INTRODUCTION

Chelation is a type of bonding of ions and molecules to metal ions. It involves the formation or presence of two or more separate coordinate bonds between a polydentate (multiple bonded) ligand and a single central atom. Usually these ligands are organic compounds, and are called chelants, chelators, chelating agents, or sequestering agents.

Chelation is useful in applications such as providing nutritional supplements, in chelation therapy to remove toxic metals from the body, as contrast agents in MRI scanning, in manufacturing using homogeneous catalysts, and in fertilizers.

Chelation in the intestinal tract is a cause of numerous interactions between drugs and metal ions (also known as minerals in nutrition). As examples, antibiotic drugs of the tetracycline and quinolone families are chelators of Fe^{2+}, Ca^{2+} and Mg^{2+} ions. Chelation of antibiotics with the ions interferes with their absorption from gastrointestinal tract.

Different classes of tetracycline antibiotitics include oxytetracycline, tetracycline, chlorotetracline, minocycline and doxycycline. They differ in their pharmacokinetic profile and chelating property. There exists paucity in literature regarding the comparative calcium chelating property of doxycycline and oxytetracycline. Therefore, this in-vitro study was designed to study and compare chelating property of oxytetracycline and doxycycline.
METHODS

Calcium chelation by Trender’s method

When test drug is treated with calcium reagent, the naphtha hydroxamic acid present in the reagent precipitates calcium as calcium naphtha hydroxamate. This precipitate is then dissolved in EDTA and treated with ferric nitrate (colour reagent). A reddish brown coloured complex is formed. The absorbance of which is measured colorimetrically at 530 nm. The intensity of the colour is directly proportional to the concentration of the calcium present. A standard calcium carbonate is treated similarly and the colour intensities are compared.

Reagents

- Calcium reagent (Naphtal hydroxamic acid)
- EDTA solution
- Colour reagent (Ferric nitrate)
- Calcium standard (CaCO₃): Concentration=0.1 mg/ml

Procedure was done according to standard methodology by P. Trinder.³

Calcium binding assay

Spectrophotometer is an instrument used to measure absorbance at various wavelengths. It is similar to the absorptiometer except that it uses diffraction gratings or glass prism to produce mono chromatic light.

- In a 1 cm quartz cuvette 0.1 ml solution of Oxytetracycline (0.1mM) was mixed with 0.8 ml methanol and 0.1 ml of 0.05 Tris buffer (pH 7.4).
- The UV absorption spectrum of the solutions was obtained initially in the absence of Ca²⁺.
- Then (10 µl) of an aqueous solution containing CaCl₂ was added to the solution and new absorption curves in the presence of Ca²⁺(0.1, 0.5 or 1 mM) were obtained.
- The changes in the UV profile in the range of 200-500 nm region and the shift of the maximum absorption after addition of CaCl₂ were recorded by using UV- spectrophotometer.
- Same procedure was done for doxycycline (0.1 mM). The UV absorption spectrum of the solutions was obtained initially in the absence of Ca²⁺ and then in presence of different concentration of Ca²⁺(0.1, 0.5 or 1 mM).

RESULTS

Calcium estimation by Trinder’s method

For estimation of calcium chelation, EDTA method was followed (P. Trinder). Different doses of oxytetracycline (25 mg, 50 mg and 100 mg) and doxycycline (25 mg, 50 mg and 100 mg) were used. The procedure was done according to the standard methodology.

Table 1: Effect of oxytetracycline and doxycycline on calcium estimation by Trinder’s method.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Oxytetracycline</th>
<th>Doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD</td>
<td>Ca conc. mg/dl</td>
</tr>
<tr>
<td>25 mg</td>
<td>2.90</td>
<td>2.4</td>
</tr>
<tr>
<td>50 mg</td>
<td>3.34</td>
<td>2.8</td>
</tr>
<tr>
<td>100 mg</td>
<td>3.89</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Oxytetracycline

The base line calcium concentration was found to be 10 mg/dl. On adding oxytetracycline at different doses of (25 mg, 50 mg, 100 mg) the % chelation of calcium was found to be 24 %, 28 % and 34 % respectively as compared to baseline (Table 1).

Doxycycline

Similarly, on adding doxycycline at different doses (25 mg, 50 mg, 100 mg) the % chelation of calcium was found to be 10 %, 17 % and 35 % respectively as compared to baseline (Table 1).
The intensity of colour appear to be increased with increase in dose of the oxytetracycline (25 mg, 50 mg, 100 mg) as the concentration of calcium binding increases. But in doxycycline intensity of colour was more with 100 mg as compared with 25 mg and 50 mg as depicted in Figure 1.

**Calcium chelation assay**

The calcium binding activity of oxytetracycline and doxycycline was investigated and are depicted in Table 2.

<table>
<thead>
<tr>
<th>Calcium Concentration</th>
<th>Oxytetracycline (OD)</th>
<th>Doxycycline (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without calcium</td>
<td>1.459</td>
<td>0.534</td>
</tr>
<tr>
<td>0.1 mM Calcium</td>
<td>1.444</td>
<td>0.513</td>
</tr>
<tr>
<td>0.5 mM Calcium</td>
<td>1.369</td>
<td>0.533</td>
</tr>
<tr>
<td>1.0 mM Calcium</td>
<td>1.427</td>
<td>0.564</td>
</tr>
</tbody>
</table>

Calcium chelation assay was done using different concentration of calcium and in absence of calcium. The UV absorption spectrum of solution of oxytetracycline (1mM) was changed after the addition of CaCl₂ to provide different concentration of Ca²⁺ (0.1, 0.5 and 1.0 mM) (Figure 2).

