Table 4). The 90% CIs of the pharmacokinetic parameters included unity, which strongly supports that the differ-
Table 3. Correlation, slope, $R^2$, and intercept for sildenafil.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cmax (ng/mL) #</th>
<th>Tmax (h)</th>
<th>$T_{1/2}$ (h)</th>
<th>AUC (ng/mL·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sildenafil</td>
<td>131.12 ± 33.82</td>
<td>0.5</td>
<td>1.13</td>
<td>520.96 ± 64.04</td>
</tr>
<tr>
<td>Sildenafil with pomegranate 2 mL</td>
<td>121.32 ± 20.71</td>
<td>0.5</td>
<td>1.04</td>
<td>451.69 ± 37.22</td>
</tr>
<tr>
<td>Sildenafil with pomegranate 4 mL</td>
<td>124.61 ± 29.66</td>
<td>1</td>
<td>1.20</td>
<td>483.42 ± 38.86</td>
</tr>
<tr>
<td>Sildenafil with pomegranate 6 mL</td>
<td>129.11 ± 30.12</td>
<td>1</td>
<td>1.40</td>
<td>564.19 ± 54.46</td>
</tr>
<tr>
<td>Sildenafil with pomegranate 8 mL</td>
<td>127.35 ± 27.96</td>
<td>1.5</td>
<td>1.44</td>
<td>547.78 ± 39.12</td>
</tr>
</tbody>
</table>

Table 4. Pharmacokinetics data of sildenafil. *P < 0.05 (significant). #P > 0.05 (insignificant), mean ± standard deviation. The difference between Cmax is insignificant while the difference between AUC is significant.

Figure 9. Mean plasma concentration-time profile of Sildenafil after oral administration of water (control) and pomegranate in dose-dependent to 30 female rats.
was no single study covers the interaction of pomegranate interaction with sildenafil in a dose-dependent manner. Even though there were few studies investigated the interaction between pomegranate and sildenafil on the level of both reports and clinical trials, eventually, they were not intimately performed in a prospective of food-drug interaction. Senthilkumaran et al. reported the development of low flow priapism in three patients related to simultaneous consumption of sildenafil with pomegranate juice. In addition, Mansouri et al. reported an influence of eligic acid (one of pomegranate contents) uptake on sildenafil levels in the blood as the AUC has increased up to 42%.

On the other hand, several studies have investigated the interaction between pomegranate and different drugs. In an interesting study, pomegranate affected the permeability of nitrendipine perhaps by inhibiting the efflux p-glycoprotein pumps in a single-pass intestinal perfusion model, the absorption was merely affected after a single pass in the perfusion model. Sildenafil is metabolized by hepatic cytochrome P450 CYP2C9 and CYP3A4 to N-desmethyl sildenafil, in humans. Pomegranate juice may inhibit intestinal CYP3A4 as observed previously with grapefruit juice.

In our study, the pomegranate juice did not cause a massive increase in the sildenafil concentration, but despite this result, pomegranate juice may cause a synergistic effect when administered with sildenafil. This pomegranate-sildenafil interaction may be caused by competition of pomegranate on the free binding sites of CYP450 in liver hence the absorption of pomegranate was faster than sildenafil until the saturation has been reached. Another possible reason is the delay in the absorption time as we note a shift in the values of Tmax to the left of the scale which in turn causes a prolonged physiological action of sildenafil. We hypothesize the activity exerted from pomegranate on ED as a reasonable explanation for the cases reported by Senthilkumaran et al., there is a synergistic activity of sildenafil in the presence of pomegranate on ED through the inhibition of free oxidants formation followed by the physiologic mechanism of penile erection involves by releasing of nitric oxide (NO) into the corpus cavernosum during sexual stimulation. NO then activates guanylate cyclase, which increases cyclic guanosine monophosphate (cGMP). cGMP relaxes smooth muscle in the corpus cavernosum, allowing for blood inflow and penile erection. Whereas, it is well known, sildenafil possesses its pharmacological activity through phosphodiesterase-5 (PDE-5) inhibition. PDE-5 is an enzyme that causes degradation of cGMP. By administration of the PDE-5 inhibitors, cGMP is increased, allowing for the cascade leading to penile erection.

CONCLUSION
We show there is a food-drug interaction between pomegranate and sildenafil; therefore, there should be awareness and to include a list of limitations for sildenafil admission with juices, hence the ingestion of pomegranate with sildenafil has caused an increase in the bioavailability of sildenafil in the blood stream which will cause a prolonged physiological effect in the presences of such juices. Especially, these days, there are pomegranate extracts formed in pharmaceutical forms used for ED treatment.

Conflict of interests. The authors declare that there is no conflict of interest.

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REFERENCES