POMEGRANATE JUICE AFFECTS ON PHARMACOKINETIC PARAMETERS OF METRONIDAZOLE BY USING HPLC-MS

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ABSTRACT
Objective: To quantify Metronidazole in rat serum by using HPLC/MS and to study the effect of pomegranate fresh juice on the Metronidazole pharmacokinetics Methods: The serum samples were prepared by using protein precipitation method with Clindamycin in methanol solution. A gradient mobile phase system and ACE 5 C18 column were used for the analysis. Rats (n=4) were treated with a single ordinary dose (5ml/kg) of freshly squeezed pomegranate juice 30 min before orally administered-Metronidazole while other groups of rats (n=4) were treated with a multiple dose of the juice twice daily for two days versus DW pretreated rats (n=2). The study protocol was approved by the ethical committee of Graduate Studies at Faculty of Pharmacy and Medical Sciences of University of Petra/Amman-Jordan (March, 2012). Key findings: The analytical method was linear with acceptable recovery, precision and accuracy. The results show that there was a significant increase in the Metronidazole pharmacokinetic parameters (Cmax and AUC, P<0.05) after pretreatment with multiple dose of pomegranate juice. Conclusions: Pomegranate juice with a multiple dose pre-treatment could be a good intestinal but not hepatic enzymes inhibitor for Metronidazole metabolism.

KEYWORDS: Pomegranate, Metronidazole, Pharmacokinetic, HPLC/MS.
1. INTRODUCTION

Metronidazole is one of commonly used antibiotics since 1970s for treatment of gram-negative anaerobes infections such as bacteroides or gram-positive anaerobes infections such as clostridia [1, 2]. Recently, MTZ, which is inexpensive, is frequently used in hospitals as a prophylaxis against anaerobic infection after bowel surgery, treatment of colitis caused by Clostridium difficile, and wound abscess treatment [3, 4]. MTZ is used in combination with other drugs for treatment of peptic ulcer diseases (PUD) caused by Helicobacter pylori (H. pylori) which is the major cause of gastritis with a risk factor for stomach cancer [5, 6]. MTZ is considered as a substrate and an inhibitor to CYP2C9 and CYP3A4 enzymes and as an inhibitor to CYP2C8 enzyme [7].

Recently, pomegranate PJ has been widely consumed around the world especially in the Middle Eastern countries. According to legend, PJ juice has been used as a medicine for thousands of years [8]. In laboratory tests, PJ shows antiviral, antibacterial, and antioxidant properties [9-11]. Some reports talked about a decreasing effect of PJ in cardiovascular diseases by inhibition of low-density lipoprotein oxidation [12, 13]. Furthermore, therapeutic properties of the fruit have been suggested for use in cases of breast cancer [14] and prostate cancer [15].

The interaction between a drug with a product consumed as food or a botanically-derived nutrient may affect health status due to altered Pharmacokinetic (PK) and/or pharmacodynamic (PD) of the drug by increasing drug’s bioavailability with the risk of adverse events and toxicity, or decreasing its bioavailability, leading to therapeutic failure [16]. Such interaction may be due to induction or inhibition of one or more CYP iso-forms which are the predominant enzymes involved in phase I drug metabolism [17].

Several studies have been explained the effect of wine, beer, tea, fruit juices, and their specific constituents on enteric-enzyme activity in vitro [18-48] but still clinical studies are limited. Moreover, though beverages have become highly recommended and over-the-counter supplements for prevention and treatment of common diseases, their interactions with medications remain misunderstood area of pharmacotherapy. Previous studies for the effect of pomegranate juice on medication’s pharmacokinetics are also still few or premature (e.g. its effect on carpamazepine, midazolam, and tolbutamide [25-28, 48]).

In addition, the use of complimentary or alternative remedies is on the increase globally, because most people believe that the natural agents are safer than the conventional therapeutic agents. Based on these findings, higher pomegranate consumption could allow for
an increased possibility of pomegranate-drug interaction. Furthermore, the effect of PJ juice on the enteric CYP isoenzymes or the other transporters involved in drug disposition is not fully addressed yet. Therefore, it is important to evaluate the interaction between pomegranate and medications. Our present study therefore attempts at elucidating the possible interaction between the Metronidazole and the pomegranate natural juice vis-a-vis the effect of pomegranate on the pharmacokinetic study of Metronidazole.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

2.1.2 Instrumentation
An API Mass spectrometer was used and consisted from the following: Degasser (Agilent 1260). Solvent delivery systems pump (Agilent 1200). Autosampler (Agilent 1200). Thermostat column compartment (Agilent 1200). API 3200 Mass Spectrometer. ACE 5, C18 (50 x 2.1 mm), 5µm. Computer System, Windows XP, SP3, Data Management Software. Other instruments used was: Bath Sonicator Elmasonic PH500EL. Sartorius pH meter. Sartorius Balance. Sartorius Centrifuge.