**DISCUSSION**

Tetracyclines were developed as a result of the screening of soil samples for antibiotics. The first of these compounds, chlortetracycline, was introduced in 1947. Tetracyclines were found to be highly effective against various pathogens including rickettsiae, as well as both gram-positive and gram-negative bacteria, thus becoming the first class of broad-spectrum antibiotics. Many other interesting properties, unrelated to their antibiotic activity, have been identified for tetracyclines which have led to widely divergent experimental and clinical uses.

Tetracyclines have a high affinity to form chelates with polyvalent metallic cations such as Fe³⁺, Fe²⁺, Al³⁺, Mg²⁺ and Ca²⁺. Many of these tetracycline-metal complexes are either insoluble or otherwise poorly absorbable from the gastro-intestinal tract. Milk and other dairy products, antacids containing polyvalent cations, as well as various iron salts ingested simultaneously with tetracycline derivatives, might interfere with their absorption by 50 to 90% or even more.
Tetracycline antibiotics in apolar solvents chelate calcium in a different conformation from that of the Mg chelate. Evidence for this different conformation is added from the fluorescence, absorption and circular dichroism spectra of the antibiotic bound to Ca and Mg respectively. The conformation of the antibiotic chelated to Ca is a high affinity form. Only those divalent cations of a size similar to or greater than that of Ca are able to induce this conformation.6

Leyden JJ in his study observed that absorption of both antibiotics was significantly decreased by administration with iron (77% inhibition with minocycline and 81% with tetracycline), milk (27% inhibition with minocycline, 65% with tetracycline), and food (13% inhibition with minocycline and 46% with tetracycline). The inhibitory effect on absorption with food and milk was significantly greater for tetracycline than for minocycline.7

In other study, conducted by Martin SS showed that metal ion form complexes with both the fully-deprotonated and mono-protonated forms of the tetracycline.8

Tetracycline antibiotics include oxytetracycline, tetracycline, chlortetracycline, minocycline and doxycycline. They differ in their pharmacokinetic profile. The older tetracyclines are incompletely absorbed from gastrointestinal tract. Doxycycline and minocycline are completely absorbed irrespective of food. Tetracyclines form complexes with calcium and other metals. Absorption of tetracycline is significantly decreased by administration with iron milk and food.7 Tetracyclines are widely distributed in the body. The plasma protein binding of oxytetracycline is low where as that of Doxycycline is high. Most tetracyclines are excreted in urine except doxycycline, which is excreted in bile. Marked alteration of intestinal flora is seen with tetracycline as compared with doxycycline. Incidence of of phototoxicity is on high side with doxycycline as compared with other tetracyclines. In study a conducted by Wessels et al the results obtained suggest that Ca2+ forms a 1:2 ligand: metal complex with TC.9

In current study, by both the methods we found that oxytetracycline chelates calcium and these findings are comparable with earlier studies. The base line calcium concentration was found to be 10 mg/dl. On adding oxytetracycline at different doses of (25 mg, 50 mg, 100 mg) the % chelation of calcium was found to be 24 %, 28 % and 34 % respectively as compared to baseline. (Table1). The UV absorption spectrum of solution of Oxytetracycline (1mM) was changed after the addition of CaCl2 to provide different concentration of Ca2+ (0.1, 0.5 and 1.0 mM) Figure 2.

Similar findings were observed by Uris MR et al. They analysed calcium complexes of oxytetracycline by use of frog heart method with improved apparatus employing a vertical shutter, photoelectric transducer, bridge circuit, and recording system. This method was readily applicable to complex biologic solutions and produced results comparable to those in the preceding article obtained by spectrophotometry on pure solutions.10

Authors Button C and Mulders MS, studied the effect of oxytetracycline in propylene glycol on blood ionized calcium and plasma total calcium in sheep and found that ionized calcium concentrations in whole blood significantly depressed after oxytetracycline IV.

In the present study, by both the methods we noted that doxycycline chelates calcium and these findings are comparable. On adding doxycycline at different doses (25 mg, 50 mg, 100 mg) the % chelation of calcium was found to be 10 %, 17 % and 35 % respectively as compared to baseline (Table 1). With minor shift in the absorption coefficient and no shift in wavelength were observed for doxycycline (Figure 3).

Doxycycline has been reported to have less binding with ions such as calcium.11 Schach von Wittenau noted that doxycycline levels 1 hour after drug administration were lower when the drug was given after full breakfast as compared to when it was given on an empty stomach.11 Kshirsagar NA et al significant noted that there was difference in the plasma values determined 4, 6 and 8 hours after drug administration, AUC and C-max being also significantly lower after administration of doxycycline with food.12

In present study, we observed that oxytetracycline chelates more of calcium in comparison with doxycycline. The onset of action of doxycycline was slow as compared to oxytetracycline. The intensity of colour appear to be increased with increase in dose of the oxytetracycline (25 mg, 50 mg, 100 mg) as the concentration of calcium binding increases. But in doxycycline intensity of colour was more with 100 mg as compared with 25 mg and 50 mg as depicted in Figure 1.

CONCLUSION
This in-vitro study concludes that oxytetracycline has more calcium chelating property than doxycycline.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES
3. Trinder P. Colorimetric microdetermination of calcium in serum. 1960. Available from: