BIOLOGICAL ASSAY OF DOXYCYCLINE TETRACYCLINE
GENTAMICIN & ERYTHROMYCIN SUBJECTED TO ACCELERATED
STABILITY STUDIES

A Thesis Submitted
by

Makarim Mohammed KHALIL
(B.Pharm University of Khartoum)

In Fulfillment of the Requirements
For the Degree of Master of Pharmacy

Under Supervision of

Dr. El-Amin Ibrahim El-Nima

Department of Pharmaceutics

Dean of Faculty of Pharmacy
University of Khartoum

April 2003
Dedication

To little Doaa’

&

To my parents
# List of Content

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of content</td>
<td>i</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>xi</td>
</tr>
<tr>
<td>Arabic Abstract</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>iv</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Drug Stability</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1. Chemical Degradation of Pharmaceutical Products and methods of reducing it.</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1.1. Hydrolysis</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1.1.1. Solvolysis</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1.2. Methods of reducing hydrolysis</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1.3. Oxidation</td>
<td>4</td>
</tr>
<tr>
<td>1.1.1.4. Methods of reducing oxidation</td>
<td>5</td>
</tr>
<tr>
<td>1.1.1.5. Isomerization</td>
<td>6</td>
</tr>
<tr>
<td>1.1.1.7. Decarboxylation</td>
<td>7</td>
</tr>
<tr>
<td>1.1.1.8. Photolysis</td>
<td>8</td>
</tr>
<tr>
<td>1.1.1.9. Methods of reducing photochemical degradation</td>
<td>8</td>
</tr>
<tr>
<td>1.1.2. The Applications of Chemical Kinetics to Decompositions of</td>
<td>8</td>
</tr>
<tr>
<td>Pharmaceutical Products</td>
<td></td>
</tr>
<tr>
<td>1.1.2.1. Definitions</td>
<td>9</td>
</tr>
<tr>
<td>1.1.2.2. Derivation of Simple Order of Reaction.</td>
<td>10</td>
</tr>
<tr>
<td>1.1.2.2.1. First order reaction.</td>
<td>10</td>
</tr>
<tr>
<td>1.1.2.2.2. Psuedo First Order Reaction.</td>
<td>11</td>
</tr>
<tr>
<td>1.1.2.2.3. Second order reaction.</td>
<td>11</td>
</tr>
<tr>
<td>1.1.2.2.4. Zero order reaction.</td>
<td>12</td>
</tr>
<tr>
<td>1.1.2.2.5. Third order reaction.</td>
<td>12</td>
</tr>
<tr>
<td>1.1.2.3. Temperature effect on the rate of reaction.</td>
<td>12</td>
</tr>
<tr>
<td>1.1.3. Physical factors influencing chemical degradation</td>
<td>13</td>
</tr>
</tbody>
</table>
1.1.3. 1. Temperature  
1.1.3. 2. Moisture  
1.1.3. 3. Light  
1.1.3. 4. Radiation  
1.1.4. The influence of packing material on dosage form stability.  
1.1.4.1 Glass  
1.1.4.2. Plastics  
1.1.4.3. Metals  
1.1.4.4. Rubber  
1.1.5. Physical degradation of pharmaceutical products.  
1.1.5.1. Loss of volatile constituents  
1.1.5. 2 Loss of water  
1.1.5. 3 Absorption of water  
1.1.5. 4. Crystal growth  
1.1.5. 5. Polymorphic changes  
1.1.5. 6. Colour changes  
1.2. Stability testing  
1.2.1. Introduction  
1.2.2. Common High stresses or challenges  
1.2.2.1. Temperature  
1.2.2.2. Humidity  
1.2.2.3. Light  
1.2.3. Definitions  
1.2.4. Purpose of stability testing  
1.2.5. Accelerated stability studies  
1.2.5.1. Stability testing protocols  
1.2.5.2. Design of stability studies  
1.2.5.2. 1. Test conditions  
1.2.5.2. 2. Test sample  
1.2.5.2. 3. Analytical Methods  
1.3. Biological Testing  
1.3. Biological assay of antibiotics.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3.1. Historical.</td>
<td>27</td>
</tr>
<tr>
<td>1.3.2. Literature review.</td>
<td>28</td>
</tr>
<tr>
<td>1.3.3. Purpose of the assay.</td>
<td>31</td>
</tr>
<tr>
<td>1.3.4. Basic techniques and principles.</td>
<td>31</td>
</tr>
<tr>
<td>1.3.4.1. The plate assay.</td>
<td>32</td>
</tr>
<tr>
<td>1.3.4.2. The tube assay.</td>
<td>32</td>
</tr>
<tr>
<td>1.3.4.3. Reference standard.</td>
<td>33</td>
</tr>
<tr>
<td>1.3.4. Agar diffusion method.</td>
<td>34</td>
</tr>
<tr>
<td>1.3.4.1. Introduction</td>
<td>34</td>
</tr>
<tr>
<td>1.3.5.2. Approach to accurate assay</td>
<td>34</td>
</tr>
<tr>
<td>1.3.5.2.1. Kinds of agar assay:</td>
<td>34</td>
</tr>
<tr>
<td>1.3.5.3. Pouring of agar.</td>
<td>35</td>
</tr>
<tr>
<td>1.3.5.4. Incubation temperature.</td>
<td>36</td>
</tr>
<tr>
<td>1.3.5.5. Dosing.</td>
<td>36</td>
</tr>
<tr>
<td>1.3.5.6. Measuring zones.</td>
<td>36</td>
</tr>
<tr>
<td>1.3.5.8. Dose-response lines</td>
<td>37</td>
</tr>
<tr>
<td>1.3.6. Tube assay.</td>
<td>38</td>
</tr>
<tr>
<td>1.3.6.1. General principle.</td>
<td>38</td>
</tr>
<tr>
<td>1.3.6.2. Scattering of light by the microorganism.</td>
<td>39</td>
</tr>
<tr>
<td>1.3.6.3. Instrumentation.</td>
<td>39</td>
</tr>
<tr>
<td>1.3.6.4. Automated system for turbidimetric assay.</td>
<td>40</td>
</tr>
<tr>
<td>1.3.6.5. Response curves- commonly used form of expressions.</td>
<td>41</td>
</tr>
<tr>
<td>1.3.7. Requirements for accurate assay.</td>
<td>42</td>
</tr>
<tr>
<td>1.3.8. Causes of assay errors.</td>
<td>42</td>
</tr>
<tr>
<td>1.4. Antibiotics</td>
<td>45</td>
</tr>
<tr>
<td>1.4.1. Tetracycline</td>
<td>45</td>
</tr>
<tr>
<td>1.4.1.1. Antimicrobial action and resistance of Tetracycline.</td>
<td>45</td>
</tr>
<tr>
<td>1.4.1.2. Mechanism of action of Tetracycline</td>
<td>45</td>
</tr>
<tr>
<td>1.4.1.3. Physical properties of Tetracycline</td>
<td>46</td>
</tr>
<tr>
<td>1.4.1.4. Stability of Tetracycline</td>
<td>46</td>
</tr>
<tr>
<td>1.4.2. Doxycycline</td>
<td>47</td>
</tr>
<tr>
<td>1.4.2.1. Antimicrobial action and resistance of Doxycycline</td>
<td>47</td>
</tr>
</tbody>
</table>
1.4.2.3. Stability of Doxycyline
1.4.2.2. Physical properties of Doxycyline:
1.4.2.3.1. Solutions of Doxycyline
1.4.2.3.2. Effect of relative humidity on Doxycyline:
1.4.2.3.3. Effect of freezing on Doxycyline
1.4.3. Erythromycin:
1.4.3.1. Physical properties of Erythromycin:
1.4.3.2. Antimicrobial action of Erythromycin
1.4.3.3. Mechanism of actions of Erythromycin
1.4.3.4. Stability of Erythromycin
1.4.3.4.1. Effect of freezing and thawing on Erythromycin:
1.4.4. Gentamicin.
1.4.4.1. Physical properties of Gentamicin
1.4.4.2. Antimicrobial action and resistance of Gentamicin
1.4.4.3. Mechanism of action of Gentamicin
1.4.4.4. Stability of Gentamicin
1.5. Aim of Work
2. Experimental
2.1. Materials
2.1.1. Microorganisms
2.1.2. Chemicals
2.1.3. Instruments
2.1.4. Media:
2.1.5. Buffer Solutions
2.2. Experimental Work
2.2.1. Storage of The Samples.
2.2.2. Preparation of stability cabinet.
2.2.3. Preparation of Test Organisms.
2.2.4. Preparation of The Culture Media.
2.2.5. Preparation of The Inoculums
2.2.6. Preparation of The Buffers and Other Solutions.
2.2.7. Sterilization of Glassware and Other Tools 60
2.2.8. Preparation of The Standards. 60
2.2.9. Preparation of The Samples. 61
2.2.10. Procedures. 62
2.2.10.1. Procedure of Agar diffusion Method. 62
2.2.10.2. Procedure of Turbibmetric Method. 62
2.2.11. Calculations. 63
2.2.11.1. Calculations in Agar diffusion method. 63
2.2.11.2. Calculations in Turbibidimetric method. 64
2.2.12. Dissolution Test of Teteracyline Capsules & Doxycycline Tablets. 64
2.2.13. Disintegration Test of Teteracyline Capsules & Doxycycline Tablets. 64

2.3. Statistical Analysis

3. Results 65
4. Discussion 88
   4.1. Introduction 88
   4.2. Tetracycline capsules. 89
   4.3. Erythromycin suspension. 91
   4.4. Doxycycle hyclate tablets. 92
   4.5. Gentamicin ampoules. 93

5. Conclusion 95
6. References 96
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1:</td>
<td>Types of drug stability</td>
<td>20</td>
</tr>
<tr>
<td>Table 2:</td>
<td>The main climatic conditions found in the climatic zones all over the world</td>
<td>23</td>
</tr>
<tr>
<td>Table 3:</td>
<td>The main objectives of stability testing and the requested type of stability testing.</td>
<td>23</td>
</tr>
<tr>
<td>Table 4:</td>
<td>Examples of accelerated stability testing</td>
<td>25</td>
</tr>
<tr>
<td>Table 5</td>
<td>Samples used in the 1st Run of the study</td>
<td>57</td>
</tr>
<tr>
<td>Table 6</td>
<td>Samples used in the 2nd Run of the study</td>
<td>57</td>
</tr>
<tr>
<td>Table 7</td>
<td>Antibiotics and their test conditions including test organisms, media, solvents, serial dilutions, and incubation conditions</td>
<td>58</td>
</tr>
<tr>
<td>Table 8</td>
<td>Samples used in the study</td>
<td>65</td>
</tr>
<tr>
<td>Table 9</td>
<td>Results of Accelerated Stability Study of Sample 1</td>
<td>66</td>
</tr>
<tr>
<td>Table 10</td>
<td>Statistical analysis of the results of stability study of sample 1</td>
<td>66</td>
</tr>
<tr>
<td>Table 11</td>
<td>Statistical analysis of the results of dissolution test of sample 1</td>
<td>68</td>
</tr>
<tr>
<td>Table 12</td>
<td>The comparison between the results of biological assay and dissolution test of sample 1</td>
<td>68</td>
</tr>
<tr>
<td>Table 13</td>
<td>Results of accelerated stability study of sample 2</td>
<td>70</td>
</tr>
<tr>
<td>Table 14</td>
<td>Statistical analysis of the results of stability study of sample 2</td>
<td>70</td>
</tr>
<tr>
<td>Table 15</td>
<td>Statistical analysis of the results of dissolution test of sample 2</td>
<td>72</td>
</tr>
<tr>
<td>Table 16</td>
<td>The comparison between the results of biological assay and dissolution test of sample 2</td>
<td>72</td>
</tr>
<tr>
<td>Table 17</td>
<td>Results of accelerated stability study of sample 3</td>
<td>74</td>
</tr>
<tr>
<td>Table 18</td>
<td>Statistical analysis of the results of the stability study of sample 3</td>
<td>74</td>
</tr>
<tr>
<td>Table 19</td>
<td>Results of accelerated stability study of sample 4</td>
<td>76</td>
</tr>
<tr>
<td>Table 20</td>
<td>Statistical analysis of the results of stability study of sample 4</td>
<td>76</td>
</tr>
<tr>
<td>Table 21</td>
<td>Results of accelerated stability study of sample 5</td>
<td>78</td>
</tr>
<tr>
<td>Table 22</td>
<td>Statistical analysis of the results of stability study of sample 5</td>
<td>78</td>
</tr>
<tr>
<td>Table 23</td>
<td>Results of accelerated stability study of sample 6</td>
<td>80</td>
</tr>
<tr>
<td>Table 24</td>
<td>Statistical analysis of the results of stability study of sample 6</td>
<td>80</td>
</tr>
<tr>
<td>Table 25</td>
<td>Results of accelerated stability study of sample 7</td>
<td>82</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Table 26</td>
<td>Statistical analysis of the results of stability study of sample 7</td>
<td>82</td>
</tr>
<tr>
<td>Table 27</td>
<td>Results of 1st month assay of sample 4</td>
<td>84</td>
</tr>
<tr>
<td>Table 28</td>
<td>Results of zero time assay of sample 7</td>
<td>84</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1</td>
<td>The structural formula of Tetracycline hydrochloride</td>
<td>45</td>
</tr>
<tr>
<td>Fig. 2</td>
<td>The structural formula of Doxycycline hydrochloride</td>
<td>47</td>
</tr>
<tr>
<td>Fig. 3</td>
<td>The structural formula of Erythromycin ethyl succinate</td>
<td>48</td>
</tr>
<tr>
<td>Fig. 4</td>
<td>The structural formula of Gentamicin sulphate</td>
<td>50</td>
</tr>
<tr>
<td>Fig. 5</td>
<td>Presentation of alternative positions of sample and standard in the petri dish</td>
<td>62</td>
</tr>
<tr>
<td>Fig. 6</td>
<td>Presentation of calculation of the concentration of the sample from the curve of the standard</td>
<td>64</td>
</tr>
<tr>
<td>Fig. 7</td>
<td>The mean of content of doxycycline (in mg) per tablet in sample 1 assayed by agar diffusion method using <em>Bacillus cereus</em> as test organism</td>
<td>67</td>
</tr>
<tr>
<td>Fig. 8</td>
<td>The mean of percentages of dissolved amount from sample 1</td>
<td>69</td>
</tr>
<tr>
<td>Fig. 9</td>
<td>Comparison between the percentage content of doxycycline assayed by biological assay and the amount dissolved in the dissolution test of sample 1</td>
<td>69</td>
</tr>
<tr>
<td>Fig. 10</td>
<td>The mean of content of tetracycline per capsule in mg in sample 2 assayed by turbidimetric method using <em>Staphylococcus aureus</em> as test organism</td>
<td>71</td>
</tr>
<tr>
<td>Fig. 11</td>
<td>The mean of percentage of dissolved amount from sample 2</td>
<td>73</td>
</tr>
<tr>
<td>Fig. 12</td>
<td>Comparison between the percentage content of doxycycline assayed by biological assay and the amount dissolved in the dissolution test of sample 2</td>
<td>73</td>
</tr>
<tr>
<td>Fig. 13</td>
<td>The mean of content of tetracycline per capsule (in mg) in sample 3 assayed by turbidimetric method using <em>Staphylococcus aureus</em> as test organism</td>
<td>75</td>
</tr>
<tr>
<td>Fig. 14</td>
<td>The mean of content of tetracycline (in mg) per capsule in sample 4 assayed by turbidimetric method using <em>Staphylococcus aureus</em> test organism</td>
<td>77</td>
</tr>
<tr>
<td>Fig. 15</td>
<td>The mean of content of gentamicin (in mg) in sample 5 assayed by agar diffusion method using <em>Bacillus pumilus</em> as test organism</td>
<td>79</td>
</tr>
<tr>
<td>Fig. 16</td>
<td>Mean of content of gentamicin (in mg) in sample 6 assayed by agar diffusion method using <em>Bacillus pumilus</em> as test organism</td>
<td>81</td>
</tr>
<tr>
<td>Fig. 17</td>
<td>Mean of content of erythromicn in sample 7 assayed by agar diffusion method using <em>Bacillus pumilus</em> as test organism</td>
<td>83</td>
</tr>
<tr>
<td>Fig. 18</td>
<td>The standard curve of tetracycline (transmittance against concentration) by turbidimetric method using <em>Staphylococcus aureus</em>.</td>
<td>85</td>
</tr>
<tr>
<td>Fig. 19</td>
<td>The standard curve of tetracycline by turbidimetric method using <em>Staphylococcus aureus</em> as test organism</td>
<td>86</td>
</tr>
<tr>
<td>Fig. 20</td>
<td>Curve of standard and sample of erythromycin assayed by agar diffusion method using <em>Bacillus pumilus</em></td>
<td>87</td>
</tr>
</tbody>
</table>
Acknowledgment

Al Hamdulillah. Thank to Allah the Creator of the Heavens and the Worlds; He who Helped us on every way of life.

I am really grateful and would like to express my thanks and appreciation to my supervisor Dr. Alamin Ibrahim Elneima for help, advice and supervision of this thesis.

Sincere thanks are extended to Dr. Abubakr Abdelraowf for his great assistance in the experimental work.

Thanks are also due to Dr. Alawia Imam for her help in collecting samples, and to the local pharmaceutical factories Amipharma, General Medicines Company (GMC), Wafra Pharma, and to El Shifa who generously supplied me the samples used in the study.

Thanks are also extended to Drug Control & Research Directorate for providing laboratory, materials, and other facilities and to my colleagues in the National Health Laboratory. Thanks are also conveyed to WHO and Ministry of Health for the financial support.

Finally great thanks are due to my mother and my family for their continuous encouragement and support.

Abstract
This study was carried out to determine the stability of antibiotics after they were exposed to drastic conditions of high temperature and high humidity by the use of microbial methods of analysis. Sudan is classified as zone IV country (hot and humid) based on the classification of the WHO of climatic zones all over the world, and the drugs are exposed to very hard conditions during transport and storage, so drugs must be highly stable in order to withstand these conditions. Accelerated stability studies are used to estimate the stability of the drug formulation when used under normal conditions during its real shelf life. The study was done using four antibiotics, doxycycline, tetracycline, gentamicin, and erythromycin in different dosage forms, tablets, capsules, injections, and oral suspensions. The samples were stored in stability cabinet at temperature of 50°C and relative humidity of 75% for six months. Samples were removed every month for the first three months and at the sixth month. These samples were assayed by the microbial methods and examined for changes occurred in their physical appearance. Other tests like dissolution and disintegration of tablets and capsules were also done. The microbial assay was done by two methods. Agar diffusion method was used for the assay of doxycycline tablets, gentamicin injections, and erythromycin suspensions, while tetracycline capsules were assayed by the use of turbidimetric method. In agar diffusion method the melted agar media were inoculated with suitable test organisms then poured into sterile petri dishes and the antibiotic was applied in stainless cylinders put on the surface of the solidified media, then the petri dishes were incubated for 18 hours and the diameters of zones of inhibition were measured. For turbidimetric method, liquid media was inoculated with suitable test organism and added to the antibiotics in sterile test tubes, then incubated for 2-4 hours and the turbidity caused by growth of the microorganism was read in the form of transmittance by the use of spectrophotometer. In both methods the assay was based on comparison of the response (zone diameter or transmittance) of the samples with the response of standard of known potency. Serial dilutions of both samples and standards were used, the response was measured, and the content was calculated by statistical methods. Media, test organisms, serial dilutions, and other test conditions were obtained from the different Pharmacopoeias. The results of the assays in every month were obtained and statistically analysed by comparing the content of active ingredient at the zero time, (before storage) and its content in each following month. The statistical analysis showed that no statically significant changes had occurred in the content of active ingredient in all samples, but on observation of the results, it was found that the contents of doxycycline and tetracycline were clearly decreased while gentamicin and
erythromycin were not greatly affected. Also the colours of doxycycline, tetracycline and the erythromycin suspension were changed. The study concluded that doxycycline and tetracycline are not stable under the conditions of accelerated stability testing, while gentamicin and erythromycin are stable. The study gave some recommendations with regard to packaging materials and some excipients used in the formulations of the drugs.