2.1.3 Experimental animals
Spargue Dawley (S.D) adult male rats supplied by the Animal House, University of Petra, with a weigh range of 200-250 g were used in the experiments. They were placed in air-conditioned environment (20-25 C°) and exposed to a photoperiod cycle (12 hours light / 12 hours dark) daily. The study protocol was approved by the ethical committee of Graduate Studies at Faculty of Pharmacy and Medical Sciences of University of Petra/Amman-Jordan (March, 2012).

2.2 Methods

2.2.1 Preclinical study design
To find out the effect of PJ on MTZ, four groups of twelve hour fasted- rats (six rats in each group) were administered by a single dose of PJ (5ml/kg) 30 min before MTZ administration,
while in case of multiple dose PJ administration, the same group numbers of rats were administered with PJ twice daily for two days then treated as well as the single dose administration at the third day (i.e. day of the experiment). In addition, another two groups of rats were administered with DW that which were used as controls. The blood sample at certain time interval of each group represents the collection of blood drops from six rats in one eppendorf tube which were obtained from rat’s tail then centrifuged for 5 minutes to obtain serum (100-125 µl) which was placed in labeled eppendorf tubes and stored in freezer at (-30°C) until the time of analysis.

2.2.2 Preparation of Metronidazole solution to be injected orally to rats
After considering the maximum therapeutic daily dose of MTZ in human which is 1000 mg/70 kg, therefore the dose for 0.25 kg (rat’s average weight) should be 3.6 mg. By dissolving 0.18 g of MTZ raw material in DW, a total volume of 50.0 ml and a concentration of 3.6 mg/ml will be obtained. The oral dose of each rat was calculated according to its weight by the following equation: oral dose = rat’s weight x dose in human (14.3 mg/kg) / prepared concentration (3.6 mg/kg).

2.2.3 Preparation of pomegranate juice to be injected orally to rats
Pomegranate fruit was cut into two pieces, squeezed by orange squeezer, and then filtered to get clear juice to be used freshly.

2.2.4 Preparation of stock solutions for the analytical method validation
2.2.4.1 Preparation of stock solution for Clindamycin as an internal standard (IS)
10 mg of Clindamycin working standard was dissolved in 10.0 ml methanol to get concentration 1.0 mg/ml stock solution.

2.2.4.2 Preparation of 2 µg/ml Clindamycin IS in methanol (Precipitating agent)
1000.0 µl was taken from Clindamycin 1.0 mg/ml stock solution and complete to 500.0 ml of methanol in dispenser bottle to obtain 2.0 µg/ml Clindamycin.

2.2.4.3 Preparation of stock solution of Metronidazole
60 mg of MTZ working standard was dissolved in 10.0 ml by methanol (100%) to get concentration of 6.0 mg/ml as stock solution.
2.2.4.4 Preparation of working solution for Metronidazole

0.50 ml was taken from MTZ stock solution and diluted to 10.0 ml by 50% methanol to obtain a concentration of 300.0 µg/ml as working solution.

2.2.5 Preparation of the mobile phase

The mobile phase consisted from aqueous solvent which was formic acid as buffer and organic solvent which was absolute methanol. Preparation of 0.1% formic acid by taking 2.50 ml from 100% formic acid completed to 2.50 L deionized water, mixing then sonication for 10 min.

2.2.6 Preparation of Metronidazole standard calibration curve serial dilution and spiked serum.

Samples of standard curve in serum were prepared by spiking 500.0 µl from serial solution into 4.50 ml of serum, using seven concentrations, not including zero to attain standard calibration curve Metronidazole concentrations of: 0.300, 0.600, 1.200, 2.400, 6.000, 12.000, and 18.000 µg /ml in serum. Each concentration of the serum sample was divided to 50.0 µl in 1.50 ml eppendorf tube and kept at (-30°C). Standard samples were given daily together with the quality control samples. As showed in (Table 1).

2.2.7 Preparation of Metronidazole quality control serial dilution and spiked serum.

Quality control (Q C) samples in serum were prepared by spiking 500.0 µl from serial solution into 4.50 ml of serum to attain quality control Metronidazole concentrations of: 0.9, 9.0, and 14.4 µg /ml. Each concentration of the QC serum sample was divided to 50.0 µl in 1.50 ml eppendorf tube and kept at (-30°C). QC samples were given daily together with the standard samples. As showed in (Table 1).

2.2.8 Method of extraction

To 0.050 ml of serum sample, 500.0 µl of internal standard (2 µg/ml Clindamycin in methanol) was added in a 1.50 ml eppendorf tube, vortex-mix for 2.0 min, centrifugation for 15 min at 14000 r.p.m., and then the supernatant was transferred into auto-sampler vials.

2.2.9 Analytical method validation

The validation of the method was performed in three separate days. In each day, seven standard calibration levels (not including zero) was prepared. Serum samples of method validation represented blank, zero, standard calibration curve, six replicates of QC samples (Q.C. Low, Q.C. Mid, and Q.C. High). The validation parameters (linearity, intra and inter-
day accuracy and precision, sensitivity and recovery) should not exceed the limits by the Food and Drug Administration (FDA) Guidance for Industry and United state pharmacopeia (USP) \(^{[49, 50]}\). All of the chromatographic and mass detector conditions are mentioned in (Table 2).

3. RESULTS

3.1 Validation

Validation of this analytical HPLC-MS method was performed in order to be evaluated in terms of recovery, linearity of response, precision, accuracy, and sensitivity for quantification of MTZ. The lower limit of quantification (LLOQ) was 0.300 µg/ml. Intra-day accuracy data showed an accuracy range of 91.4% - 113.2%, 95.3% - 111.8%, and 82.0% - 106.0% for the first, second, and third day of validation, respectively with a linear relationship which was observed between the concentration and the peak area of MTZ (correlation coefficient, \(R^2 = 0.999\)). In addition, the recovery of MTZ from its biological matrix in this bioanalytical method was (96.0-102.0%).

The plot of linearity of calibration curve levels for MTZ quantification against their analytical response and regression linear equation that represents all three days of validation was done by plotting the calculated mean of the measured concentrations versus the calculated mean of the AUC ratio for each standard point showed in (Figure 1).

3.2 Effect of a single and multiple dose of pomegranate juice on Metronidazole

After single dose administration of PJ with MTZ, the pharmacokinetic parameters of MTZ are represented in (Table 3). As seen there were not any significant changes in these parameters when compared with the control groups (groups that administered with DW and drug).

After multiple dose administration of PJ with MTZ, the pharmacokinetic parameters of MTZ are represented in (Table 4). Cmax, AUC, and \(t^{1/2}\) were increased by (43.40%), (132.30%), and (27.00%), respectively, with decreasing in the elimination rate constant by (25.00%) while Tmax was not changed.

The plot that represents the comparison between DW administration, single and multiple dose administration of PJ with MTZ is showed in (Figure 2).
4. DISCUSSION

The analytical method is one or even the most critical step in the pharmaceutical research for determination of the drug plasma concentration and identifying its pharmacokinetic profile.\(^{[51]}\) The combination between the separation capacity of HPLC along with the sensitivity and specificity of mass spectrometry is considered one of the most powerful technologies for identification and quantification of drug substances.\(^{[52]}\)

Comparing with the accepted criteria which is for QC levels 85.0-115% and for LLOQ range which should be 80-120\%, the accuracy and LLOQ range data obtained from the first, second, and third day of validation are within the required range. In addition, precision (CV\%) is not exceed 20\% for LLOQ, and 15\% for the other concentrations which prove the closeness of the measurements when repeatedly applied the method of analysis to multiple aliquots of a single homogenous volume of the serum. In addition, a high and acceptable recovery \% at the studied concentrations was obtained.

As said by bacteriological studies, the pharmacokinetic parameters of antibiotics have been shown to be coordinated with their ability to eradicate bacteria; as a result any significant changes in such parameters could affect their clinical outcome and even could be developed to a bacterial resistance.\(^{[53]}\) Therefore, it is very important to take in consideration the food-antibiotics interactions since these interactions can affect antibiotic pharmacokinetic parameters.

According to our knowledge, the effect of pomegranate juice on Metronidazole has not been studied previously. In the current study, in comparison with DW-feed rats, pharmacokinetic parameters of Metronidazole after single dose pre-treatment with pomegranate juice showed insignificant decline and this could be due to a very small sample size or to a low dose of PJ. In contrast, two doses a day of normal-strength pomegranate fresh juice that was administered to rats for two days followed by a single dose on the third day, showed a considerable increase in the pharmacokinetic parameters (Cmax and AUC) \((P<0.05)\) of Metronidazole (Table 4). From this finding we may consider that a multiple dose of pomegranate juice pre-treatment is a good Metronidazole- intestinal enzyme inhibitor. Moreover, \(t^{1/2}\) and the elimination rate constant were not significantly changed which means that the hepatic enzymes could be not affected.
Figure 1: Plot of linearity of calibration curve levels for Metronidazole quantification against their analytical response and regression linear equation.

Figure 2: Line chart showing the changes in mean plasma Metronidazole concentration with time after drug administration with DW, with single dose of juice, and with multiple dose of juice.

From previous studies, the ability of pomegranate juice to inhibit the activity of human CYP2C9 was investigated using human liver microsomes. Pomegranate juice was shown to be a potent inhibitor of human CYP2C9. In addition, investigation’s results for the effect of pomegranate juice on the pharmacokinetics of tolbutamide (substrate for CYP2C9) in rats showed that the area under the concentration-time curve was approximately 1.2-fold greater when pomegranate juice was injected before oral administration of the tolbutamide. The elimination half-life of tolbutamide was not altered by pomegranate juice administration.
These findings suggest pomegranate juice ingestion inhibits the intestinal metabolism of tolbutamide without inhibiting the hepatic metabolism in rats \(^{[48]}\). A more recent study evaluated the effect of repeated commercially available pomegranate juice consumption on the CYP3A-mediated metabolism of midazolam\(^{[27, 28]}\) and showed that pomegranate juice did not significantly alter midazolam PK. Since MTZ is considered as a substrate and an inhibitor to CYP2C9 and CYP3A4 enzymes, therefore, pomegranate juice may affect CYP2C9, but not CYP3A4-mediated metabolism of Metronidazole.

Table 1: Preparation of Metronidazole standared calibration curve serial dilution and the quality control solutions and spiked serum.

<table>
<thead>
<tr>
<th>Sol. No:</th>
<th>Vol. taken (ml) from working sol.</th>
<th>Total vol. (ml)</th>
<th>Conc. of MTZ serial sol. (µg /ml)</th>
<th>Conc. of MTZ in serum (µg /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>0.01</td>
<td>1.00</td>
<td>3.00</td>
<td>0.30</td>
</tr>
<tr>
<td>S2</td>
<td>0.02</td>
<td>1.00</td>
<td>6.00</td>
<td>0.60</td>
</tr>
<tr>
<td>S3</td>
<td>0.04</td>
<td>1.00</td>
<td>12.00</td>
<td>1.20</td>
</tr>
<tr>
<td>S4</td>
<td>0.08</td>
<td>1.00</td>
<td>24.00</td>
<td>2.40</td>
</tr>
<tr>
<td>S5</td>
<td>0.20</td>
<td>1.00</td>
<td>60.00</td>
<td>6.00</td>
</tr>
<tr>
<td>S6</td>
<td>0.40</td>
<td>1.00</td>
<td>120.00</td>
<td>12.00</td>
</tr>
<tr>
<td>S7</td>
<td>0.60</td>
<td>1.00</td>
<td>180.00</td>
<td>18.00</td>
</tr>
<tr>
<td>S8(QC Low)</td>
<td>0.03</td>
<td>1.00</td>
<td>9.00</td>
<td>0.90</td>
</tr>
<tr>
<td>S9(QC Mid)</td>
<td>0.30</td>
<td>1.00</td>
<td>90.00</td>
<td>9.00</td>
</tr>
<tr>
<td>S10(QC High)</td>
<td>0.48</td>
<td>1.00</td>
<td>144.00</td>
<td>14.40</td>
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</table>


Table 2: Chromatographic and mass spectrometric conditions.

<table>
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<tr>
<th>HPLC conditions</th>
<th>Pump flow rate mL/ min</th>
<th>Auto sampler injection volume/ µl</th>
<th>Auto-sampler temp/°C</th>
<th>Column oven temp/°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>1.0</td>
<td>5</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>Chromatography</td>
<td>Total Time</td>
<td>FA 0.1%</td>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>Mobile phase</td>
<td></td>
<td>Min. A%</td>
<td>B%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.51</td>
<td>0</td>
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<td>1.50</td>
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<td></td>
<td>1.51</td>
<td>100</td>
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<tr>
<td></td>
<td>3.00</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Column type</td>
<td>ACE C18 column (50 X 2.1 mm), 5µ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retention times</td>
<td>MTZ</td>
<td>Clindamycin (IS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(minutes)</td>
<td></td>
<td>0.27</td>
<td>1.62</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Comparing Cmax, Tmax, AUC, T½, and the elimination rate constant between: Metronidazole with DW and (Metronidazole + single dose of juice).

<table>
<thead>
<tr>
<th></th>
<th>MTZ+ DW</th>
<th>MTZ + single dose of PJ</th>
<th>Difference</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax(µg/ml)</td>
<td>13.35±0.35</td>
<td>12.62±0.30</td>
<td>-0.73*</td>
<td>-5.50</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>1.00</td>
<td>1.00±0.15</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>AUC0→∞ (µg/ml*hr)</td>
<td>94.13±6.04</td>
<td>86.73±1.09</td>
<td>-7.40*</td>
<td>-7.86</td>
</tr>
<tr>
<td>T½ (hr)</td>
<td>4.47</td>
<td>3.91</td>
<td>-0.56*</td>
<td>-12.53</td>
</tr>
<tr>
<td>Ke</td>
<td>0.16</td>
<td>0.18</td>
<td>+0.02*</td>
<td>+12.50</td>
</tr>
</tbody>
</table>

Abbreviations: DW, Distilled Water. Cmax, Mean Maximum concentration. Tmax, Median time to Maximum Plasma Concentration. T½, Half Time. Ke, Rate of Elimination Constant. AUC, Area Under the Curve. *P>0.05 (insignificant).

Table 4: Comparing Cmax, Tmax, AUC, T½, and the elimination rate constant between: Metronidazole with DW and (Metronidazole + multiple dose of juice).

<table>
<thead>
<tr>
<th></th>
<th>MTZ+ DW</th>
<th>MTZ + multiple dose of PJ</th>
<th>Difference</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax(µg/ml)</td>
<td>13.35±0.35</td>
<td>19.15±1.60</td>
<td>+5.80**</td>
<td>+43.40</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>1.00</td>
<td>1.00±0.35</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>AUC0→∞ (µg/ml*hr)</td>
<td>94.13±6.04</td>
<td>218.68±12.07</td>
<td>+124.55**</td>
<td>+132.30</td>
</tr>
<tr>
<td>T½ (hr)</td>
<td>4.47</td>
<td>5.68</td>
<td>+1.20*</td>
<td>+27.00</td>
</tr>
<tr>
<td>Ke</td>
<td>0.16</td>
<td>0.12</td>
<td>-0.04*</td>
<td>-25.00</td>
</tr>
</tbody>
</table>

Abbreviations: DW, Distilled Water. Cmax, Mean Maximum concentration. Tmax, Median time to Maximum Plasma Concentration. T½, Half Time. Ke, Rate of Elimination Constant.
Abbreviations: DW, Distilled Water. Cmax, Mean Maximum concentration. Tmax, Median time to Maximum Plasma Concentration. T1/2, Half Time. Ke, Rate of Elimination Constant. AUC, Area Under the Curve. **P<0.05 (significant), *P> 0.05 (insignificant).

5. CONCLUSION
An analytical method with high resolution was used for quantification of Metronidazole. The method was validated and all of obtained data was within the acceptance criteria according to United State Food and Drug Administration and European Medicines Agency guidelines. In comparison with distilled water-feed rats, pre-treatment with multiple doses of normal-strength pomegranate fresh juice administration showed a considerable increase in the pharmacokinetic parameters (Cmax and AUC) (P<0.05) of Metronidazole. Based on previous studies [27, 28, 48], we could conclude that pomegranate juice may affect CYP2C9-mediated metabolism of Metronidazole. On the other hand, there are different enteric metabolic enzymes involved in the orally administered drug metabolism; therefore, further in vitro and in vivo investigations are needed to confirm these findings.

6. DECLARATIONS
Conflict of interest
The Author(s) declare(s) that they have no conflicts of interest to disclose.

7. FUNDING
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8. ACKNOWLEDGMENT
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