ملخص

هذه الدراسة قدمت لنيل درجة ماجستير الصيدلة في مجال علم الأحياء الدقيقة الصيدلاني وموضوعها ( استعمال طرق التحليل الميكروبية في تحليل بعض المضادات الحيوية في إطار دراسات الثبات المُسْرَع) والمضادات الحيوية هي دوكسيسايكلين, نتراسيكلين, جنتاميسين, وإيرثرومايسين في مختلف الأشكال الصيدلانية من أقراص, كبسولات حرق وشراب.
يعتبر السودان من المناطق الحارة الرطبة وقد وضعته منظمة الصحة العالمية ضمن دول المنطقة المناخية الرابعة حسب تقسيمها للمناطق المناخية في العالم حيث تتعرض الأدوية لظروف متأصلة قاسية خلال الترحيل والتخزين لذلك يجب أن تكون الأدوية والمستحضرات الصيدلية على درجة عالية من التثبيت حتى تستطيع أن تتحمل هذه الظروف القاسية. تجري دراسات للكثاب المسرع لكي تتوفر بما ويستخدم للظروف العامة خلال فترة صلاحيتها الفعالة.

تم خلال الدراية تعريض عينات من المضادات الحيوية المذكورة أعلاه لدرجة حرارة عالية (50 درجة مئوية) وطريقة نسبية عالية (75%) لمدة 6 أشهر حيث تسبح جزء من العينات كل شهر خلال الثلاثة أشهر الأولى ثم في الشهر السادس. تم تحليل العينات المسحوبة بالطرق الميكروبيا كم تمت ملاحظة التغييرات الفيزيائية التي حدثت لها. كذلك تم إجراء بعض الاختبارات الأخرى للأعراض والكسولات مثل اختبار反抗ية زمن التنقية. تم التحليل الميكروبي بواسطة طريقة قياس درجة العكورة والانتشار في الأجار. في طريقة قياس العكورة تم تزييد البكتريا في أوساط سائلة وأضيفت إلى المضادات الحيوية في أنابيب اختبار تم تخزينها لمدة 2 إلى 4 ساعات ثم قياس العكورة الناتجة من نمو البكتريا بواسطة جهاز الطيف الضوئي. أما طريقة الإشارة في الأجار، فقد تم تزييد البكتريا في الإجار المسال ثم صب في أطباق بترى ثم ترك حتى تجمد بعد ذلك تم وضع المضادات الحيوية في إسطوانات من الحديد الجيد على سطح الإجار المجتمد حيث تضرب خلال الإسلام. تم تحضير الطبق لمدة 18 ساعة ثم قياس قطر المنطقة الخالية من النمو البكتيري. المبدأ الأساسي للتحليل هو مقارنة بين تأثير العينة المحلية على البكتريا وتأثير شاهد عياري معنوي على نفس البكتريا وذلك باستعمال تركيز متساوي لخلع من العينة المشعة البكتريا المستعملة مع مضادات حيوية والمركزي المتسلسلة من كل من العينة والشاهد العياري البكتريا المستعملة مع مضادات حيوية والمركزي المتسلسلة وظروف التحليل الأخرى ثم الحصول عليها من دسائير الأدوية المختلفة. تم حساب تركيز العينات في كل شهر وتم تحليل النتائج إحصائيا مقارنة تركيز المضادات الحيوية قبل بدء الدراية وتركيزه في الشهر التالي. كذلك تم تمت ملاحظة التغييرات الفيزيائية التي حدثت للعينات. بعد التحليل الإحصائي وجد أن لم يحدث أي تغيير معنوي في كمية المادة الفائضة من الناحية الإحصائية إلا أنه بمقدار النتيجة وجد أن هناك تفاوت واضح في تركيز مادتي الدوسيسينكول والتراسيلكين بينما لم يحدث تغيير يذكر في تركيز الجانبيين الإبروميسينين. كذلك حدث تغيير في اللون في عينات الدوسيسينكول والتراسيلكين تجاوب الإبروميسينين.

توصلت الدراية إلى أن مادتي الجانبين الإبروميسينين والتراسيلكين تمتعت بالثبات عند تعرضها لظروف الأثار المسرع لمنطقة المناخ الرابعة بينما مادتي الدوسيسينكول والتراسيلكين لم تتمتع بالثبات الكافي عند تعرضها لظروف ما. كذلك قامت الدراية ببعض التوصيات الخاصة بمواد التغذية والمواد المضافة المستعملة في الصيغ التركيبي الخاصة بالمستحضرات المستعملة في الدراية.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association Of Analysis Communities</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BDH</td>
<td>British Drug House</td>
</tr>
<tr>
<td>BP</td>
<td>British Pharmacopoeia</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>g.p.s.</td>
<td>Growth promoting substance</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>International Pharmacopoeia</td>
</tr>
<tr>
<td>I.R.</td>
<td>Infra Red</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Culture Type Committee</td>
</tr>
<tr>
<td>RH</td>
<td>Relative Humidity</td>
</tr>
<tr>
<td>S</td>
<td>Standard</td>
</tr>
<tr>
<td>U</td>
<td>Unknown</td>
</tr>
<tr>
<td>U.V.</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopoeia</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
1. Introduction

1.1. Drug Stability

A study of the stability of pharmaceutical products and of stability testing techniques is essential for many reasons. Firstly it is important from the point of view of the safety of the patients. The present trend in the pharmaceutical industry is towards production of highly specific, chemically complex, potent drugs. It is important, therefore, that the patient receives a uniform dose of drug throughout the whole of the shelf-life of the product. In addition, although a drug may have been shown to be safe for use, this is not necessarily true of the decomposition product(s). It is the duty of the manufacturer to minimize, or if possible prevent decomposition of the product.

Secondly, consideration must be given to the relevant legal requirements concerned with the identity, strength, purity and quality of the drug and finally such a study is important to prevent the economic repercussions of marketing an unstable product. The sale of such a product is hardly the best advertisement for a manufacturer, and subsequent withdrawal and reformulation of the drug may lead to considerable financial loss. (Bentyles, 1982)

1.1.1. Chemical Degradation of Pharmaceutical Products and methods of reducing it:

Pharmaceutical products differ considerably in their composition, so naturally they are subject to different forms of chemical degradation, and in addition there, may be several simultaneous decomposition reactions occurring in a product. By obtaining information on each isolated degradation process, it is possible to establish reliable methods of reducing, or even eliminating, the causes of instability (Bentyles, 1982).

1.1.1.1. Hydrolysis

Hydrolysis is considered to be the major cause of deterioration of drugs, especially for those in aqueous solution. It may be defined as the reaction of a compound with water, and one may distinguish between ionic and molecular forms of hydrolysis. Ionic hydrolysis occurs when the salt of weak acids e.g. potassium acetate, and bases, e.g. codeine phosphate, interact with water to give alkaline and acidic solutions, respectively. This is an instantaneous equilibrium process. On the other hand molecular hydrolysis is a much slower, irreversible process involving cleavage of the drug molecule. This form of hydrolysis is responsible for the decomposition of pharmaceutical products. Esters, e.g. the local anaesthetic amethocaine and benzocaine, and amides, e.g. the sulphonamides and nitriles, are all substances that are liable to decompose by hydrolysis. The reaction is frequently catalysed by hydrogen (H⁺) or...
hydroxyl (OH\(^-\)) ions and is therefore said to be specific acid- or specific base catalysed. Although hydrolysis will occur principally with drugs in aqueous solution, suspensions and solid dosage forms are also susceptible to hydrolytic attack. Moisture rapidly absorbed to the surface of aspirin particle causing a solution of a portion of drug in water layer around the particle. As the aspirin in solution hydrolyses, more of the solid materials dissolve and decomposition continues.

1.1.1.1. Solvolysis
Where the reacting solvent is not water, then the break down of the drug is termed solvolysis. Furthermore the definition may extend to include any change in solvent polarity (usually measured as dielectric constant) as a result of an increased ionic strength, which is equivalent to an increased solvent polarity. For example, phenobarbitone is considerably more stable in preparations containing water-miscible solvents whereas aqueous solvents degrade aspirin further, which undergoes extensive hydrolysis. Both effects are directly related to the dielectric constant (polarity) of the solvent. In general if the compound produces degradation products which are more polar than itself then the addition of a less polar solvent will stabilize the formulation. If the degradation products are less polar, then the vehicle should be more polar to improve stability. With the hydrolysis of neutral non-polar drugs, e.g. steroids, the transition state will be non-polar and with no net charge. In this case solvents will not affect the rate of decomposition and can be used with impunity to increase solubility. (Aulton, 1991)

1.1.1.2. Methods of reducing hydrolysis:
1.1.1.2.1. Adjustment of pH
It will be recalled that when hydrolysis is catalysed by hydrogen and/or hydroxyl ions, the rate of decomposition is critically dependant upon pH. Often, plotting the relationship between the logarithms of the reaction velocity, and pH can show this constant for decomposition. If the reaction exhibits specific acid-base catalysis, a minimum in the curve is observed. It follows that a useful method for reducing hydrolysis of a drug exhibiting specific acid-base catalyses is to adjust the pH to the point corresponding to the minimum in the relationship. In many instances, however, pH affects the solubility and the therapeutic activity of the drug as well as its stability. It is necessary to take all three factors into account when attempting to limit decomposition.

1.1.1.2.2. Choice of solvent
Non-aqueous solvents, e.g. alcohol and propylene glycol, have often been used to replace a portion, or all, of the water in a solution in order to reduce hydrolysis of a drug. For example,
an elixir of pentobarbitone sodium contains considerable quantities of glycerin and alcohol, while aspirin, which is very unstable in aqueous solution, has been formulated in alcohol-polypropylene solvent (Shwartz et al, 1958). The assumption is widespread that replacement of water by a non-aqueous solvent automatically enhances the stability of product. Although this is true in many instances, there are cases where a non-aqueous solvent may increase the instability of the product. For example, cyclamic acid in aqueous solution hydrolyses at a very slow rate, whereas in alcoholic solution the compound degrades at markedly faster rates (Talmage, et al, 1968). Only with knowledge of the chemical kinetics of the process, and the reactants species involved, it is possible to make a valid prediction of the effect that a non-aqueous solvent system will have.

1.1.1.2.3. Production of an Insoluble Drug Form
Hydrolysis only occurs with that portion of a drug, which is only in aqueous solution. Therefore, if the majority of the drug is present as suspension with a minimal amount in solution, there will be a reduction in the amount of hydrolysis. The solubility of a drug may sometimes be reduced by adjustment of the pH of the aqueous vehicle. This has been achieved with β-cyclopentyl-propionylsalicylic acid which hydrolyses in suspension by only 1 per cent a year at pH values less than 3.5 (Garrett, 1957). In the instances, solubility may be reduced by a careful selection of the drug vehicle or by producing an insoluble salt of the drug. This latter technique has been successfully used to produce the insoluble procaine salt of benzylpenicillin (Swintosky et al, 1956)

1.1.1.2.4. Presence of surface active agent
Surface active agents are widely used in pharmacy as solubilizing and emulsifying agents. Hence it is important to determine the effect they may have upon drug hydrolysis especially when present in concentrations in excess of their critical micelle concentrations. The presence of surface-active agent can result in a significant improvement in stability, but this is by no means the general rule. Their effect is dependant on a variety of factors including the water solubility of the drug, the type, the chain length and concentration of the surface-active agent, and the type of hydrolytic species, i.e. H⁺ or OH⁻ ions, present.

1.1.1.2.5. Presence of a complexing agent.
The concept of protecting a drug from hydrolysis by addition of chemical stabilizer was introduced by Higuchi and Lachman (1955). They postulated that adding a compound which would form a water soluble complex with the drug might in some measure decrease the rate of decomposition.
To this end they determined the effect that caffeine has upon base-catalysed hydrolysis of the local anaesthetics benzocaine, procaine and amethocaine. In all cases caffeine was found to decrease the rate of decomposition. Also caffeine stabilizes riboflavin. Polyvinylpyrrolidone, desoxycholic and 1-ethyltheogromine, all have a similar but less marked effect than caffeine upon local anaesthetics. (Bentyles, 1982)

1.1.1.3. Oxidation

Decomposition of pharmaceutical preparations due to oxidation is nearly as prevalent as that due to hydrolysis. Morphine, adrenaline, fixed oils, volatile oils and phenols are example of products that oxidize. Oxidation can be defined as the removal of electropositive radical or atom, or the addition of an electronegative atom, or radical. One may make a distinction between those oxidation processes that proceed slowly under the influence of atmospheric oxygen, and those that involve the reversible loss of electrons without the addition of oxygen. The latter type of oxidative change is less frequently encountered than those involving atmospheric oxygen, but is nevertheless important. For example adrenaline, ferrous salts such as ferrous phosphate, riboflavin, and ascorbic acid are all prone to this form of oxidation.

Autoxidation is the term applied to those oxidation processes that slowly proceed under the influence of atmospheric oxygen (Ostendorf, 1964). Oils and fats containing unsaturated linkages in the molecules are particularly susceptible to autoxidation. Volatile oils e.g. clove and cinnamon, change in colour, consistency and odour. Fixed oils, e.g. the oily injections vehicles ethyl oleate and arachis oil, develop an unpleasant odour and taste, and are said to be ‘rancid’. In majority of cases, the oxidation process is a chain reaction involving the formation of free radicals, and comprises three distinct steps, initiation, propagation and termination.

An organic compound (RH) is converted into an active free radical (R•) during the initiation step as a result of the influence of some factors such as heat, light, presence of trace metals or other free radicals.

$$\text{RH} \rightarrow \text{R}^* + \text{H}^*$$

Organic compound Free Radical

The propagation step is the chain reaction during which the free radical absorbs a molecule of oxygen to form a peroxy radical (ROO•). The peroxy radical then abstracts hydrogen from another molecule of RH to form a hydroperoxide (ROOH) and a new free radical R•. R• will absorb a molecule of oxygen and thus continue the reaction.
\[ R^\bullet + O_2 \rightarrow ROO^\bullet \]

Peroxy radical

\[ ROO^\bullet + RH \rightarrow ROOH + R^\bullet \]

Hydroperoxide

The primary products of autoxidation are the odourless and tasteless hydroperoxides. They however, break down to yield aldehydes, ketones and short-chain fatty acids that are responsible for the rancid nature of the oil or fat.

In theory, the propagation step can continue until either all of the organic compound, or the oxygen, has been consumed. However, in practice, a termination step intervenes since certain of the free radicals can combine to form inactive product, which breaks the chain reaction.

\[ ROO^\bullet + ROO^\bullet \rightarrow \text{inactive} \]

\[ ROO^\bullet + R^\bullet \rightarrow \text{inactive} \]

\[ R^\bullet + R^\bullet \rightarrow \text{inactive} \]

Several factors can affect the rate of autoxidation.

1. The degree of unsaturation of the organic compound: Highly unsaturated compounds are more susceptible to autoxidation.

2. The presence of free fatty acids: Free fatty acids oxidize more rapidly than, for example, esters because of the presence of a free carboxylic acid group.

3. Dilution: Dilution of an oxidizable compound with an inert solvent will decrease the rate of oxidation.

4. Temperature: The rate of oxidation of an organic compound is increased with increase in the temperature of storage, in addition, the rate at which the hydroperoxides break down into aldehydes, ketones and fatty acids is also accelerated at temperatures in excess of 50°C.

5. The presence of pro-oxidants: Pro-oxidants, by definition, accelerate the rate of autoxidation. Heavy metals, e.g. copper and iron, also have a pro-oxidant effect.

6. The physical state of the oxidizable compound: The oxidation rate of a solid fat is normally very slow in comparison with that occurring in the liquid state.

### 1.1.1.4. Methods of reducing oxidation

#### 1.1.1.4.1. The presence of antioxidants:

The decomposition of many readily oxidizable substances e.g. fats, may be reduced by adding a small quantity of a substance that will retard the autoxidation process. Such substance is called antioxidant. The antioxidant must be effective in low concentration even for long period of storage and adequately soluble in the oxidizable products. Also it has to be non
toxic or irritant, odourless, tasteless and impart no colour. In addition to that it should be neutral, effective over a wide range of pH and does not react chemically with other constituents present. Also it has to be non volatile to ensure that loss does not occur during storage.

1.1.1.4.2. Presence of reducing agent:
Oxidation of pharmaceutical preparation may be retarded by the addition of a reducing agent. Reducing agents are effective against oxidizing agent as well as atmospheric oxygen and they act by being oxidized in preference to the drug they are protecting. The most commonly used reducing agents in pharmacy are sodium and potassium metabisulphite, bisulphite and sulphites.

1.1.1.4.3. Adjustment of pH
Many oxidative decompositions involving a reversible oxidation-reduction process are influenced by the hydrogen ion concentration of the system. In many instances, decreasing the pH (increasing the hydrogen concentration) results in an increase in the E value and hence an increased resistance to oxidation.

1.1.1.4.4. Removal of Oxygen
By limiting contact of the drug with the atmosphere, those oxidative decompositions dependent upon atmospheric oxygen may often be minimized. Sometimes it is only necessary to store the drug in well-filled airtight container. If more stringent precautions are needed, packing in sealed container under vacuum is recommended. With single dose containers, oxidation is reduced by displacing the air with an inert gas. Nitrogen is frequently used for this purpose.

1.1.1.4.5. Presence of surface active agents
Oxidizable material such as oil-soluble vitamins, essential oils and unsaturated oils, have been formulated as solubilized and emulsified products. In general the rate of autooxidation of a drug in solubilized system is dictated by the concentration of the drug in the oil and/or micellar pseudophase. The exact effect of the surface active agent depends on the water solubility of the drug, the type and the concentration of the surface active agent, and the nature of the phase in which the drug is preferentially soluble. As the concentration of surface active agent was increased, the rate of solubilisation increased until the point was reached where the compound was completely solubilized. Beyond this point, addition of more surface-active agent resulted in a decrease in the rate of decomposition.
1.1.1.5. Isomerization
Isomerization is the conversion of an active drug into a less active, drug or inactive, isomer having the same structural formula but differing in stereochemical configuration.

1.1.1.5.1. Optical Isomerization: Optical isomerization as a cause of drug deterioration may be further divided into the processes of racemization and epimerization.

1.1.1.5.1.1. Racemization: in solution involves the conversion of an optically active form of a drug into its enantiomorph. For example, (-)-hyoscyamine is readily converted by the action of heat or alkali to atropine, which is the racemic mixture of (+) and (-)-hyoscyamine.

1.1.1.5.1.2. Epimerization: Can occur with a compound having more than one asymmetric atom in the molecule. For example, upon the prolong storage, solution containing the alkaloid ergometrine (the 2-amino 1-propanol derivative of D-(+)-lysergic acid), as well as decomposing by hydrolysis, undergo isomerization to ergometrinine (Foster et al, 1949). Ergometrinine is the D (+)-isolysergic acid derivative, which has little physiological activity.

1.1.1.5.2. Geometrical Isomerization: loss of activity produced by geometrical isomerization is due to the difference in potency exhibited by cis and trans isomers of some organic compounds. For example, the most active form of the vitamin A molecule has the all-trans configuration. However, in aqueous solution as a component of a multivitamin preparation, in addition to oxidation, vitamin A palmitate isomerizes and forms the 6-mono-cis and 1,6 di-cis isomers, both of which have much lower potencies (Lehman et al, 1960)

1.1.1.6. Polymerization
Degradation of pharmaceutical products by polymerization involves the combination of two or more identical molecules to form a much larger and more complex molecule. Such a reaction is not often the initial cause of drug decomposition, but does occur as further degradation process of primary decomposition products. Polymerization however, is the prime cause of degradation of the antiseptic formaldehyde. On standing, particularly in the cold, the aldehyde forms paraformaldehyde, which is thrown out of solution as a white deposit, in order to prevent polymerization, 10 to 15 percent of methyl alcohol is added as a stabilizer. (Bentyles, 1982).

1.1.1.7. Decarboxylation
As the word implies, decarboxylation is the elimination of carbon dioxide from a compound. This problem is most commonly encountered when parenteral solutions of sodium bicarbonate are autoclaved. In order to minimize the decomposition of bicarbonate, carbon dioxide is passed into the solution for one minute and the container are sealed so as to be gas-tight prior to autoclaving.
The absorption of carbon dioxide from the atmosphere by a pharmaceutical product is a more frequent occurrence than the loss of carbon dioxide by decarboxylation. Solution of potassium hydroxide, sodium hydroxide, calcium hydroxide and lead subacetate become turbid due to the formation of insoluble carbonates. (Bentyles, 1982).

1.1.1.8. Photolysis
Oxidation and to some extend hydrolysis, is often catalysed by light. The energy associated with the radiation increases as its wavelength decreases, so that the energy of U.V. >visible >I.R., and is independed of temperature. When molecules are exposed to electromagnetic radiation they absorb light (photons) at characteristic wavelengths, which causes an increase in the energy state of the compound. This energy can:

a- Cause decomposition
b- Be retained or transferred
c- Be converted to heat
d- Result in emission of light at a new wavelength e.g. (fluorescence, phosphorescence).

Natural sunlight lies in the wavelength range 290-780 of which only the higher energy (U.V.) range causes photo degradation of drugs. Fluorescent lighting tubes emit visible light and potentially deleterious U.V. radiation in the range of 320-380 nm, whilst conventional tungsten filament light bulbs are, safe. Emitting radiations > 390nm. (Aulton, 1991)

1.1.1.9. Methods of reducing photochemical degradation
The photochemical degradation of a sensitive material can be reduced by protecting it from light. This is achieved by storing the product in a clear glass container and then either placing it in the dark or enclosing it in an opaque wrapper. Alternatively, light resistant container may be used. Since degradation is chiefly due to absorption of light of shorter wavelength, the British Pharmaceutical Codex defines a light-resistance container as one that does not transmit more than 15 per cent of incident radiation between 290 and 450nm. Yellow-green amber glasses are satisfactory since they transmit very little light below 400 nm.

The inspection of solutions for any sign of precipitation or discoloration is difficult in coloured containers, and for this reason many parentral solution are packed in clear glass containers and placed in a lightproof enclosure. (Bentyles, 1982).

1.1.2. The Applications of Chemical Kinetics to Decompositions of Pharmaceutical Products:
A study of the reaction kinetics of decomposition enables a quantitative assessment to be made of the rate at which the drug is destroyed. In addition, information concerning the
number and the type of intermediate steps, which eventually produce the decomposition products, can often be obtained. The application of kinetics also helps us to understand how such factors as concentration of the reactants, temperature, composition of solvent system, and the presence of catalysts may affect the rate of reaction (Bentyles, 1982).

Chemical kinetics theory is based upon the Law of Mass Action proposed by Guldberg and Waage in 1863. According to this law, the rate of chemical reaction is proportional to the molar concentrations of the reacting substances each raised to the power equal to the number of g-moles of that substance involved in the reaction. Therefore for a reaction of general form:

$$uA + vB + wC \rightarrow \text{product} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldoto
stoichiometric equation for the reaction. Order of reaction expresses the experimentally
determined dependence of the rate upon the reactant concentrations. The order of reaction
with respect to a single reaction is equal to the power to which the concentration term of the
reactant is raised in the experimental rate equation. It is evident that, in contrast to
molecularity, it is possible for the order of reaction to assume fractional or zero values.

1.1.2.1.4. Half-life:
The rate or chemical reaction can be expressed in terms of the time taken for 50 per cent of
the reaction to occur. This is called the half-life of the reaction, \( t_{1/2} \) and depends, in general,
upon both the initial concentrations of the reactants and the reaction velocity constant.

1.1.2.2. Derivation of Simple Order of Reaction:
1.1.2.2.1. First order reaction:
Where the rate of the reaction is proportional to the concentration of the reactant.
\[
\frac{dx}{dt} = k(a-x) \hspace{1cm} \text{(4)}
\]
Where \( a \) is the initial reactant concentration.
\( x \) is the amount reacted in time \( t \).
\( k \) is the first – order reaction velocity constant.

By rearrangement of the equation then,
\[
\frac{dx}{a-x} = k \hspace{1cm} \text{dt} \hspace{1cm} \text{(5)}
\]

and upon integration of equation
\[
\int \frac{dx}{a-x} = \int k \hspace{1cm} \text{dt}
\]
Then
\[
-ln(a-x) = kt + \text{integration constant} \hspace{1cm} \text{(6)}
\]
When \( t = 0, x = 0 \) the integration constant = \( \ln a \).

By substitution in equation (6) then,
\[
k = \frac{1}{t} \ln \frac{a}{a-x} \hspace{1cm} \text{(7)}
\]

By changing to common logarithms rather than natural logarithms
\[
k = \frac{2.203}{t} \log \frac{a}{a-x} \hspace{1cm} \text{(8)}
\]
The plot obtained by change of the reactant concentration with respect to the time will give a
straight line whose slope is \(-k/2.303\). The first order reaction constant can be calculated and
have the dimensions of time \(^{-1}\).

Half-life of the first order reaction:
If the initial concentration of the reactant is \( a \), then the concentration at time \( t_{1/2} \) will be \( a/2 \)
and the rate equation will be
\[
k = 2.303 \times \log2 / t_{1/2} \hspace{1cm} \text{(9)}
\]
And
\[ t_{1/2} = 0.693/k \]  \hspace{1cm} (10)
Thus for a first order reaction, the half-life is independent of the initial reactant concentration, and depends solely on the reaction velocity constant.

1.1.2.2.2. Pseudo First Order Reaction:
A chemical reaction obeys pseudo first order reaction kinetics when the rate of the process is proportional to the concentration of only one of the reactants even though the reaction involves several reactant species, this occurs if one or more of the reactants may be present in such large excess that there is no measurable change in its concentration during the course of the reaction, or if the reaction has several component steps, and if one of these is considerably slower than the rest, in this event the kinetics and the order of the reaction will be those of the rate determining step. Also if one of the reactants is acting as a catalyst in the reaction so its concentration will not change as the reaction proceeds.

1.1.2.2.3. Second order reaction:
The rate of the second order reaction is proportional to the concentration of two reacting species. The rate of reaction is given by:
\[ \frac{dx}{dt} = k(a-x)(b-x) \]  \hspace{1cm} (11)
Where \( a \) & \( b \) are the initial concentration of reactants \( A \) & \( B \), \( x \) is the decrease in concentration after time \( t \) by integration of equation (11) and evaluation of integration constant:
\[ k = \frac{2.203}{t(a-b)\log b(a-x)/a(b-x)} \]  \hspace{1cm} (12)
The value of \( k \) can be obtained by plotting the function:
\[ \frac{1}{(a-b)\log b(a-x)/a(b-x)} \] against \( t \)
The slope of the straight line will be equal to \( 2.303/k \). The reaction velocity constant will have the dimension of concentration\(^{-1}\)time\(^{-1}\).
If the initial concentration of the reactants is equal then:
\[ \frac{dx}{dt} = k(a-x)^2 \]  \hspace{1cm} (13)
And by integration
\[ k = \frac{1}{t \cdot x} / (a-x) \]  \hspace{1cm} (14)
Half-life of second order reaction:
By substitution in equation (14) then:
\[ t_{1/2} = \frac{1}{k \cdot a} \]  \hspace{1cm} (15)
Hence, it is evident that the half-life is dependant upon both the reaction velocity constant and the initial concentration of the reactant.
1.1.2.2.4. Zero order reaction:

Zero order reaction is obeyed when the rate of reaction is independent of the concentration of any of the reactants and does not alter with time.

\[ \frac{dx}{dt} = k \] .......................... (16)

By integration

\[ \int dx = \int k \, dt \] .......................... (17)

And

\[ kt = x \] .......................... (18)

A plot of decrease in concentration against time will give straight its slope is equal to \( k \). The dimensions of the reaction velocity constant is concentration \(^{-1}\).

Half-life of a zero order reaction:

\[ t_{1/2} = \frac{a}{2k} \] .......................... (19)

so the half life is proportional to the initial concentration of the reactant and inversely proportional to the reaction velocity constant.

1.1.2.2.5. Third order reaction:

If the reaction is dependent on the collision of three molecules, it is called third order reaction. It is an unusual occurrence, and the kinetic equations expressing the rates of reaction are complex.

1.1.2.3. Temperature effect on the rate of reaction:

The rate of decomposition increases as the temperature is raised. It has long been recognized that the rate of a chemical reaction increases by a factor between two and three folds for every 10° in temperature in the region of room temperature. (Bentyles, 1982)

Experimentally (Connors, 1986) the reaction rate constant is observed to have an exponential dependence on temperature:

\[ K = A \exp \left( -\frac{E_a}{RT} \right) \] .......................... (20)

Where \( k \) is the reaction rate constant of any order, \( A \) and \( E_a \) are constants and \( T \) is the absolute temperature (\( t^\circ C + 273.16^\circ C \)). \( E_a \) is called the activation energy of the chemical reaction. The equation is called Arrhenius equation and can also be written

\[ \log k = \log A - \frac{E_a}{2.303 RT} \] .......................... (21)

Or

\[ \log \frac{k_2}{k_1} = \frac{E_a}{2.303R} \left( \frac{1}{T_2} - \frac{1}{T_1} \right) \] .......................... (22)

\[ \log \frac{k_2}{k_1} = \frac{E_a}{2.303} \frac{T_2 - T_1}{T_2 T_1} \] .......................... (23)
Where \( k_1 \) and \( k_2 \) are the velocity constants at \( T_1 \) and \( T_2 \) respectively. As the reaction proceeds from reactants to products the system must pass through a state whose energy is greater than that of the initial reactants. This barrier is what prevents the reactants from immediately becoming products; the activation energy \( E_a \) is a measure of this barrier. (Connors, 1986).

From equation (21) (Aulton, 1991) a straight line graph can be obtained from \( \log k \) against \( 1/T \) and the constant \( E_a \) can be obtained from the graph slope which is equal to \( -E_a / 2.303 R \). This plot is called Arrhenius plot.

1.1.3. Physical factors influencing chemical degradation

1.1.3. 1. Temperature

The rates of chemical reactions increase with rise in temperature. It is, important to be aware of this when formulating pharmaceutical products for use in tropical area, or when a product has to be heat sterilized before use. Autoclaving aqueous injections, e.g. dextrose injection, or sterilizing oils, e.g. ethyl oleate, and powders, e.g. sulphanilamide, by dry heat methods can cause decomposition problems when the drug is thermolabile. The observed variation of reaction rate with temperature is an important aspect of the collision theory of chemical reaction. According to this theory, a chemical reaction only takes place when molecules collide. The thermal energy of the colliding molecules is converted into energy, which is necessary to break chemical bonds and enable the reaction to take place. However, the number of molecular collisions greatly exceeds the number of molecules reacting per second. It has been postulated that reaction only occurs upon collision of molecule possessing a certain minimum amount of energy (the energy of activation). As the temperature of the system is raised, the proportion of molecules having this minimum energy increases. It follows that, at higher temperatures, a greater number of the collisions will result in reaction of molecules, that gives rise to the observed greater rate of reaction.

There are however, a few instances where low temperature storage may have a tendency to accelerate decomposition, e.g. increased rate of polymerization of formaldehyde at temperatures below 15°C. (Bentleyes,1982)

1.1.3. 2. Moisture

Moisture absorbed on to the surface of a solid drug will often increase the rate of decomposition if it is susceptible to hydrolysis. This is particularly true of aspirin, the penicillins and other antibiotics such as tetracycline and streptomycin. The presence of moisture may in some instances, also increase the rate of oxidation of a susceptible product, since oxygen is dissolved in the water layer surrounding the drug particles. For example, ferrous sulphate crystals are more rapidly oxidized in moist air than in dry surroundings. It is
therefore important that such drugs should be stored in as dry a condition as possible. When manufacturing solid dosage preparation from drugs whose chemical stability is affected by moisture, it may be necessary to work in an environment of controlled humidity. This technique has to be used when processing sodium ampicillin and potassium propicillin (Gore & Ashwin 1967). Also excipients chosen for the product must have moisture content low enough to prevent transfer of moisture to the active drug.

1.1.3.3. Light
Numerous texts in pharmaceutical literature refer to the instability of many products when exposed to strong sunlight. Although, in some instances, the instability is due to the heat accompanying sunrays, light is a form of energy that can initiate and accelerate decomposition. In some way that increased temperature can accelerate a thermal reaction, exposure of a light-sensitive drug (photolabile) to sunlight supplies sufficient energy of activation to enable decomposition to take place. Only the light radiation absorbed by the drug is effective in producing photochemical reaction. Since a portion, or all, of the light energy may be converted into heat, it should be borne in mind that absorption of light by a compound does not necessarily indicate that a photochemical reaction is preceding. However, if a photochemical reaction is taking place, this will be independent of the temperature of the system.

1.1.3.4. Radiation
Ionizing radiation can be a useful technique for the sterilization of certain pharmaceutical products, especially those that are thermolabile. Unfortunately, radiation treatment can also produce deleterious changes in the products since the procedure also causes ionization in the irradiated material. The ions formed in the initial stage of the process are subsequently converted into atoms and free radicals, which become involved in chemical reactions. In 1960, a preliminary investigation was undertaken to assess the stability of a wide range of pharmaceutical products to $\gamma$-irradiation from a $^{60}\text{Co}$ source of radiation for the sterilization of pharmaceutical products. Antibiotics e.g. polymyxin sulphate and streptomycin sulphate, alkaloids, e.g. atropine sulphate, steriods e.g. progesterone, sulphonamides and biological products, e.g. insulin and heparin, were all irradiated at dose levels of 2.5 M rad and also 25 Mrad in order to identify possible decomposition products. The effect varied from change of colour as in progesteron to almost loss of activity as with insulin.

1.1.4. The influence of packing material on dosage form stability:
Faulty packaging of pharmaceutical dosage forms can invalidate the most stable formulation. Consequently, it is essential that the choice of container materials for any particular product
be made only after a thorough evaluation has been made of the influence of these materials on the stability of the product and of the effectiveness of the container in protecting the product during extended storage under varying environmental conditions of temperature, humidity and light (Lachman, 1984).

1.1.4.1 Glass

Glass has been the container of choice for pharmaceutical products because of its resistance to decomposition by atmospheric conditions or by solid and liquid contents of different chemical compositions. Also by change of chemical composition of glass it is possible to adjust the chemical behavior and radiation properties of the glass.

Release of alkali and release of insoluble flakes to liquids stored in glass containers, are the two principal faults of glass.

Releasing alkali can be treated by decreasing the soda content of glass or by replacing sodium oxide with other oxides. Also treatment of the surface of the glass with sulfur dioxide in the presence of water vapor and heat makes glass more resistant (Dimbley 1953). Buffering of solution of liquid dosage form can eliminate any effect due to possible change of pH if some alkali were released from the glass.

Insoluble flakes appearance depends upon the type of glass used. Flake formation always occurs in nonborosilicate glass immediately after autoclaving, whereas in borosilicate occurs in much higher temperatures.

Glass container may possess various additives such as oxides of boron, sodium, potassium, calcium, iron and magnesium, which alter physical and chemical properties of glass. Glass containers should contain a minimal amount of barium and calcium to prevent formation of insoluble inorganic salts (Boddapati, 1980). Flake formation is also likely to occur with phosphate, citrate, tartarate, and alkaline solutions. Pretreatment of the container with dilute acid solution may delay flakes formation.

Amber glass is extensively used in pharmaceutical industry; the amber colour of glass is imparted by the addition of iron and manganese oxides, cations that are known to catalyse oxidative reactions. Studies have shown that these ions are extracted from the glass and decomposition rates of several drugs, such as thiomersal, amitriptylene, (Enver, 1977) and L-ascorbic acid (Kassem, 1969) are enhanced in amber glass.

1.1.4.2. Plastics

The term plastic denotes a considerable group of high molecular weight polymers, each having different chemical and physical properties. They include polyethylene, polypropylene, polystyrene, polyvinylchloride, and several others.
The chief disadvantage of plastic compared with glass is the problem of permeation in two directions, namely from the solution in the plastic container through the plastic into the ambient environment and from the ambient environment through the plastic into the preparation. In addition material can leach from the plastic container into the liquid preparation, material from the preparation can be adsorbed or absorbed onto and into the plastic container. Also the contents of the container can react chemically or physically with plastic components of the container causing container deformation. The chemical as well as physical stability of the tablets dosage form can be influenced considerably by penetration of water vapour from the atmosphere into the container. Oxygen and carbon dioxide can permeate through plastic container catalyzing the degradation of drugs.

Plastic container when used for emulsions, thorough evaluation for physical and chemical changes of the emulsion and physical changes of the container must be carried out. Some materials of the emulsion have the tendency to migrate through the polypropylene wall causing either change of the emulsion or a collapse in the container (Chen, 1957). Air can cause emulsion to break down, owing to dehydration or oxidation of the oil phase. This phenomenon is called breathing and is the major cause of deterioration during storage in plastic containers. Loss of flavours and perfumes can occur through plastic containers. The property of plastics to sorb materials from solution can cause loss of drug, antibacterial agent or other materials from solution.

1.1.4.3. Metals
Disperse systems, having a consistency of a soft paste, gel, cream, or ointment, can be packed into collapsible tubes. Metals commonly used for tubes are tin, plastic-coated tin, tin coated lead, aluminum and coated aluminum. Tubes constructed of a single material can readily be tested for stability with a product. Tubes having coatings present additional problems, since it must be established whether the coating material is sufficiently inert for the preparation as well as whether the coating completely covers the underlying material. In addition they must be evaluated for ease of cracking and solvent resistance.

Tin and tin coated tubes are usually employed because of their unreactive properties, although it has been reported that tin tubes can be corroded by chlorides or acid conditions. Vinyl cellulose lacquers are applied to tin to increase their utility.

Coated and uncoated aluminum tubes are being used, but are not always satisfactory because aluminum reacts with fatty alcohol emulsions to form a white encrustation (Board, 1949). Also coated and uncoated aluminum are deleteriously affected when used for preparation
outside the pH range (6.5-8.0) (Stephebson, 1953). The application of epoxy lining to the internal surface of aluminum tubes was found to make them more resistant to attack.

1.1.4.4. Rubber
Rubber of varying composition is used as stopper, cap liner, and parts of dropper assemblies. Rubber is mainly used as stopper for multiple-dose vial solutions for injection. The main problem of rubber is sorption of active ingredient, preservative and other materials and extraction of one or more components of the rubber into the vial solution. If epoxy lining is applied to rubber stoppers, a considerable reduction results in the amount of extractive leach from the stopper by water for injection in the vial but has no effect on sorption of the preservative.

The presence of rubber closure extractives in the vial solutions could interfere with the chemical analysis of the active ingredient, affect the toxicity or pyrogenicity of the injectable preparation, interact with the drug or preservative to cause inactivation or loss of stability, and cause physical instability to the preparation, owing to the presence of particulate matter in the solution. (Lachman, 1984)

1.1.5. Physical degradation of pharmaceutical products:

1.1.5.1. Loss of volatile constituents:
A wide range of materials e.g. iodine, camphor and alcohol are volatile at ambient temperature and may be lost from the pharmaceutical preparations. Even tablets containing nitroglycerin may lose potency because of volatilization. (Banes, 1968). Since volatilization rate is increased with increase in temperature, it can be reduced by keeping the preparation in a well-closed container in a cool place. (Bentyles, 1982).

1.1.5.2. Loss of water
Evaporation of water from liquid dosage forms or semi-solid oil-in-water emulsions may cause cracking of these systems. Loss of water from aqueous solutions will give rise to concentration, and possibly crystallization of the solutes. Water loss depends on the temperature and humidity of the environment so can be prevented by storing the preparation in well-closed containers. (Bentyles, 1982).

1.1.5.3. Absorption of water
Absorption of water from the atmosphere is a common cause of deterioration of a variety of drug products. For example glycerin will absorb moisture and become opaque while gelatin capsules will soften. Effervescent tablets and granules will react prematurely in moist atmosphere. Storage of all such materials in well closed container will prevent them from deterioration (Bentyles, 1982).
1.1.5.4. Crystal growth
The deposition of crystal from aqueous solution occurs when the vehicle becomes supersaturated with respect to the solute. This result from a fall in temperature with a consequent decrease in solubility of the solute. Classic example of crystal growth is often with calcium gluconate injection. In suspensions of finely divided solids, growth of crystals is also undesirable. There is a tendency for crystals to bind together to form a hard cake which is difficult to redisperse. Large crystals form gritty texture, which is unacceptable in topical and ophthalmic preparations. When present in suspension for injection there is a danger of blocking the hypodermic needle.

Prevention or reduction of crystal growth may be achieved by avoiding metastable form of drug and by storing the preparation in an environment, which exhibits the minimum of temperature fluctuation. Also the use of narrow size range of crystals, addition of surface active agent and increasing the viscosity of the suspending medium, inhibit or decrease crystal growth (Bentyles, 1982).

1.1.5.5. Polymorphic changes
Many substances are found in two or more polymorphic forms. Upon storage in the dry state or in suspension interconversion of these forms may happen. These changes cause changes in solubility and crystal growth (Bentyles, 1982).

1.1.5.6. Colour changes
A change in colour of a pharmaceutical product is usually just a visual indication that some form of chemical or photochemical decomposition is occurring. Medicines are usually coloured for aesthetic reasons and colour fading is a fairly common source of instability. Some water soluble dyes e.g. indigo carmine, tend to fade in presence of reducing substance such as dextrose and lactose (Kuramoto et al, 1958). The process is also affected by the pH of the solution. Non ionic surface active agents e.g. tween 20, accelerate the rate of fading of indigo carmine, tartarazine and orange G in aqueous solution. This is either due to interaction of the dyes with polyoxyethylene groupings of the surface active agent or to the presence of impurities in these materials. Dyes also tend to fade when present in tablets exposed to light and this is reduced by incorporating an ultraviolet light-absorbing compound in the tablet formulation. Lachman et al (1962) found that tablets when coloured with brilliant blue or tartarazine were more stable when the compound 2,4 dihydroxybenzophenone was present.

Colour development in pharmaceutical products is often encountered. For example solutions of physiostigmine or adrenaline become red on exposure to light while apomorphine HCl turns green. Solid dosage forms also develop colour e.g. aspirin tablets become red and
ascorbic acid tablets will gradually change from white to yellowish-brown colour. The colour stability of ascorbic acid is affected by the presence of lubricants such as magnesium or calcium stearate (Wortz, 1967).
1.2. Stability testing

1.2.1. Introduction

Stability is defined as the extent to which a product retains, within specified limits, and throughout its period of storage and use (shelf-life), the same properties and characteristics that are possessed at the time of its manufacture. Five types of stability are generally recognized. (USP, 1995)

<table>
<thead>
<tr>
<th>Type of stability</th>
<th>Conditions Maintained Throughout the Shelf-Life of the Drug Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>Each active ingredient retains its chemical integrity and labeled potency, within the specified limits.</td>
</tr>
<tr>
<td>Physical</td>
<td>The original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability are retained</td>
</tr>
<tr>
<td>Microbiological</td>
<td>Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits.</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>The therapeutic effect remains unchanged.</td>
</tr>
<tr>
<td>Toxicological</td>
<td>No significant increase in toxicity occurs</td>
</tr>
</tbody>
</table>

Table 1: Types of drug stability

Drug instability in pharmaceutical formulation may be detected on some instances by changes in the physical appearance, colour, odour, taste or texture of the formulation whereas in other instances chemical changes may occur and are not self-evident and may only be ascertained through chemical analysis.

To assess the stability of a formulated product it is usual to expose it to high stress conditions to enhance its deterioration and therefore reduce the time required for testing. This enables more data to be gathered in shorter time, which in turn allow unsatisfactory formulations to be eliminated early and reduce the time for successful product to reach the market. (Aulton, 1991)

The objectives of the accelerated test are:

1. The rapid detection of deterioration in different initial formulations of the product in order to select the best formulation from a series of possible choices.

2. The prediction of shelf life when stored under expected or directed storage conditions.
3. The provision of a rapid means of quality control, which ensures that no unexpected changes have occurred in the stored product (Aulton, 1991).

1.2.2. Common High stresses or challenges

1.2.2.1. Temperature

An increase in temperature causes an increase in the rate of chemical reaction. The products are stored at temperatures greater than room temperature. The nature of the product often determines the range covered in the accelerated test. Samples are removed at various time intervals and the extent of decomposition is determined by analysis. (Aulton, 1991).

When determining the chemical stability of a pharmaceutical product, it is essential that the assay employed should be sufficiently specific to distinguish between the drug and its decomposition products. (Bentyles, 1982).

1.2.2.2. Humidity

Storage of products in atmosphere of high humidity will accelerate decompositions that results from hydrolysis (Aulton, 1991). This is achieved by using a number of cabinets each containing different saturated salt solution that produces the desired humidity. (Bentyles, 1982). Marked acceleration will be obtained if the naked product (i.e. not enclosed in a container) is subjected to these tests, which usually indicate the minimum humidity tolerated by the product without undue decomposition (Aulton, 1991). The final packaged product must be tested in this manner (Bentyles, 1982) and this is useful in determining the degree of protection that should be afforded by a container (Aulton, 1991).

1.2.2.3. Light

A source of artificial light is used to accelerate the effects of sunlight or skylight. The source should emit a similar distribution of radiant energy to that in sunlight because photochemical reaction involves the absorption of light definite wavelengths. Daylight florescencent lamps provide a satisfactory, and banks of such lamps may be used to accelerate the effects of light. The heating effect of these lamp is reduced by the use of glass plates. Otherwise it is difficult to separate between decomposition caused by light or increased temperatures. (Aulton, 1991).

1.2.3. Definitions

1.2.3.2. Stability:

It is the ability of a pharmaceutical product to retain its properties within specified limits throughout its shelf-life. The following aspects of stability are to be considered: chemical, physical, microbiological and biopharmaceutical. (WHO, 1994)
1.2.3.2. Stability tests
Stability tests are a series of tests designed to obtain information on the stability of pharmaceutical product in order to define its shelf-life and utilization period under specified packaging and storage conditions (WHO, 1994).

1.2.3.3. Accelerated stability testing
Studies are designed to increase the rate of chemical degradation and physical change of a drug by using exaggerated storage conditions as a part of formal stability testing programme (WHO, 1994).

These data, in addition to real time studies, may be used to assess longer-term chemical effects at non-accelerated conditions and to evaluate the impact of short-term excursions outside the label storage conditions such as might occur during shipping. Results from accelerated testing studies are not always predictive of physical changes (WHO, 1994).

1.2.3.4. Real time (long-term) stability studies:
These constitute the evaluation of experiments for physical, chemical, biological and microbiological characteristics of a drug, during and beyond the expected time of shelf-life and storage of samples at expected storage conditions in the intended market. The results are used to establish shelf-life, to confirm projected shelf-life and recommend storage conditions (WHO, 1994).

1.2.3.5. Shelf-life
The period of time during which a pharmaceutical product is expected, if stored correctly, to comply with the specification as determined by stability studies on a number of batches of the product is termed the shelf-life and is used to establish the expiry date of each batch. (WHO, 1994)

1.2.3.6. Intended Market:
The design of the stability testing programme needs to take into consideration the intended market and the climatic conditions of the area in which the drug will be used. (WHO, 1994)

1.2.3.7. Climatic zone
The concept of dividing the world into four zones is based on defining the prevalent annual climatic conditions (WHO, 1994).

Four climatic zones can be distinguished for the purpose of worldwide stability testing:
Zone I: temperate
Zone II: sub-tropical with possible high humidity
Zone III: hot/dry
Zone IV: hot/humid
<table>
<thead>
<tr>
<th>Climatic zone</th>
<th>Measured data in the open air</th>
<th>Measured data in storage room</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[°C]</td>
<td>[%RH]</td>
</tr>
<tr>
<td>I</td>
<td>10.9</td>
<td>75</td>
</tr>
<tr>
<td>II</td>
<td>17.0</td>
<td>70</td>
</tr>
<tr>
<td>III</td>
<td>24.4</td>
<td>39</td>
</tr>
<tr>
<td>IV</td>
<td>25.6</td>
<td>77</td>
</tr>
</tbody>
</table>

Table 2: The main climatic conditions found in the climatic zones all over the world.

1.2.4. Purpose of stability testing

<table>
<thead>
<tr>
<th>Objectives</th>
<th>Type of study</th>
<th>For use in</th>
</tr>
</thead>
<tbody>
<tr>
<td>To select adequate (from point of view of stability) formulation and container-closure systems</td>
<td>Accelerated</td>
<td>Development of product</td>
</tr>
<tr>
<td>To determine shelf-life and storage conditions</td>
<td>Accelerated and/or real time</td>
<td>Development of product and registration dossier</td>
</tr>
<tr>
<td>To verify that no changes have been introduced in the formulation or manufacturing process that can adversely affect the stability of the product</td>
<td>Accelerated and real-time</td>
<td>Quality assurance in general including quality control</td>
</tr>
</tbody>
</table>

Table 3: The main objectives of stability testing and the requested type of stability testing.

1.2.3.1 In the development phase

Accelerated stability studies tests are carried out to compare in short-term experiments alternative formulations, packing materials, and/or manufacturing processes. As soon as the final formulation and manufacturing process have been established, the manufacturer will carry out a series of accelerated stability testing which will permit prediction of the stability of the drug product, and determine its shelf-life and storage conditions. (WHO,1994)
1.2.3.2. For the registration dossier
The drug regulatory authority will request the manufacturer to submit information on the stability of the product from tests made of the final dosage form in its final container packaging. The data submitted result from both accelerated and real-time studies. Published and/or experimental supporting stability data may be submitted, e.g. on stability of active ingredients and related formulations.

“In use” stability data have to be submitted to support a recommended storage conditions of diluted or reconstituted products in cases where the product is to be diluted or reconstituted before being administered to the patient (e.g. powder for injection and concentrate for oral suspension).

With the approval of drug regulatory authority, a preliminary shelf-life is often established on condition that additional information from first production batches will be submitted after registration. (WHO,1994)

1.2.3.3. In post-registration period
The manufacturer will carry out on-going real-stability studies to substantiate the expiry date and the storage conditions previously projected. Data needed to confirm a tentative shelf-life have to be submitted to the registration body.

Once the product has been registered, additional stability studies are required whenever major modifications are made to the formulation, manufacturing process, packaging or method of preparation. These results have to be communicated to the receptive drug regulatory authorities. (WHO,1994)

1.2.5. Accelerated stability studies
1.2.5.1. Stability testing protocols
Accelerated stability testing requires the careful design of protocols that must define clearly the following (Aulton, 1991):

1. The temperature and humidity for storage.
2. Storage time before sampling
3. The number of batches to be sampled
4. The number of replicates within each batch
5. A suitable light challenge
6. Details of assay.
1.2.5.2. Design of stability studies

1.2.5.2 1. Test conditions

Test conditions are determined by the intended climatic zone in which the drug product will be distributed and used, as well as by the type of dosage forms. As a rule, accelerated studies are less suitable for semi-solid and heterogeneous formulations e.g. emulsions etc.

<table>
<thead>
<tr>
<th>Storage temp. [°c]</th>
<th>Humidity [%]</th>
<th>Length of period [month]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone VI for hot climatic zones or global market</td>
<td>40±2%</td>
<td>75±5%</td>
</tr>
<tr>
<td>Zone II – for temperate and subtropical climatic zone</td>
<td>40±2%</td>
<td>75±5%</td>
</tr>
</tbody>
</table>

Table 4: Shows examples of accelerated stability testing

1.2.5.2 2. Test samples

For registration purposes test samples from products containing fairly stable active ingredients are taken from two different production batches, whereas three batches should be sampled from products containing easily degradable active ingredients. The batches to be sampled should be representative of manufacturing process, of pilot plant or full-scale production. For on going studies batches from current production should be sampled in accordance with a preparation-determined schedule. The following approach may be suggested. (WHO,1994)

- One batch every other year may be tested for formulations considered to be stable, (otherwise one batch per year).
- One batch every 3-5 years may be tested for formulations when the stability profile has been established, unless a major product change has been made, e.g. to the formulation or the method of manufacture.
- Details concerning the batches should be stated in the test records, i.e. packaging of the drug product batch number, manufacture, batch size, etc.

1.2.5.2 3. Analytical Methods

A systemic approach should be adopted in the presentation and evaluation of stability information; this should include as necessary, physical, chemical, biological and microbiological test characteristics
All products characteristics likely to be affected by storage, e.g. assay, value or potency tests for product of decomposition, physicochemical tests (hardness, disintegration, particulate matters, etc.), should be determined, and for solid or semi-solid oral dosage forms dissolution test should be carried out.

Analytical methods should be validated or verified, and the accuracy as well as the precision (standard deviations) should be recorded. Assay methods should be chosen to be stability indicating. Tests for related compounds or products of decomposition used should be validated to demonstrate that they are specific to the product being examined and are of adequate sensitivity.

Test method to prove the efficacy of additives, such as antimicrobial agents, should be foreseen to see if they remain effective and unchanged throughout the projected shelf-life.

A check list, similar to the one used for the WHO Survey on Stability of Essential Drugs, could be used to identify other stability characteristics of the product. (WHO, 1994)
1.3. Biological Testing

Biological testing includes the quantitative assay of drugs by biological methods as well as the application of qualitative biological tests. Such testing utilizes intact animals, animals’ preparations, isolated living tissues, or microorganism. The majority of currently available therapeutic agents are substances of known chemical composition, which can be assayed by quantitative chemical or physical analyses. There remains, however, a limited number of useful drugs, which cannot be assayed satisfactorily by chemical or physical means. Such drugs, which are primarily of natural origin, are assayed by biological methods. Biological standardization procedures are generally less precise, more time-consuming, and more expensive to conduct than chemical assays; therefore, they are generally reserved for use mainly:

1. If the chemical identity of the active principle has not been fully elucidated.
2. If no adequate chemical assay has been devised for the active principle, although its chemical structure has been established, e.g., insulin.
3. If the drug is composed of a complex mixture of substances of varying structure and activity, e.g., digitalis, posterior pituitary hormone.
4. If purification of the crude drug, sufficient for performance of a chemical assay, is not possible or practical, e.g., the separation of vitamin D from certain irradiated oils.
5. If the chemical assay is not a valid indication of biological activity, due for example, to lack of differentiation between active and inactive isomers.

There are several situations in which factors such as specificity, sensitivity, or practicability dictate the use of a biological rather than a chemical assay procedure. (Remington, 1990)

1.3. Biological assay of antibiotics:

1.3.1. Historical:

The quantitative use of zones of inhibition in the growth of one microorganism on agar media by diffusing substances produced by another organism was in use at least as early as 1885. Florey quotes the work of Grarre on the inhibition of *Staphylococcus pyogenes* by *Pseudomonas fluorescens*. Fleming used this method in 1922 for his work on lysozyme, and it was his observation of the lysis of staphylococcus in the neighborhood of a colony of *Penicillium notatum* that led to his work on penicillin in 1929. It was natural therefore that Chain *et al.* in 1940 used inhibition zones to follow the activity of the penicillin they isolated and purified and it should be suggested that zone size should be a basis for quantitative comparison with standard penicillin for purposes of estimation. Abraham *et al.* (1941) and Foster and Woodruff (1943, 1944) investigated many of the factors influencing the results...
and compared the advantages and disadvantages of the method with the turbidimetric method. Schmidt and Moyer (1944) also examined the influence of experimental conditions on the assay, and from that time many papers have been published involving many modifications of techniques. Despite the great use made of these agar diffusion methods, it was not until the years after the war that any systematic work on the theoretical aspects involved was published in 1946 by Cooper and Woodman. (Kavanagh, 1963)

1.3.2. Literature review:
A study on validation of an analytical method for the quantitative determination of erythromycin thiocyanate formulated on an antibiotic preparation for veterinary use was carried out using the microbiological method described in the European Pharmacopoeia to analyse erythromycin thiocyanate as raw material. The microbiological method followed a linear model and was not proportional. The number of replicates needed to obtain a valid result was less than four in all cases. (Bernabeu et al, 1999).

A study on the determination of serum aminoglycoside and vancomycin concentrations by microbiological method in the presence of other antimicrobials was done by using strains with selective sensitivity to the antimicrobial to be tested or by adding crude extracts of beta-lactamase for inactivation of beta-lactam such as pencillins and cephalosporines. The selective organisms used were Staphylococcus aureus for appraisal of vancomycin when accompanying antimicrobials were gentamicin, ciprofloxacin or rifampicin, and E. faecalis, E. avium and Staphylococcus aureus when the drugs in the mixture were amikacin, lincomycin or trimethoprim-sulphamethaxazole, respectively. For appraisal of gentamicin in the presence of vancomycin or fluoroquinlones like ciprofloxacin and norfloxacin Escherichaia coli was the most suitable organism, while the use of K. pneumonia was required for the appraisal of amikacin or netilmicin. (Corso et al, 1996)

A comparison between monolayer and bilayer plates used in antibiotic assay was done using five antibiotics and AOAC-specified test organism and agar media. Antibiotics were penicillin G, erythromycin, streptomycin, tetracycline and gentamicin. Micrococcus luteus ATCC No. 9341 was the test organism for the first two antibiotics, while Bacillus subtilis ATCC No. 6633 for streptomycin, Bacillus. cereus ATCC No. 11778 for tetracycline, and Staphylococcus epidermidis for gentamicin. The standard curves were obtained and the difference in response between monolayer and bilayer was less than 10% so monolayer plates were accepted for analyses. (Reamer et al, 1998).

Different studies were done for determination and screening of antibiotics in animal tissues using microbial methods. Microbiological methods were used for detection of antibiotics in
kidneys and muscles of slaughtered animals. 95 kidneys and 76 muscles were used from 58 cattle, 36 pigs and one horse. Information on pre-slaughter medication with one antimicrobial drug was available for 63% of the carcasses. Microbiological identification was performed by agar diffusion using 17 or 18 combinations of eight test organisms, varying pH and three substances blocking the action of certain antimicrobials. The results of the study showed that microbiological method is well suited for identification of antibiotic residues. (Myllyniemi et al, 1999).

Tissue concentration of amoxicillin was detected using animal model. Induced granulomatous tissue made by implantation of polyurethane sponge in rats’ backs was used. The tissue and the animal serum were obtained after administration of different concentrations of amoxicillin and placed in plates containing Muller Hinton agar inoculated with staphylococcus aureus ATCC No. 25923. The diameters of zones of inhibition were measured after 18 hr of incubation. Results showed that this method is valid to measure the tissue concentration of amoxicillin (Groppo et al, 2000).

Bioassay was used for evaluation of the efficacy of four different antibiotics administered in a prophylactic dose to children with vesicoureteral reflux. The children were divided into four group according to the antibiotics used which included nalidixic acid, cephalexin, cotrimoxazole and cefixime. 159 Urine samples were collected from 53 children in the morning, at noon and in the evening and each sample was bioassayed for growth inhibition using Escherichaia coli. In addition the specific gravity, which reflected urinary concentration of each sample, was measured. The study revealed that urine concentration during the day is dependent on age, with older children having more concentrated urine in the latter part of the day. Growth inhibition is enhanced by concentrated urine. Also they found that cotrimoxazole and cefixime produce a sustained bactericidal effect for about 60% of a 24-hour day compared to nalidixic acid and cephalexin due to the longer half-life. (Pomeranz et al, 2000)

Rapid determination of neomycin was done by microbiological agar diffusion assay using triphenyltetrazolium chloride. The standard quantitative determination of neomycin requires incubation period of bout 18 to 24 hours. The use of triphenyltetrazolium indicator reduced this period to only 7 hours. This indicator produced a physical response related to the biological one and the zones appeared much earlier. The test organism used was Staphylococcus epidermidis. There was no significant difference between the standard curves for 7 and 24 hours. Comparative assays for some pharmaceutical drugs containing
neomycin in different forms showed that it is possible to reduce incubation time. (Yamamoto et al, 1996).

Interlaboratory study comparing the microbiological potency of spiramycins I, II, and III. was carried out. The method used was agar diffusion using *Bacillus subtilis* as test organism and turbidimetric assay using *Staphylococcus aureus*. Six laboratories from three countries participated. Experimental procedure was according to European Pharmacopoeia. The activity of spiramycin I was markedly higher than that of spiramycins II and III. The interlaboratory relative standard deviation varied from 3.6 to 16.3% in diffusion method and from 2.6 to 7.7 in turbidimetric method. (Lui et al, 1999).

Diffusion method commonly used for biological assay of antibiotics were applied to the identification and assay of antiseptic substances used as preservatives. (Bernard et al, 1977).

Many studies were performed to compare microbiological assay with other techniques such as HPLC (high performance liquid chromatography), radioimmunoassay and others. Analysis of isepamicin in human plasma was done using microbiological assay, HPLC and radioimmunoassay. Regression analysis indicated good correlations among the three methods. (Lin et al, 1997). Also comparison between biological and chemical assays was done for quantification of rifapentine in human plasma. The bioassay was found to overestimate antibiotic plasma concentration when compared to HPLC assay because of the presence of other biologically active metabolite in the plasma. (Kenny et al, 1997). A study was done for the detection of aminoglycoside using universal ELISA (enzyme linked immunosorbent assay) binding procedure compared to microbiological agar diffusion assay and HPLC analysis. Five samples of tobramycin were assayed using the three different techniques. ELISA and HPLC gave equivalent results while microbiological agar diffusion assays gave overestimated results. (Sachetelli et al, 1998). A comparison between microbiologic assays and HPLC assay was done in determination of plasma concentration, pharmacokinetics and bioavailability of erythromycin base in plasma of foals after intravenous administration of erythromycin lactobionate and intragastric administration of erythromycin base. Pharmacokinetic data obtained by the 2 methods revealed substantial differences in results, values for area under the curve and area under first moment of the curve were substantially higher when determined by the bioassay indicating overestimation of plasma concentration. The study concluded that bioassay overestimated plasma concentration of erythromycin compared to HPLC. (Lakritz et al, 1999).

Many papers have been published involving different techniques in microbiological assays of antibiotics. Rippere, (1979) explained some principles of microbiological turbidimetric

1.3.3. Purpose of the assay:
While the need to determine the potency may almost always be ultimately related to health services, the immediate purpose of particular assays may vary. The type and the design of assay used may vary according to this purpose.

In research and development on new substances, samples ranging through fermentation broths, crude extracts, and partially purified substances may be assayed using several test organisms. Chromatographic techniques such as the bioautograph may supplement the conventional potency comparisons. Economic production of antibiotics and some vitamins that are made by a fermentation process necessitates process control procedures, which are often microbial. Active ingredient levels are relatively high and so sensitivity is not a requirement for the assay. Speed is probably important. In contrast, studies of absorption and excretion rate of an antibiotic by animals and human need more sensitivity than in mass production because of low concentration of the antibiotic. Rapid and sensitive methods are needed for toxic antibiotics like gentamicin in body fluids during the course of clinical treatments to control the dose. Also bioavailability studies of antibiotics and determination of their metabolites after their administration are always tested by biological methods. For assessment of the quality of the finished refined active substance, neither speed nor sensitivity is important. A reliable estimate of potency, however, is necessary and so precise and bias-free assay procedure must be employed. Similar considerations apply in the batch control and long term stability testing of pharmaceutical dosage form. (Hewitt, 1977)

1.3.4. Basic techniques and principles:
The two commonly used methods of microbiological assay will be referred to as the plate (or agar diffusion) method and the tube method. The basis of both methods is the quantitative comparison of the effect of two substances on the growth of a suitable microorganism in a nutrient medium. The two substances are a standard and a sample whose potency is to be determined. The effect may be to inhibit the growth, as in the case of antibiotic, or to promote the growth, as the case of vitamin and amino acids (Hewitt, 1977).

1.3.4.1. The plate assay:
Nutrient agar is melted and its temperature reduced to 48°C. A small volume of suspension of sensitive organism is added by pipette and gently but well mixed to give a uniform dispersion in the agar medium. A suitable volume (15-20 ml) of the seeded agar is pipetted in petri dishes to give a layer of uniform thickness (about 3-5 mm). After solidification, the agar plates are ready for use and may be refrigerated until required (Hewitt, 1977).
Two or more concentrations from both standard and test samples are applied to a reservoir at appropriately spaced positions on the plate. These positions may be in accordance with randomized pattern for each plate in a set comparing on assay. The reservoir is made either by removing small cylinders of agar using cork borer or 8 cm stainless steel punch. Specially designed stainless steel cylinders placed on the surface of the agar can also be used as reservoir. Small filter paper discs or fish spine beads can be used after dipping them in the test solution and surplus liquid is drained and then placed on the surface of the agar (Hewitt, 1977).

For the first two procedures, a standard volume of test is added to each reservoir. The solution is allowed to diffuse into the agar at room temperature or lower for an hour or perhaps more, and then the plates are incubated, usually overnight. (Hewitt, 1977)

After incubation, clear zones surround the points of application of the antibiotic, whereas in other parts of the plate growth of the microorganism causes turbidity. Zones boundaries are usually clearly defined, although the sharpness of definition varies according to test organism, the density of the inoculum and the antibiotic. (Hewitt, 1977)

Inhibition zone diameters are measured. The relationship between mean response (zone diameter) to each test solution and the concentration of that test solution is the quantitative basis of the assay. (Hewitt, 1977)

**1.3.4.2. The tube assay:**

A series of concentrations of the test standard solution are prepared, as well as one or more solutions of sample within the same concentration range as the standard. On one series of tubes 1 ml of each solution is added to a separate test tube. This is followed by 9 ml of a nutrient medium inoculated with a suspension of a sensitive organism. Usually two or more series of tubes are included in each assay. The tubes are incubated for about 4 hours; then the growth is stopped in all tubes at the same time by immersion in water bath at 80°C. The growth of the organism is estimated by the turbidity measured in a suitable photometer. The mean inhibition of growth corresponding to each test solution in the set is the basis of calculation of potencies. Lower turbidity corresponds to higher concentration of antibiotic.

Despite of widely differing techniques, these two methods have common basis in that they depend on the following principles:

1. Comparison of a sample of unknown potency with a standard substance of known defined activity.
2. Measurement of the inhibiting effect on the multiplication of the test organism.
3. The existence of some form of quantitative relationship between concentration of active substance and response.

4. This quantitative relationship is the same for the sample as for the standard.

Both plate and tube assays of growth-promoting substance (g.p.s) such as vitamins and amino acids differ from assays of growth-inhibiting substances in that the response is opposite. In plate assays a turbid zone of exhibition contrasting with its relatively clear surroundings surrounds the point of application of the test. In the tube assay increasing doses of test solution cause increasing growth of the organism. (Hewitt, 1977)

1.3.4.3. Reference standard:

As the basis of any microbiological assay is the comparison of a sample with a reference standard, it is clear that the standard is of fundamental importance. Any difference between the standard used in different laboratories or successive occasions in the same laboratory will lead to changing biases in the estimated potency of a single sample (Hewitt, 1977).

Ideally standard reference material should be:

1. Available in sufficient quantities for all assays over a long period.
2. Completely homogeneous.
4. Qualitatively identical with substance to be tested.

5. Preferably a standard should be a single substance, but if it is a mixture, then the various components should be present in the same proportions as the product to be tested. (Hewitt, 1977).

International reference standards are available from official organizations such as the World Health Organization, the United States Pharmacopoeia, the National Formulary of the American Pharmaceutical Association and others (Kavanagh, 1972). These official standard may consist of 1 kg packed into ampoules each containing 50-150 mg. Thus the quantity available for distribution to any individual laboratory is strictly limited. For routine use each laboratory concerned with antibiotic potency testing needs for each antibiotic a “working standard” that is available in adequate quantities and whose potency has been accurately determined relative to an official standard. (Hewitt, 1977).

1.3.4. Agar diffusion method:

1.3.4.1. Introduction

Inhibition zones in inoculated agar media have been used quantitatively for many decades to demonstrate antibacterial activity. Quantitative measurements were made by Chain and his
colleagues in 1940 to monitor purification processes in the isolation of penicillin. (Hewitt, 1977).

The relationship between applied dose of antibiotic and the size of the resulting inhibition zone has remained for over thirty years as basis for the comparison of samples of unknown potency with standard reference substances. The agar diffusion method is now used extensively in quality control laboratories throughout the world. It is a method potentially capable of yielding reliable potency estimates. This potential is achieved when assay and practical techniques take into consideration the many factors other than dose of applied antibiotic that influence zone size. (Hewitt, 1977). Kavanagh (1972) has stated, “The antibiotic diffusion assay is not a biological assay. It is a physiochemical method in which a microorganism is used as indicator. Most of the observed variations are caused by neglect of the physicochemical aspects and not by biological variation (Hewitt, 1977).

Antibiotic inhibition zones have been extensively used in order to estimate the amount of antibiotics at all stages in the isolation and purification of new antibiotics, from the early crude biological products up to the assessment of the activity of pure substance. They have been used in addition for estimation of antibiotics in body fluids, animal and plant tissues and other situations where environmental conditions vary greatly (Kavanagh, 1972). A plant from Puerto Rico with antimycobacterium tuberculosis properties was tested primarily using disc diffusion method (Frame et al, 1999).

1.3.5.2. Approach to accurate assay

1.3.5.2.1. Kinds of agar assay:

Agar diffusion assay can be put in one of three categories. One is one-dimensional diffusion such as diffusion in test tubes or in capillary tubes. Another is two-dimensional diffusion such as the radial diffusion from cups in the agar. The third is three-dimensional diffusion from cylinders, beads or discs placed on the surface of the agar. (Kavanagh, 1972).

1.3.5.2.2. Special methods:

1.3.5.2.2.1. Double and triple layer plates:

The use of layers of uninoculated agar below and above the inoculated layer enables the growth layer to be made thin. This is because nutrients can diffuse from the uninoculated layer and ensures that oxygen availability is standardized. Zone size differs at different depth. The zones are made sharp, but the theory made is complicated, the further diffusion of antibiotic through the uninoculated layers and subsequent growth outside the zone may be modified. Zones are also increased in size. (Kavanagh, 1963). Five antibiotics were determined in an antibiotic assay using bilayer and monolayer plates. Difference in standard
curves and inhibitory responses obtained with monolayer and bilayer plates were <10%.
Thus, the monolayer plates are acceptable for use in analyses of antibiotics residues, with
saving in laboratory resources and time. (Reamer et al, 1998)

1.3.5.2.2.2. Surface inoculation:
This is a complicated method because the inoculum size cannot be expressed per milliliter of
agar, and the critical population for dense surface colonies or confluent growth is unknown.
Zones are larger than in deep seeded agar plates, but the critical concentration is greater.
(Kavanagh, 1972).

1.3.5.2.2.3. Heated cultures:
The killing of part of the inoculum by heat may decrease the scatter of variables, such as
sensitivity and germination times of individual cells in the populations. Sharp zones are
achieved in this way but only at the expense of the introduction of more factors to control.
(Kavanagh, 1972).

1.3.5.2.2.4. Spores:
The use of spore suspensions in place of vegetative organisms seems to give very
reproducible results. A function of germination is thus added to the initial lag period. It must
be remembered that factors affecting germination (nutrients, temperature, pretreatment) will
differ from those affecting vegetative growth. These factors must be controlled and made as
identical as possible for both standard and tests (Kavanagh, 1963).

1.3.5.3. Pouring of agar:
Zone size is a function of thickness and therefore attention must be paid to achieving a
uniform layer. Thin layers give larger zones than thick layers. Nonuniformity in the thickness
causes error in zone size. The theoretical equations indicate that percentage change in zone
size will be somewhat smaller than the percentage change in thickness of the agar responsible
for the change in zone size. The layers are about 4.4 mm thick when 25 ml of agar are put
into a 10 cm petri dish. Regardless of the type of dosing arrangement, the agar layer should
be uniform in thickness and be the same for all dishes in a test. This can be achieved only by
supporting the dish on an accurately leveled flat surface and adding accurately measured
volume of molten agar to it. (Kavanagh, 1972)

1.3.5.4. Incubation temperature:
One of the important causes of inaccurate diffusion assays is nonuniform incubation
temperature. Temperate of incubation influences both zone and the slope of the dose-
response line. A temperature difference of 0.1°C between incubation temperature of standard
and sample could make a measurable difference. Differences of (4°C-5°C) between adjacent
areas in air incubators are not uncommon. Not only terminal temperature, but also the rate of heating of the content of the plate influences zone size and the slope. Many manufacturers developed advanced incubators that make homogeneous temperature in all parts of the incubators (Kavanagh, 1972).

**1.3.5.5. Dosing:**
Dosing is an operation with an unavoidable inherent error. Current practice is to measure the dose accurately with an automatic pipette. The doses are put into a hole in the agar, in a cylinder or on a disc. The inherent error is caused by the time that elapses between the first application and the last one. The time becomes prediffusion time the moment the first dose is applied. In effect each dose has a different slope of its prescribed amount-response line. Only when agar diffusion is mechanized that all samples and standards are treated in an identical manner with respect to time and temperature will the potential potency of this assay be achieved (Kavanagh, 1972).

**1.3.5.6. Measuring zones:**

**1.3.5.6.1. Manual method:** zone diameter may be measured directly by a millimeter scale, vernier caliper, Fisher-Lilly Antibiotic Zone Reader or after projection. Only the last three devices are suitably accurate for assaying. All three could be fitted by position transducer to obtain automatic recording of zone size. For petri dishes the most common zone reader used is the Lilly antibiotic zone reader. Its scale is graduated in units of 0.2 mm. The operator estimates the reading to 0.1 mm. The resolution of measurement thus becomes 0.1 or more. Different operators have different concepts of the zone edge. For this reason one operator should measure all zones in a test to reduce measuring bias.

**1.3.5.6.2. Automatic methods:** automatic measuring of zone sizes has the theoretical advantage of eliminating human judgment and human errors. The Zone Comparator made by Technical Controls Inc. has been used for a number of years with both petri dishes and large plates. Leving (1968) had the same large plate of neomycin assay four times to the test machine. The error was less than 1%. (Kavanagh, 1972)

**1.3.5.7. Sharpness of the zone:**
The edge of the zone dividing inhibition and growth may vary from very sharp to complete gradation over a considerable distance. Difficulty in the estimation of the edge will therefore decrease the accuracy of the assay. No complete theory of sharpness and visibility of the zone edge is available (Kavanagh, 1972).
The amount of growth and its opacity after the formation of the zone prior to the zone being measured will depend on media composition and atmospheric oxygen and carbon dioxide. Diffusion of unused nutrients from the zone of inhibition may cause enhancement of growth at the edge. Microaerophilic conditions may abolish sharpness of the edge or give zones of different size. The morphology of colonial growth, the opacity of confluent growth, capsule formation, sporulation, motility and surface configuration may all affect visibility. Secondary effect of the antibiotic such as lysis may further increase zone size. The mode and intensity of illumination will affect visibility. The amount of transmitted, scattered, and reflected light from the agar surface and the bacterial growth will determine the contrast between the zone and the growth culture, (Kavanagh, 1972). Difference of sensitivities of individual organisms in the population to the antibiotic may lead to sublethal concentration of the antibiotic different from the normal (Kavanagh, 1972).

1.3.5.8. Dose-response lines:
Dose response lines are constructed by plotting on graph paper, by calculation of the best straight line through the points, and point-to-point calculation. The calculation may be manual, or by computer from data acquired on punched cards. A small computer can acquire the data directly from the zone reader, thereby eliminating the recording step. (Kavanagh, 1972)

Application of incorrect dose-response line to an assay can nullify the work of the best operator. Dose-response lines of diffusion assays are usually of one of two types:

1. In general case the square of the diameter of the zone of inhibition ($X^2$) is proportional to the logarithm of the concentration ($\log C$) of the substances causing the response.

2. If the range of the assay is short, the diameter of the zone of inhibition ($X$) is proportional to the logarithm of the concentration of the active substance ($\log C$).

The linear ($X \ vs. \ \log C$) dose-response line is an accurate representation when the largest concentration of the active ingredient is only four times the smallest concentration. Usually ($X^2 \ vs. \ \log C$) give more accurate line than ($X \ vs. \ \log C$) when the range is more than fourfold. (Kavanagh, 1972)

1.3.6. Tube assay:
Photometric methods of assay have been used since the earliest days of the penicillin program. Assays were needed at each step in the production, isolation, purification, and investigation of pharmacological activity of penicillin. Methods in which the effect of
antibiotics upon growth of a test organism in liquid is measured photometrically have several advantages. Among these are rapidity, ease of operation, objective measurement of response to the drug, absence of diffusion effect, and accuracy. The disadvantages are minor ones, including complexity of equipment and requirements that the sample not be grossly contaminated and not contribute measurable colour to the assay medium, (Kavanagh, 1963). Generally turbidimetric methods for determining the potency of antibiotics are inherently more accurate and precise compared with agar diffusion procedures. But assays conducted in liquid media are subject to degradation to much greater extent than agar diffusion methods. (Rippere, 1979)

Photometric assays are very simple in principle: the test substance is added to a suspension of the test organism in a nutrient medium, the mixture incubated, and the response of the test organism is measured. (Kavanagh, 1963). The test organism may be any organism that will give a uniform suspension, bacteria, fungi protozoa, yeast and algae. (Kavanagh, 1963).

1.3.6.1. General principle:
The basis of estimation of the potency is the relationship between applied dose of antibiotic and the resulting growth of the test organism. The nature of the response is increasing the doses of the antibiotic causing a reduction in the final numbers of cells in each tube as compared with the zero dose control. (Hewitt, 1977)

Factors which may bias the estimated potency include:
1. The possible presence of other inhibiting substances:
Perhaps other antibiotics that occur naturally in a mixture or compounded in a pharmaceutical dosage form or animal feed supplements in the case of sample but that are not present in the standard. Also inadequately cleaned tubes may be contaminated with absorbed traces of detergent or chromic acid.

2. The possible presence of nutrient substances in the sample, which increase growth rate by enriching the medium, these substances include sugars or vitamins in pharmaceutical formulation and animal feed supplements. (Hewitt, 1977).

Other factors that affect the tube assay:
1. Temperature and time of incubation:
Practical procedure should be designed to aim for the idea that all tubes be rapidly brought to the same incubation temperature at the same time and that growth be terminated in all tubes at the same time. In practice chilled inculcated broth is used so that growth does not begin until racks of tubes are immersed in the incubation bath, also the use of tubes of uniform shape and thickness of glass so heat transfer may be uniform. In addition to that use of large
capacity well-stirred incubation bath ensures that tubes are heated at the same rate regardless of position in the bath. Growth termination is done by immersion of racks in a high capacity water bath at 80°C. To balance out residual differences in incubation conditions, each rack should include tubes representing samples and standards in a randomized arrangement. (Hewitt, 1977)

2. The size and phase of growth of inoculum:
With heavy inoculums the incubation period may be as low as 3 hours. Also inoculum in logarithmic phase growth shortens the incubation period. However, use of chilled inoculums (which is in the lag phase) has the advantage that the incubation period is more easily controlled.

3. Nature of the medium:
Day to day differences in batches of apparently identical media may contribute to substantial variation. These may be difference in pH or there may be partial decomposition of essential nutrient of varying degree according to condition of autoclaving and cooling.

13.6.2. Scattering of light by the microorganism:
Light is scattered by colloidal particles of which suspensions of latex particles, sulphur and gold sols, smoke, dust and suspension of bacteria are just several of the innumerable examples. Light scattered by microbes has been the topic of several important investigations in the last decades. Most of the light scattered by microbes is at angles only slightly deviated from the light beam. The importance of angular distribution of scattered light to analytical microbiology is in its effect upon transmittance measured by usual photometric systems. (Kavanagh, 1972)

13.6.3. Instrumentation:
Responses in tube assays are most commonly measured in terms of optical properties of resulting cell suspension. The concentration of cell suspension may be estimated in two ways:

1. By measurement of the selected light band scattered by the suspended cells (nephelometry).
2. By measurement of the proportion of the selected light band that transmitted through the suspension to a detector in the light path (absorptiometry). (Hewitt, 1977).

Since the process is essentially one of scattering rather than absorbance, the former principle seem the more appropriate. Rather surprisingly, absorptiometric measurements appear to be widely used for the following reason:
1. The availability of absorptiometers that have already been purchased with colorimetric methods in mind.

2. Due to the empirical manner in which turbidimetric assay responses are so widely interpreted, consideration of the nature of the detection system has seemed irrelevant to most analysts, (Hewitt, 1977).

**1.3.6.3.1. Characteristics of photometers used in microbiological assay:**

Raw data generated by a microbiological turbidimetric assay cannot be any better than the quality of the instrument used to obtain the data, (Rippere, 1979).

In general, the more sophisticated the optical system, the greater the accuracy of measured observation will be. The accuracy and precision of the spectrophotometer increases as its bandwidth decreases, as the amount of stray light decreases, and the half angle of acceptance of light by the detector decreases. As all these factors decreases the spectrophotometer becomes more sensitive to both the presence of light scattering particles, such as bacteria, and the varying populations of such particles, (Rippere, 1979).

An investigator can use any spectrophotometer to its greatest advantage with optimal selection of operating parameters. (Rippere, 1979).

Variations on observed results also arise due to the following sources of error in optical measurements:

1. Nonuniformity of tubes (if the measurements are made in the original tubes and not by transferring to a standard cell).
2. Inadequate dispersion of the suspension.
3. Variation in time between shaking to disperse the organism and measuring its optical properties; too short a time may cause interference from minute suspended air bubbles; too long time may result in settling of organism. (Hewitt, 1977)

Any photometer may be used to measure turbidity of bacterial suspension. When used with calibration curve prepared from an organism grown under specified conditions, it may be used with considerable accuracy at some future time to estimate concentrations of the same organism grown under the same conditions. (Kavanagh, 1972)

**1.3.6.4. Automated system for turbidimetric assay:**

Automation of critical steps in the turbidimetric assay resulted in a significant increase in the accuracy and precision of assays, (Kavanagh, 1975). Turbulence after pouring the rod shaped organism suspension into the cuvette causes fluctuations in measurements until the organism has returned to its completely random orientation state, this may take up to 20 seconds. This
problem is overcome in the automated Autoturb system, which employs a flow cell for optical measurements. (Hewitt, 1977)
Connection of spectrophotometer to the computer dedicated to analytical services affected a further increase in accuracy and precision. The on-line computer had a further advantage of providing a typed report of assay results within 5 minutes after the last assay tube had been measured. (Kavanagh, 1975).

1.3.6.5. Response curves- commonly used form of expressions:
The most commonly used experimental designs for turbidimetric antibiotic assays are based on interpolation from a standard curve. Apart from log dose versus cell concentration line, a variety of empirical procedures have been proposed. Pharmacopoeias or authoritative publications have adopted some of these procedures. (Hewitt, 1977). Proposed methods include:

1. The average of transmittances is calculated and mean responses are plotted on semi log paper with doses on the log scale and responses on the arithmetic scale, then smooth curve is drawn through the points.
2. USP XVII suggests plotting the response as 100 minus percent transmittance against log dose. This is not different from the previous method. The curve has the same characteristics but the direction of slope is reversed.
3. Absorbencies are plotted against log dose. The United States Code of Federal Regulations recommends this for most assays.
4. The relative cell concentrations, which are also called the corrected absorbance, are plotted against log dose.
5. USP XVII suggests either three-dose levels of standard and sample in geometrical progression or a five dose level standard curve with one dose level of sample.
6. The European Pharmacopoeia, volume II, referring to both diffusion and turbidimetric assays requires that dose-response lines for standard and sample be shown to be both linear and parallel within a given probability levels. The method of calculation would depend on the optical apparatus used, but a suitable transformation of response should be found so as to give linearity of the dose-response lines. The pharmacopoeia does not make any specific suggestions of suitable transformations for this type of assay nor does it give an example of calculation.
7. Transmittances are plotted against dose.

1.3.7. Requirements for accurate assay:
1.3.7.1. Estimated potency of the sample:
It is fundamental in any analytical procedure that it is preferable for a sample and standard to
be weighed, extracted, and diluted by the same operator. Thus variables due to cleaning and
type of glassware, laboratory conditions, use of pipettes, burettes, and chemical glassware,
diluents, storage of the test solutions and of balances are all eliminated. (Kavanagh, 1963)

1.3.7.2. Volume and nature of sample:
Successful assay is always best performed on samples of large volume. The pipetting of 10
ml aliquots is well known to be less subject to error than that of smaller aliquot. (Kavanagh,
1963)

1.3.7.3. Number of samples received for assay:
This factor often governs all other considerations and unless control is exercised two
main sources of error will occur. A laboratory employing almost exclusively one type
design and size of plate will merely alter the daily workload per assistant to meet the
requirements. Gross daily variation should be avoided at all costs and nothing should be
allowed to interfere with the normal throughput of work with its known standard error of
assay. Personal interest tends to be lost in the face of heavy pressure of work. Work-study
considerations generally tend to break down the assay procedure whereby each assistant
helps in the entire process and again personal interest is lost. (Kavanagh, 1963)

1.3.7.4. Quality of training of assay laboratory staff:
Successful assay, whatever the precision, is based very firmly on the ability of staff to
develop personal interest, appreciation and understanding of the techniques.

1.3.8. Causes of assay errors:

1.3.8.1. Sampling:
Inhomogenity of sample is a common cause of error. Crude mixtures such as animal feed
which is an excellent example of large numbers of components with greatly different particle
sizes and particle densities. Taking large amount of such samples, grinding, and mixing may
solve this problem. Pharmaceutical powders are expected to be well mixed. Liquids can be
mixed if there is any doubt about uniformity of compositions. Proper sampling requires
understanding the problem and constant vigilance. (Kavanagh, 1972).
If the material filtered, attention must be paid to possible loss of the material removed by the
filter. Extraction also may affect the sample and lead to loss of the active ingredient.
(Kavanagh, 1972).

1.3.8.2. Bias of chemical origin:
Bias is a nonrandom error caused by an asymmetry in the assay system. When the potency is changed with dilution of the sample assayed, the bias is called drift. (Kavanagh, 1972).

A common cause of drift is the lack of identity of the standard and sample. Bacitracin and neomycin are mixtures of several related compounds and their assays are notorious for drifts. (Kavanagh, 1972). Another cause of drift is the presence of an unrelated active substance in the same sample; an example of this is penicillin in feed sample being assayed for tylosin. (Kavanagh, 1972). Also sample containing several biologically active degradation compounds may show considerable drift.

Drift caused by the dose-response line of the sample being different from that of the standard. Assay at one concentration cannot detect drift. Drift can be detected only by assaying the sample at two or more concentrations (Kavanagh, 1972). Absence of drift does not guarantee absence of a second active substance or even that the standard and sample contain the same active drug. Potencies obtained by a diffusion assay may be less susceptible to presence of a second compound. A second substance that causes severe interference in a turbidimetric assay may be innocuous in a diffusion assay. (Kavanagh, 1972).

A bias distinct from drift is the large difference in potencies of a sample when assayed by different methods employing the same or different standard. Assay of certain mixtures of antibiotics, like bacitracin and neomycin, are known to have this bias as well as drift. A bacitracin sample containing nine active ingredients may be assayed against a standard containing only four. This problem can be solved by using at least three standard preparations that are substantially different in composition, (Kavanagh, 1972).

1.3.8.3. Bias caused by operations:

Operational bias may be caused by ununiform temperature in the incubator, ununiform inoculation, incorrect design of the assay, deviation from time schedule in diffusion assay, and interaction of antibiotic and test organism in the assay tubes before start of incubation period. (Kavanagh, 1972).

Both diffusion and turbidimetric assays are sensitive to nonuniformity of final temperature and to nonuniformity of rate of rise of temperature of the plates or tubes. Different assay systems show different responses to temperature. Erythromycin and tylosin are closely related macrolides and yet assay for the former is much more sensitive to temperature inhomogenity than the latter, in one test, a standard of 1000 µ/ml measured 840 when placed at the beginning of the test and 1460 at the end. Tylosin assays incubated in the same bath gave acceptable results. (Kavanagh, 1972).
1.3.8.4. Systemic errors:

A common systemic error is the high answer caused by a decayed standard. Most of the antibiotics are unstable in aqueous solution and begin to lose when prepared. Storage in refrigerator reduces the rate of decay. Some of antibiotics like penicillin is made as needed. Others, such as neomycin and streptomycin, are stable for long time. (Kavanagh, 1972).
1.4. Antibiotics

1.4.1. Tetracycline

Tetracycline is a bacteriostatic antibiotic with a broad spectrum of activity against bacteria, and also some antiprotozoal properties. It is used particularly in the treatment of chlamydial, rickettsial, mycoplasmic, and some spirochaetal infections, as well as in infections due to gram positive and gram negative pathogens, although the emergence of resistance due to widespread use has limited its value in some cases. It has been given long term in the management of moderate to severe acne. Also it has a role in the treatment of multidrug resistant malaria and some other protozoal disease such as balantidiasis. Tetracycline is also administered as a sclerosant in the treatment of malignant effusions and pneumothorax. It is usually given by mouth, although intramuscular or intravenous administration has occasionally been used and has also been applied topically. Adverse effects include gastrointestinal disturbances, renal dysfunction (mainly in existing renal impairment), hepatotoxicity, raised intracranial pressure, skin reaction, and superinfection. Tetracycline is deposited in developing teeth and bones with detrimental results, and should be avoided in pregnant women and children. (Martindale, 1993).

1.4.1.1. Antimicrobial action and resistance of Tetracycline:

The tetracycline is mainly bacteriostatic, with a broad spectrum of antimicrobial activity including chlamydiae, mycoplasma, rickettsias, and spirochaetes, and also many aerobic and anaerobic gram-positive and gram-negative pathogenic bacteria and some protozoa. (Martindale, 1993).

1.4.1.2. Mechanism of action of Tetracycline:

Tetracyclines are taken up into sensitive bacterial cells by an active transport process. Once within the cell they bind reversibly to the 30S subunit of the ribosome, preventing the binding
of aminoacyl transfer RNA and inhibiting protein synthesis and hence cell growth. (Martindale, 1993).

**1.4.1.3. Physical properties of Tetracycline:**

Tetracycline is a yellow, odourless crystalline powder. Tetracycline hydrochloride is a yellow, hygroscopic crystalline powder, odourless with bitter taste. (The Pharmaceutical Codex, 1994).

**1.4.1.4. Stability of Tetracycline:**

Degradation pathways:

Tetracycline is rapidly inactivated in pH less than 2 and is slowly destroyed at pH 7 and above. In aqueous solutions, tetracycline and its hydrochloride degrade by epimerisation and dehydration to yield 4-epitetracycline (low antimicrobial activity) and anhydrotetracycline respectively, which subsequently dehydrate and epimerise respectively to yield the toxic product 4-epianhydrotetracycline. Epimerisation (Vej-Handen et al, 1978) is a reversible, first order reaction that occur between pH 2.5 and 6 whereas dehydration (Gieber et al, 1974 & Vej-Handen et al, 1978) at carbon 5a, occurs at very low pH. Tetracycline can also degrade by oxidation. (Leeson et al, 1969 & Vej-Handen et al, 1978)

In the form of powder and crystals, tetracycline and its hydrochloride darken in strong sunlight in a moist atmosphere. In the solid state no degradation was detected of tetracycline during storage in well-closed container at 37° and 50° for 27 months and 16 months respectively. (Dihudi et al, 1982). At 70°C a decrease in tetracycline content was noted with a slight increase in the amount of anhydrotetracycline present but there was no change in amounts of epitetracycline or epianhydrotetracycline. A t₁₀ of 19 months at 70° was predicted. Walton et al (1970) demonstrated that after storage of tetracycline hydrochloride powder at 37° and relative humidity 66%, about 10% of the initial potency was lost in 70 days.

Microbiologic analysis of samples of tetracycline hydrochloride, stored for eight weeks at 37° or 20° indicated that 9% and 7% respectively, had decomposed (Shrestha et al, 1986). However spectrophotometric analysis of the same samples, at three wavelengths, did not reveal any decomposition and was not recommended by the authors as a dependable method. Photo-oxidation of tetracycline in aerated aqueous solution was studied by measurement of oxygen uptake during irradiation with ultraviolet light (Wiebe et al, 1977). Below pH 8, oxygen uptake was insignificant. At pH 9, uptake of oxygen was accompanied by a yellow to pink, red or brown discolouration. Anhydrotetracycline, epianhydrotetracycline and peroxides were not detected. (The Pharmaceutical Codex, 1994).
1.4.2. Doxycycline

\[
\text{C}_{22}\text{H}_{24}\text{N}_{2}\text{O}_{8}\cdot\text{H}_{2}\text{O} \quad 462.5 \quad \text{Doxycycline}
\]

Fig. 2 the structural formula of Doxycycline

Doxycycline is a tetracycline derivative with bacteriostatic properties against a broad spectrum of bacteria and also some antiprotozoal properties. It is used for the treatment of chlamydial, reikittsial, mycoplasma, and spirochaetal infections as well as infections due to Gram-positive and Gram-negative pathogens, although the emergence of resistance due to widespread use of tetracyclines has limited its value in some cases. It has been given long term in the management of moderate to severe acne, and has been used for the prophylaxis of malaria. It is usually given by mouth, although intravenous administration has occasionally been used. Unlike other tetracyclines it can be given to patients with renal impairment, but its other effects including gastrointestinal disturbances skin reactions and deposition in developing bones and teeth are similar to that of tetracycline. (Martindale, 1993).

1.4.2.1. Antimicrobial action and resistance of Doxycycline:

Doxycycline is more active than tetracycline against many species including the enterococci and various anaerobes. Cross-resistance is common although some tetracycline resistant \textit{staphylococcus aureus} strains respond to doxycycline. MICs for the most sensitive organisms range from about 0.1µg to 2 µg per ml, but organisms with MICs up to about 4 µg per ml are considered sensitive and those with MICs between 4 and about 12.5 µg per ml of moderate sensitivity. (Martindale, 1993).

1.4.2.2. Physical properties of Doxycycline:

Doxycycline is a yellow crystalline powder. Doxycycline hydrochloride is a yellow crystalline, hygroscopic powder with an ethanolic odour and a bitter taste. (The Pharmaceutical Codex, 1994).

Pharmacopeial status: BP (doxycycline hydrochloride); USP (doxycycline hydrochloride, doxycycline hydrate)

1.4.2.3. Stability of Doxycycline:

1.4.2.3.1. Solutions of Doxycycline: The degradation of doxycycline in aqueous solution follows first order kinetics and is subjected in general to acid base catalysis (Pawelczyk et al
1985). It has been show to be most stable in strongly acid solutions t10% at pH 1.11 was 295 days. (The Pharmaceutical Codex, 1994)

1.4.2.3.2. Effect of relative humidity on Doxycyline:
Hard gelatin capsules containing doxycyline hydrochloride alone and in formulation with magnesium stearate, lactose, or both, were kept for up to 78 weeks at 40%, 60%, and 85% relative humidity in desiccators protected from daylight (Özol et al, 1984). The presence of magnesium stearate or lactose increased degradation at all levels of relative humidity. From formulations containing both lubricant and diluent, the loss of doxycyline at 40% RH, was 9.28% by 78 weeks, at 60% was 9.77% by 68 week, and at 85%, was 9.84% by 20 weeks (The Pharmaceutical Codex, 1994).

1.4.2.3.3. Effect of freezing on Doxycyline:
Doxycyline hydrochloride for injection (Pfizer) was stored at –20°C for up to 8 weeks following reconstitution in sterile water for injections or 5% glucose (Petrick et al, 1978), no significant decomposition was detected by either ultraviolet spectrophotometry or by microbiological turbidimetric measurements. There were no visually perceptible changes in clarity or colour of the solution and no significant changes in pH. (The Pharmaceutical Codex, 1994)

1.4.3. Erythromycin:

\[
\text{C}_{43}\text{H}_{75}\text{NO}_{16} \quad 862.06 \quad \text{Erythromycin ethyl succinate}
\]

*Fig. 3 The structural formula of Erythromycin ethyl succinate*

Erythromycin is a macrolide antibiotic with a primarily bacteriostatic action against a broad range of bacteria. It is used in condition such as diphtheria, pertussis, legionnaire’s disease, and other respiratory tract infections as being used as an alternative to penicillin in penicillin-allergic patients and as alternative to tetracycline in chlamydial infections and some other conditions. It is given by mouth, usually as its salts or esters such as the estolate, ethyl succinate, or stearate which are more stable to gastric acid and better absorbed; it is also given intravenously as the gluceptate or lactobionate. It is used topically in the treatment of
neonatal conjunctivitis, and in acne. Adverse effects include gastro-intestinal disturbances (due in part to a direct stimulant effect on the gastro intestinal tract) hepatotoxicity, transient deafness, and thrombophlebitis following intravenous administration. (Martindale, 1993).

1.4.3.1. Physical properties of Erythromycin:

1.4.3.1.1. Erythromycin ethyl succinate:
A white or slight yellow odourless or almost odourless hygroscopic crystalline powder. BP limits not less than 780 units per mcg and the USP specifies a potency equivalent to not less than 765 µg of erythromycin per mg both calculated on anhydrous basis. (Martindale, 1993).

1.4.3.2. Antimicrobial action of Erythromycin:
Erythromycin is a macrolide antibiotic with a broad and essentially bacteriostatic action against many Gram-positive and to lesser extent some Gram-negative bacteria as well as other organisms including mycoplasma, spirochaetes, chlamydiae, and richettsiae. (Martindale, 1993).

1.4.3.3. Mechanism of actions of Erythromycin:
Erythromycin and other macrolides bind reversibly to the 50S subunit of the ribosome, resulting in blockade of the transpeptidation or translocation, inhibition of protein synthesis and hence inhibition of cell growth. (Martindale, 1993).

1.4.3.4. Stability of Erythromycin:
The stability of erythromycin base in aqueous solution is affected by pH. Maximum stability occurs in the pH range 7 to 7.5. Decomposition in both acid and alkaline media follows first order kinetics. The activation energy for the hydrolysis of erythromycin at pH 7.0 has been reported to be 77.8 kJ/mol. Following treatment with weak acid (glacial acetic acid), erythromycin6,9hemiketal was produced and further treatment with a stronger acid (methanolic acetic acid ) yielded the spiroketal anhydroerythromycin. Treatment with strong acid (hydrochloric acid pH 3, 30 minutes) directly yields spiroketal form following the loss of one molecule of water from the active erythromycin. The reaction appeared to be irreversible. Further acid treatment of anhydroerythromycin produced erythralosamine and cladinose. Greater instability was demonstrated in acid media than in alkaline media. In an extensive review of the properties and degradation of erythromycin, Flynn (1954) reported that, in alkaline media, erythromycin yielded an unidentified Zwitterionic product that contained one extra molecule of water compared with the base, and had an acidic group that had pkₐ of 4.3 and a basic group of pkₐ 9.1. A 2,2-disubstituted-1,3- diketone was also suggested to be a possible degradation product. Workers in Belgium identified the degradation products of erythromycin (at pH values greater than 8) as pseudoerythromycin enole ether and
pseudoerythromycin hemiketal. Erythromycin, in solid state and in solution (pH 4 and pH 8), is photostable. (The Pharmaceutical Codex, 1994)

**1.4.3.4.1. Effect of freezing and thawing on Erythromycin:**
The stability of erythromycin as lactobionate infusions (Abbott) was not affected by storage for 12 months at –20°C, followed by microwave thawing. The infusion solutions remained chemically and physically stable even when thawed after six months and subjected to three freeze-thaw cycles. (Swell *et al*, 1991)

**1.4.4. Gentamicin:**

![Gentamicin sulphate](image)

Gentamicin sulphate

Gentamicin is an aminoglycoside antibiotic that is active against many strains of gram negative bacteria, including pseudomonas, and also against many *Staphylococcus aureus* strains. *Enterococci* and *streptococci* are insensitive to gentamicin alone, but it exhibits synergy with beta lactam against these organisms. It is not absorbed by mouth and is usually given intramuscularly or intravenously in severe systemic Gram-negative infections, often in combination with another agent such as a beta lactam. The main adverse effects are ototoxicity and monitoring of plasma concentrations is important to avoid toxic concentrations. Other adverse effects include neuromuscular blockade, hypersensitivity blood dyscrasias and central and peripheral neurotoxicity. (Martindale, 1993).

**1.4.4.1. Physical properties of Gentamicin:**
Gentamicin is a white, amorphous powder. Gentamicin sulphate is a white or almost white powder; odourless. (The Pharmaceutical Codex, 1994).

**1.4.4.2. Antimicrobial action and resistance of Gentamicin:**
Gentamicin is an aminoglycoside antibiotic and has a bactericidal action against many gram-negative aerobes and against some strains of staphylococci. (Martindale, 1993).
1.4.4.3. Mechanism of action of Gentamicin:
Aminoglycosides are taken up by an active transport process, which is inhibited, in anaerobic, acidic, or hyperosmolar environments. Within the cell they bind to the 30S, and to some extent to the 50S subunits of the bacterial ribosome, inhibiting protein synthesis and generating errors in the translation of the genetic code. The manner in which cell death is brought about is imperfectly understood, and other mechanisms may contribute, including effect on membrane permeability. (Martindale, 1993).

1.4.4.4. Stability of Gentamicin:
Gentamicin sulphate exhibits good stability as a raw material and in formulated products. In moderately acidic to strongly alkaline aqueous solutions it is chemically stable and shows little decomposition in boiling aqueous buffers (pH 2-14). Decomposition can be induced by gamma irradiation. Freeman et al (1979) obtained good recoveries of gentamicin from spiked injection vehicle subjected to adverse treatments (for example 20 days at 85°). Injectable and topical preparation degraded by less than 10% following storage for 2.5 to 3 years at 37°. Gentamicin sulphate in aqueous solutions is described as moderately oxygen sensitive, (Chrai, et al, 1977) commercial injections protected from oxygen by gas barrier and by inclusion of an antioxidant. (The Pharmaceutical Codex, 1994).
1.5. Aim of Work

Biological assays are known methods of analysis used for the assay of many pharmaceutical substances. The microbial assay methods are usually used for the assay of antibiotics; these methods are affected by many factors and need many precautions in order to get reliable results. This study aims to practice the different methods of microbial assay and to manage the different techniques used in this field. Since Sudan is large country and has many climatic zones and drugs are transported to different parts of the Sudan and stored in different places most of them deviate the ideal storage conditions of drugs, there must be studies that confirm that the drug are stable enough to withstand the drastic conditions of transport and storage in the Sudan. This study is also aims to detect the effect of the drastic storage conditions (High temperature and high humidity) on the stability of the formulations of doxycycline, tetracycline, gentamicin and erythromycin with regard to physical appearance and the content of active ingredients. The microbial methods of analysis are used to detect the content of the four antibiotic used in the study.
2. Experimental Work

2.1. Materials

2.1.1. Microorganisms

- *Bacillus cereus* NCTC No. 10320
- *Bacillus pumilus* ATCC No. 8241
- *Staphylococcus aureus* ATCC No. 9144

2.1.2. Chemicals

- Peptone (OXOID)
- Pancreatic digest of casein (OXOID)
- Yeast extract (OXOID)
- Beef extract (OXOID)
- Agar No. 1 (OXOID)
- Dibasic potassium phosphate (Analar) (BDH)
- Monobasic potassium phosphate (Analar) (BDH)
- Sodium hydroxide (Analar) (BDH)
- Phosphoric acid (BDH)
- Hydrochloric acid (BDH)
- Methanol (BDH)
- Manganous sulphate (BDH)
- Glucose monohydrate (OXOID)

2.1.3. Instruments

- Balance (Mettler AE 240)
- pH meter (Hanna Instrument HI 8417)
- Autoclave (Astell Laboratory services)
- Oven (Fisher Isotemp 500 series)
- Oven (nuve FN 400)
- Stability Cabinet (EHRET)
- Incubator (Gallenkamp Economy Incubator size 2)
- Zone Reader (Fisher Scientific)
- Hot Plate (STUART Scientific Hot plate SHI)
- Water Bath (nüve SB 401)
- Laminar Flow (Envirco)
• Spectrophotometer (Perkin Elmer uv/vis Lamda 2S)
• Disintegration Apparatus (SOFTAX DT3)
• Dissolution Apparatus (Erweka DT6)
• Automatic Pipette (Brownes CK 15412)
• Shaker (Vortex Genie 2)
• Shaker (Edmudn Bhler KS 10)
• Refrigerator (White-Westinghouse)
• Hygrometer (Casella London Ltd.)

2.1.4. Media:
Medium No.1 (USPXXIII), A (BP93), Cm1 (IP Vol. 1)
Peptone 6.0g
Pancreatic digest of casein 4g
Yeast extract 3g
Beef extract 1.5g
Dextrose 1g
Agar 15g
Water 1000ml
pH after sterilization 6.6 ± 0.1

Medium No.11 (USPXXIII)
Peptone 6.0g
Pancreatic digest of casein 4g
Yeast extract 3g
Beef extract 1.5g
Dextrose 1g
Agar 15g
Water 1000ml
pH after sterilization 8 ± 0.1

Medium No.3 (USP XXIII)
Peptone 5.0g
Yeast extract 1.5
Beef extract 1.5g
Sodium chloride 2.1.5.5g
Dextrose 1g
Dibasic potassium phosphate 2.1.68g
Monobasic potassium phosphate 1.32g
Water 1000ml
pH after sterilization 7 ± 0.05

Medium No.32 (USPXXIII)

Peptone 6.0g
Pancreatic digest of casein 4g
Yeast extract 3g
Beef extract 1.5g
Dextrose 1g
Manganese sulphate 0.3g
Agar 15g
Water 1000ml

2.1.5. Buffer Solutions:-

Buffer No.3 0.1M, pH 8 (USPXXIII)

Dibasic potassium phosphate 16.73g
Monobasic potassium phosphate 0.523g
Water To1000ml

Adjust the pH with 10N potassium hydroxide or 18N phosphoric acid

Buffer for doxycycline (BP93)

Monobasic potassium phosphate 12.1.6g
Water To1000ml
2.2. Methods

2.2.1. Storage of the Samples:

The samples used for the study were brought from local manufacturers except Gentamicin samples, which were manufactured abroad (table 5& 6). All the samples were stored in stability cabinet at temperature of 50°C and relative humidity of 75% R.H. for 6 months. For tablets and capsules 250 tablets were stored, for ampoules 50 ampoules and for suspensions 25 bottles were used.

For each drug, some of the samples were removed each month for the first 3 months and in the 6th month, and analysed. For tablets and capsules about 40 to 50 tablets, were taken for the analysis, for ampoules about 10 ampoules and for suspension 5 bottles. Biological analysis, detection of physical appearance and some other tests were done. Each group of samples was labeled with its date of analysis.

2.2.2. Preparation of stability cabinet:

An oven (Fisher Isotemp 500 series) was used as stability chamber. Its temperature was adjusted at 50°C. the humidity was maintained by saturated sodium chloride solution contained in a large dish at the bottom of the oven. The relative humidity was measured by hygrometer (Casella London Ltd.) and the temperature and humidity were recorded regularly.

2.2.3. Preparation of Test Organism (IP,Vol):

2.2.3.1. Bacillus cereus and Bacillus pumilus

The test organism (Bacillus cereus or Bacillus pumilus) was grown on the surface of 250 ml slant of medium Cm1 to which 0.3 g/litre of manganese sulphate were added (Antibiotic Medium No. 32 (USP)) for seven days in an incubator (Gallenkamp Economy Incubator size 2) at 37°C. The growth, which consisted mainly of spores, was washed with about 50 ml of saline and kept in well-closed sterile 100 ml glass bottle. Then the suspension of the spores was heated at 70° in water bath to kill all vegetative cells. The suspension was stored at 4° in the refrigerator.

2.2.3.2. Staphylococcus aureus

The test organism (Staphylococcus aureus) was grown on the surface of 14 ml slant of medium Cm1 (Antibiotic medium No. 1 (USP) for 24 hours in an incubator (Gallenkamp Economy Incubator size 2) at 37°C. The growth was then washed with 5 ml of saline and diluted to prepare a suspension having a transmittance of about 25% at 580 nm using spectrophotometer (Perkin Elmer lamda 2)
2.2.4. Preparation of the Culture Media:

2.2.4.1. Antibiotic Medium No. 1 (A, pH 6.6, Cm1)

The dehydrated medium [Antibiotic Medium No. 1 (OXOID)] was reconstituted according to the instructions of the manufacturer and sterilized by autoclaving at 121° for 15 min. and stored in the refrigerator.

2.2.4.2. Antibiotic Medium No. 11(A, pH 8)

The dehydrated medium [Antibiotic Medium No. 1 (OXOID)] was reconstituted according to the instructions of the manufacturer and, the pH of the medium was adjusted to 8 with few drops of saturated NaOH solution, using pH paper. Then the medium was sterilized by autoclaving at 121° for 15 min. and stored in the refrigerator.

2.2.4.3. Antibiotic Medium No. 32

The needed amount of the dehydrated medium [Antibiotic Medium No.1 (OXOID)] was weighed and a weight equivalent to 0.3 g/ litre of manganese sulphate was added and the media was reconstituted according to the instructions of the manufacturer and sterilized by autoclaving at 121° for 15 min. and stored in the refrigerator.

2.2.4.4. Antibiotic medium No.3

On the day of analysis, the dehydrated medium [Antibiotic Medium No. 3 (OXOID)] was reconstituted according to the instructions of the manufacturer and sterilized by autoclaving at 121° for 15 min.

2.2.5. Preparation of The Inoculum:

2.2.5.1. Preparation of the Inoculum used for the assay of doxycycline, gentamicin, and erythromycin:

On the day of analysis the medium was melted and inoculated with about 1% v/v with the suspension of the test organism.

2.2.5.2. Preparation of the Inoculum used for the assay of tetracycline

The freshly prepared medium was inoculated with about 1% v/v of the suspension of the test organism.

2.2.6. Preparation of the Buffers and Other Solutions:

4.6.1. Buffer NO. 3 (USP 23):

An amount of 16.73 g of dibasic potassium phosphate and 0.523 g of monobasic potassium phosphate were weighed and dissolved in 1 litre volumetric flask and completed to volume. Using pH meter (Hanna Instrument HI 8417), the pH of the solution was adjusted to 8 ± 0.1 using few drops of concentrated phosphoric acid.
2.2.6.2. Buffer solution used for assay of doxycycline (BP 93):
An amount of 13.6 g of monobasic potassium phosphate (KH₂SO₄) powder was weighed and dissolved in 1 litre of water and completed to volume. The solution was divided into suitable containers and sterilized by autoclaving for 15 min at 121°C.

2.2.6.3. 0.1N HCl (BP 93):
A volume of 8.5 ml of concentrated HCl (37%) was diluted to 1000 ml in 1 litre volumetric flask.

2.2.7. Sterilization of Glassware and Other Tools:
All the glassware and stainless-steel cylinders were sterilized by heating in an oven (nüve FN 400) at 180°C for 2 hours. The plastic tips of the automatic pipettes were sterilized by autoclaving at 121°C for 15 min.

2.2.8. Preparation of the Standards:

2.2.8.1. Preparation of Doxycycline Standard:
A weight of the standard doxycycline HCl equivalent to 25 mg doxycycline was dissolved in 0.1N HCl in 25 ml volumetric flask and completed to volume. 5 ml of this solution was further diluted to 50 ml in volumetric flask using the phosphate buffer (1.36% KH₂SO₄ solution). From the last solution, in 3 different 50 ml volumetric flasks, 0.25, 0.5, and 1.0 ml were taken and completed to volume with the buffer to gain three serial dilutions of 0.5µg/ml (S₁), 1µg/ml (S₂) and 2µg/ml (S₃) of doxycycline.

2.2.8.2. Preparation of Gentamicin Standard:
A weight of the standard gentamicin sulphate equivalent to 25 mg gentamicin was dissolved in the phosphate buffer in 25 ml volumetric flask and completed to volume. From this solution, in 3 different 50 ml volumetric flasks, 0.25, 0.5, and 1.0 ml were taken and completed to volume with the buffer to gain three serial dilutions of 5.0µg/ml (S₁), 10.0 µg/ml (S₂) and 20.0 µg/ml (S₃) of gentamicin.

2.2.8.3. Preparation of Erythromycin Standard:
A weight of the standard erythromycin ethyl succinate equivalent to 25 mg erythromycin was dissolved in methanol in 25 ml volumetric flask and completed to volume. From this solution, in 3 different 50 ml volumetric flasks, 0.25, 0.5, and 1.0 ml were taken and completed to volume with the phosphate buffer to gain three serial dilutions of 5.0 µg/ml (S₁), 10.0 µg/ml (S₂) and 20.0 µg/ml (S₃) of erythromycin.

2.2.8.4. Preparation of Tetracycline Standard:
A weight of the standard tetracycline HCl equivalent to 25 mg was dissolved in 0.1N HCl in
25 ml volumetric flask and completed to volume. 5 ml of this solution was further diluted to 100 ml in volumetric flask. From this solution, in 5 different 100 ml volumetric flasks, 0.213, 0.32, 0.48, 0.72 and 1.16 ml were taken and completed to volume with sterile water to gain five serial dilutions of 0.106 µg/ml (S₁), 0.16 µg/ml (S₂), 0.24 µg/ml (S₃), 0.36 µg/ml (S₄) and 0.58 µg/ml (S₅) of tetracycline respectively.

2.2.9. Preparation of the Samples:

2.2.9.1. Preparation of the sample of Doxycycline tablets:
10 tablets from the sample were weight and ground. From the powdered tablets, a weight assumed to be equivalent to 25 mg of doxycline (according to the label) was dissolved in 0.1N HCl in 25 ml volumetric flask, completed to volume and filtered. 5 ml of the filtrate was further diluted using the phosphate buffer (1.36% KH₂SO₄ solution) to 50 ml in volumetric flask. From the last solution, in 3 different 50 ml volumetric flasks, 0.25, 0.5, and 1.0 ml were taken and completed to volume using the buffer to gain three serial dilutions assumed to contain 0.5 µg/ml (U₁), 1 µg/ml (U₂) and 2 µg/ml (U₃) of the sample.

2.2.9.2. Preparation of the samples of Gentamicin Injection:
The content of three ampoules was emptied in small beaker and mixed well. Volume assumed to be equivalent to 50 mg gentamicin (1.25 ml) was added to 50 ml volumetric flask and completed to volume with the phosphate buffer. From this solution, in 3 different 50 ml volumetric flasks, 0.25, 0.5, and 1.0 ml were taken and completed to volume with the phosphate buffer to gain three serial dilutions assumed to contain 5.0 µg/ml (U₁), 10.0 µg/ml (U₂) and 20.0 µg/ml (U₃) of the sample.

2.2.9.3. Preparation of the samples of Erythromycin Suspension:
The dry powder for suspension was reconstituted as the instructions of the manufacturer. Volume assumed to contain 125 mg erythromycin (5 ml) was dissolved in methanol and the volume was completed to 125 ml. From this solution, in 3 different 50 ml volumetric flasks, 0.25, 0.5, and 1.0 ml were taken and completed to volume with the phosphate buffer to gain three serial dilutions assumed to contain 5.0 µg/ml (U₁), 10.0 µg/ml (U₂) and 20.0 µg/ml (U₃) of the sample.

2.2.9.4. Preparation of the samples of Tetracycline Capsules:
Ten capsule were weighed then their contents were emptied in small beaker and mixed well, empty shells of the capsules were washed with absolute ethanol, dried and weighed. The weight of the powder content of the capsule was calculated. Weight of powder assumed to be equivalent to 25 mg tetracycline HCl (according to the label) was dissolved in 0.1N HCl in 25...
ml volumetric flask and completed to volume. Five ml of this solution was further diluted to 100ml in volumetric flask. From this solution, in 100 ml volumetric flasks, 0.48 ml were taken and completed to volume with sterile water to gain a solution of 0.24 µg/ml (U₁) of the sample.

2.2.10. Procedures:

2.2.10.1. Procedure of Agar diffusion Method:
Six suitable sterile petri dishes were placed on a levelled place (laminar flow (Envirco)). To each petri dish 22 to 25 ml of the prepared inoculum were poured carefully and allowed to dry at room temperature for 30min. After that, in each petri dish four sterile stainless-steel cylinders were placed on the surface of the solid medium at equidistances. To each cylinder, 0.2 ml of the standard or the sample was introduced by means of pipette in alternate manner.

![Fig. 5 Presentation of alternative positions of sample and standard in the petri dish](image)

Each two petri dishes contained the same degree of dilution (Duplicate) i.e. {S₁, U₁}, {S₂, U₂}, {S₃, U₃}. The petri dishes were kept at room temperature for about 2 hours and then incubated at 37° for 18 hours in a suitable incubator (Gallenkamp Economy Incubator size 2). After the incubation period, the cylinders were removed and the diameters of zones of inhibitions were measured by means of zone reader (Fisher Scientific) and the results were tabulated and the calculations were done.

2.2.10.1.1. Tabulation of the results in Agar Diffusion Method:

<table>
<thead>
<tr>
<th>S₁</th>
<th>U₁</th>
<th>S₂</th>
<th>U₂</th>
<th>S₃</th>
<th>U₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S₁*</th>
<th>U₁*</th>
<th>S₂*</th>
<th>U₂*</th>
<th>S₃*</th>
<th>U₃*</th>
</tr>
</thead>
</table>

S₁*, U₁*, S₂*, U₂*, S₃* & U₃* were the averages of the values

2.2.10.2. Procedure of Turbidimetric Method:
Twenty-four sterile tubes (20X200 mm) were placed in tube-rack. From each dilution of the
standard and the sample, 1 ml was added to each of 3 tubes. In further 6 tubes, 1 ml of sterile water was added too. In 3 of the tubes that contained the sterile water, 9 ml of the sterile media were added to make the negative control, which would be used, as the blank when reading the turbidity of the growth. Then the medium was inoculated with about 1% v/v of suspension of the test organism and 9 ml of this inoculum was added to each of the rest of the tubes that contained the standard, the sample or the sterile water. The tubes were shaken well by Votrex shaker and incubated in an incubator (Gallenkamp Economy Incubator size 2) at 37° for 4 hours. The tubes were reshaken after the first 2 hours of the incubation. At the end of the incubation period, the tubes were removed from the incubator and to each tube 0.5ml of formaldehyde was added to stop the growth. Then the transmittance of each tube was read immediately with a spectrophotometer (Perkin Elmer) using the negative control as the blank. The results are tabulated and calculations were done.

2.2.10.2.1. Tabulation of the results in Turbidimetric Method:

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>U4</th>
<th>S5</th>
<th>U1</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1*</td>
<td>S2*</td>
<td>S3*</td>
<td>S4*</td>
<td>S5*</td>
<td>U1*</td>
<td></td>
</tr>
</tbody>
</table>

Where S1*, S2*, S3*, S4*, S5* & U1* were the averages of the values

2.2.11. Calculations (Hewitt, 1977):

2.2.11.1. Calculations in Agar diffusion method:

Difference due to dose:

\[ E = 1/4\{(U_3* + S_3*) - (U_1* + S_1*)\} \]

Difference due to preparations

\[ F = 1/3\{(U_3* + U_2* + U_1*) - (S_3* + S_2* + S_1*)\} \]

Log ratio of doses

\[ I = \log 2 = 0.3010 \]

Slope:

\[ b = \frac{E}{I} = \]

Log of potency ratio (of T/S)

\[ M = \frac{F}{b} \]

Potency ratio:

\[ \text{AntiLog } M = \]

\% CONTENT = \text{Antilog } M * 100
2.2.11. 2. Calculations in Turbibidimetric method:

The mean of the values of the transmittances were plotted against the log concentration (µg/ml) of the standard and the value of the concentration of the sample was calculated from the curve.

![Log Concentration vs Transmittance](image)

**Fig. 6** Presentation of calculation of the concentration of the sample from the curve of the standard

2.2.12. Dissolution Test of Tetracycline Capsules & Doxycycline Tablets (USP XXIII):

Three capsules or tablets were put in 3 vessels containing 900 ml distilled water and the apparatus2 (ERWEKA GT6) was operated at speed of 75 rpm for 60 minutes for tetracycline capsules or 90 minutes for doxycycline tablets. At the end of the operation time, a portion of medium was filtered and quantitatively diluted to a suitable dilution and the dissolved amount of tetracycline or doxycycline was determined by measuring the maximum absorbences of the test solutions at 276 nm (for both tetracycline and doxycycline) in comparison with tetracycline reference standard or doxycycline reference standard, diluted to the same concentration as the test solutions.

2.2.13. Disintegration Test of Tetracycline Capsules & Doxycycline Tablets (USP XXIII):

One tablet or capsule was placed in each of the six tubes of the apparatus basket (SOFTAX DT3) and the apparatus was operated using water maintained at 37±2° as immersion fluid, and the capsules or tablets were observed until they all were disintegrated and their disintegration time was recorded.

2.3. Statistical Analysis:

Results of the study were obtained and statistically analysed. Results of content of active ingredients were analysed using Wilcoxon test for comparing two related samples in the non-parametric statistical analysis. The analysis was based on comparing the content of active ingredient at the zero time and its content in other months. The statistical analysis was done using SPSS version 10, tables were designed using Microsoft Word 2000, and graphs were made using Microsoft Excel 2000 and SPSS version 10.
4. Results

Table 8:
Samples used in the study and their containers

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Generic Name</th>
<th>Mfg. Date</th>
<th>Start of the Study</th>
<th>Container</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>Doxycycline hyclate 100mg/tablet</td>
<td>9/1999</td>
<td>15/12/1999</td>
<td>10 tablets packed in strips of blister and aluminum foil.</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Tetracycline HCl 250 mg/capsule</td>
<td>10/1999</td>
<td>8/2/2000</td>
<td>8 capsules in aluminum foil strip.</td>
</tr>
<tr>
<td>Sample 3</td>
<td>Tetracycline HCl 250 mg/capsule</td>
<td>3/1999</td>
<td>21/3/1999</td>
<td>8 capsules in aluminum foil strip.</td>
</tr>
<tr>
<td>Sample 4</td>
<td>Tetracycline HCl 250 mg/capsule</td>
<td>1/1999</td>
<td>21/2/1999</td>
<td>8 capsules in aluminum foil strip.</td>
</tr>
<tr>
<td>Sample 5</td>
<td>Gentamicin sulphate 80 mg/2 ml</td>
<td>5/1998</td>
<td>8/3/1999</td>
<td>2 ml amber glass ampoule</td>
</tr>
<tr>
<td>Sample 6</td>
<td>Gentamicin sulphate 80 mg /2 ml</td>
<td>9/1998</td>
<td>8/2/1999</td>
<td>2 ml colourless glass ampoule</td>
</tr>
<tr>
<td>Sample 7</td>
<td>Erythromycin Ethyl succinate 125 mg/5 ml</td>
<td>5/1998</td>
<td>16/12/1998</td>
<td>100-ml amber glass bottle well closed with metallic cover.</td>
</tr>
</tbody>
</table>
4. Discussion

4.1. Introduction

Accelerated stability testing studies are useful techniques that would enable rapid prediction of the long-term stability of the products during development stages. These studies are done by accelerating the decomposition process and extrapolating the results to normal storage conditions. It is then possible to identify the most stable and suitable formulation without resorting to the lengthy, conventional, real time testing process. Nevertheless, accelerated storage tests cannot be expected to replace the conventional stability programmes for the finally marketed product, although they do minimize the effort and expense of testing new formulations. Registration rules in Sudan give the products that pass the accelerated stability test of zone IV, a shelf life of two years. In accelerated stability testing, both chemical and physical stability of the product will be detected. Physical stability is detected by observing changes in colour and/or other physical tests like pH and disintegration time. Chemical stability is done by detecting the change in content of active ingredient and its related substances by using chemical or microbial and other biological methods. Microbial analysis is an official method of analysis and can be used in the stability studies but it has the disadvantage of that it cannot detect the decomposition compounds of the product, it can only detect the decrease in potency if the decomposition products are microbially inactive, or increase in potency if the decomposition products are microbially active. The microbial assay is the official method of analysis for Erythromycin ethyl succinate and Gentamicin in the USP and BP. Doxycycline was assayed microbially until BP 1988 but changed into HPLC in the following editions while in USP Doxycycline is assayed by HPLC since 1990, USP XXII, but in both USP and BP all the specifications and the requirements for the microbial assay are mentioned. The official method of the assay of Tetracycline in both USP and BP is HPLC but complete descriptions of the conditions of the microbial assay are mentioned in both references. Erthromycin is classified as a substance resistant to degradation and included in the WHO index of the substance resistant to degradation, while tetracycline, doxycycline, and gentamicin are classified as degradable substances and included in the WHO index of the degradable substances. (WHO, 1986).
Results of content of active ingredients in the study were statistically analysed using Wilcoxon test for comparing two related samples in the non-parametric statistical analysis. The analysis was based on comparing the content of active ingredient at the zero time and its content in other months. The results of the samples were discussed according to the method of the analysis and chemical group of the sample, regardless to their numerical order in results chapter. Samples 2, 3, & 4 were discussed collectively together because they were tetracyclines and assayed by turbidimetric method. Then sample 7 was discussed as an example of agar diffusion method, then sample 1, and lastly samples 5 & 6 discussed together because they were both gentamicin.

4.2. Tetracycline capsule: (samples 2, 3, & 4)

Tetracycline capsules developed physical changes. Tetracycline powder in Tetracycline capsules changed from yellow powder at zero time to almost black at the sixth month (tables 13, 17, 19). Darkening of the colour is one of characteristic of Tetracycline when stored in strong sunlight and moist atmosphere (The Pharmaceutical Codex, 1994). Also this phenomenon was observed in Tetracycline when stored at high temperature and high humidity. (WHO, 1986). Another physical change occurred in Sample 2, which was complete damage of the capsules at the sixth month (fig.13). This occurred due to the high humidity of the cabinet. Hard gelatin capsules contain (12-16%) water, but their water content can vary depending on the storage conditions. When humidity is low, the capsule become brittle; if stored at high humidity, the capsules become flaccid and lose their shape. (Remington, 1985)

The content of Tetracycline in all samples decreased. The decrease in all was statistically non significant. The contents of Tetracycline in sample 4 decreased to 88.8% at the sixth month, which is less than 90% (the lower limit in USP) (table 19). The contents of active ingredient in the other two samples were found to be within the limit of USP (125%-90%) (table13 & table17) but the content of Tetracycline decreased from the upper limit (121%) as in sample 3 at the zero time to lower limit at the sixth month (92%). This decrease is due to degradation of tetracycline. Degradation of Tetracycline hydrochloride and decrease in its potency is a known phenomenon of Tetracycline when stored at high humidity and high temperature (WHO, 1986). Also when Tetracycline powder was stored at 37°C and 66% relative humidity for 70 days, 10% of its initial potency was lost (Walton et al, 1970).
degradation is due to high humidity since Tetracycline powder when stored at 37°C for 27 month and at 50°C for 16 month, no degradation was detected (Dihuidi et al., 1970). At 70°C a decrease in Tetracycline was noted with slight increase in the concentration of anhydrotetracycline but no change in epianhydrotetracycline. Degradation of Tetracycline is enhanced by moisture. Although the samples were packed in laminated aluminum foil strips, they were not protected completely from the high humidity of the stability cabinet. This problem may be solved by packing the capsule in aluminum foil laminated with thicker layer of plastic. The degradation of Tetracycline is usually accompanied with an increase in the amount of anhydrotetracycline and epianhydrotetracycline but the microbial analysis can not detect these changes, it only detect the decrease in potency.

The results of dissolution test of Tetracycline capsules showed that no statistically significant changes had occurred in the dissolved amount from the capsules in sample 2 (table15). On comparison between the dissolution test, as chemical method and the biological assay, no significant difference between the results of the two methods was detected. (table16 & fig.12) The dissolution test was done using spectrophotometric method, which was not recommended as dependable method for detection of degradation of Tetracycline (The Pharmaceutical Codex, 1994). Microbiological analysis of samples of Tetracycline hydrochloride stored for eight weeks at 37°C or 20°C indicated that 9% and 7% respectively has decomposed. However, spectrophotometric analysis of the same samples at three wavelengths did not reveal any decomposition. (Shrestha et al., 1986).

Tetracycline samples were assayed using the turbidimetric method, which is inherently more accurate and more precise than agar diffusion method (Rippere, 1979). Another advantage of turbimetric method is its short incubation period (2-4 hrs), which is much less compared to that of agar diffusion method (18 hrs).

For a valid assay procedure, the response should produce linear relationship with change in concentration. An example of turbidimetric assay is the assay of Tetracycline in the second month of sample 4 whose results were illustrated in table27, fig.18, & and fig.19. The range of values, which is obtained by subtracting the lowest value from the highest value for each concentration, was calculated. The observed range, which is the ratio of the largest range to the sum of ranges, should not exceed the appropriate critical value (0.539 for 6 ranges and 3 observations) (USP,
This value was found to be 0.2957, which is acceptable and it indicates the validity of the assay. When the doses (µg/ml) of the standard were plotted against the percentage transmittance (Fig.18), the obtained graph did not show good linearity ($r = 0.975$) with an intercept of 11.469. Theoretically, the intercept will be equivalent to the zero concentration of the assay (+ ve control), but it is known that the zero control response does not form a part of the linear portion of the response line (Rippere, 1979). To calculate the potency of the sample, better linearity is needed. This was obtained by plotting log concentration against response (transmittance). The obtained curve (Fig.19) showed good linearity ($r = 0.999$). For all of the experiments done in the study, the ($r$) value was not less than 0.99 for the log concentration vs. transmittance plots. These results confirm that the turbidimetric method is an accurate method and the concentration of the sample can be easily calculated from the curve of the standard. Although the method finally gave good results, there were many technical difficulties faced during the practical work. Since all the processes were done manually, there were difficulties in reading the transmittance of the samples because the tubes containing the suspension of the microorganism were shaken first, then with the aid of pipette about 3 ml of the sample were taken into disposable plastic cuvette and the transmittance was immediately read by the spectrophotometer. This process was time consuming, and any delay would lead to sedimentation of the suspension so the transmittance would be decreased, and if the reading was taken too fast, there would be some air bubbles due to the process of shaking that may lead to decrease of the reading too. These problems would lead to tube-to tube variation. Furthermore, there was a hazard from the spillage of the contaminated suspension into the apparatus. Although formaldehyde was added, there was still some hazard of presence of living microorganisms. These problems would be easily solved by the use of an automated system, for example, flow- cell spectrophotometer or by the use of autosampler for taking the samples.

**4.3. Erythromycin suspension (sample 7).**

The suspension of Erythromycin ethylsuccinate showed a change of colour. The colour of the suspension faded from pink to almost white at the sixth month (table25). The colouring material used in the suspension was erythrosine. Fading of colour of colouring materials was found to be first order reaction (Lachman, 1980). Fading of colour usually occurs at high intensity of light (Lachman, 1980), although the suspension was packed in amber glass, fading of colour occured because amber glass
only reduce the fading of colour but does not prevent it completely. Tablets coated with D&C (yellow # 10) faded with 6.2% everyday when exposed to exaggerated intensity of light when packed in amber glass, while it faded with 38.1% when packed in flint glass.

The samples of Erythromycin suspension were assayed using agar diffusion method. In this method, the relationship between the dose of antibiotic and the size of the resulting zone of inhibition (Response) is the basis for the comparison of samples and the reference standard. Since it is usually found that the response bears a linear relation to the log of the dose level over a certain range of the dose levels, it means that the test preparation and the standard preparation should give parallel dose - response lines, if they do not, the assay in invalid. (Kavanagh, 1963). Slight deviation from parallelism is permitted due to random error.

The first month assay of Erythromycin is an example of agar diffusion method (table.28). The observed range of data was found to be less than 0.342 the mentioned value in the USP 2000. When log concentration was plotted against zone diameter (fig.20), both sample and the standard gave linear curve (r =1) and they were parallel since their slopes were equal (b=0.178) so the assay is valid and the agar diffusion is an applicable method for the assay of Erythromycin. For the other assays of Erythromycin good linearity was obtained too (r=1- 0.991). Sometimes slight deviation from parallelism was observed which was mostly due to random error arising from preparation and dilution of the solutions of samples and standards, and other factor such as the application of the solution in the stainless steel reservoir. The time between the application of the solution in the first petri dishes and other dishes may affect the size of the zone, since this period is regarded as diffusion period. In addition, there were some difficulties in reading the diameter of the zone of inhibition. The zones boundaries were uneven this is may be due to less sensitivity of the organism to erythromycin.

4.4. Doxycycline hyclate tablets (sample 1)

Darkening of colour was the major change in the physical appearance observed in the samples of Doxycycline hyclate tablets. They became almost black at the sixth month (table9). Darkening of colour occurred in the outer coating and the core tablet. The colouring agent used in the coating of tablets was D&C # 10 (yellow TS) which should be stored at temperature below 30°C in a well closed container (Pharmaceutical Excipients,1994). Colouring materials are generally affected by light
and temperature. Darkening of colouring agents follows either zero or first order reaction (Lachman, 1984). The core tablet of Doxycycline also darkened, darkening of doxycycline occurs when stored in high temperature and high humidity. (WHO, 1986). The content of Doxycycline in Doxycycline hyclate tablets also decreased gradually and showed good linearity (\( R = 0.897 \)) (fig.7). The statistical analysis of the results showed that no statistically significant change had occurred in the content of Doxycycline (table10) but on observation of the results, it was found that the potency of the samples decreased from 107% at the zero time to about 92% at sixth month. USP range of the content of Doxycycline in Doxycycline hyclate tablets is from 120% to 90%. There was clear decrease in the content of Doxycycline. Doxycycline hyclate is known to be non stable when exposed to high humidity and high temperature. (WHO, 1986). Also the presence of magnesium stearate and lactose in the composition formula increases Doxycycline degradation at high relative humidity. (Codex,1994). With reference to the registration documents, the composition formula of the tablet used in the study contains lactose, which enhances the degradation of doxycycline. This decrease of potency in sample 1 is due to the presence of lactose and high humidity. The results of dissolution test for Doxycycline tablets showed that no significant changes had occurred in the dissolved amount of Doxycycline from the tablets (table11). On comparison between the results of dissolution test, as chemical method, and that of biological assays (table12 & fig.9), no significant difference was observed between the results of the two methods.

The samples were assayed using agar diffusion method. When the log concentration and zone diameter was plotted against the zone diameter, the graphs of the sample and the standard showed good linearity (\( r=0.999-0.997 \)). Sometimes slight deviation from parallelism was observed which was mostly due to random error. The boundaries of the zone of inhibition were sharp and uniform and the zone diameters were read easily.

4.5. Gentamicin ampoules (samples 5 & 6)

Gentamicin samples showed good stability. No change of colour or other physical changes was observed (tables21 & 23). Gentamicin powder when stored at 50°C and 100% relative humidity for 30 days changed from white to cream powder, into dark brown liquid (WHO,1986) and its water content increased by more than 40% which indicates that change of colour is due to high humidity. Since our samples were
already in the form of liquid packed in sealed glass ampoules, they were not affected by the humidity.

No significant decrease in the content of active ingredient was observed in the results of gentamicin samples (tables 22 & 24). Results of Sample 5 showed the best linearity (r=0.91) (fig. 15), also there was slight increase in the content of active ingredient at the last month in sample 6 (table 26), this increase is statistically non significant and may be due to non homogeneity of the samples within the same batch. No change in the content of Gentamicin had occurred in Gentamicin injection when stored for 20 days at 85°C (Freeman et al, 1979). Injectable and topical preparations of Gentamicin degraded by less than 10% following storage for 2.5 to 3 years at 37°C (Codex, 1994). No loss of potency of Gentamicin sulphate injections when diluted from 40 mg/ml to 10 mg/ml with sodium chloride (0.9%) injection and repackaged in glass syringes and stored for 12 weeks at 4°C (Nahata et al, 1987). Gentamicin exhibits good stability in the form of raw material and different formulations (The Pharmaceutical Codex, 1994).

Samples of Gentamicin were assayed by agar diffusion method, which is the official method of assay of Erythromycin and Gentamicin in both of British Pharmacopoeia and United State Pharmacopoeia. The boundaries of the zone were sharper than those of Erythromycin, but the linearity is less than that of Erythromycin (r=1-0.98).
5. Conclusions

Conclusions drawn from the study:

- Gentamicin and erythromycin are stable when exposed to the conditions of accelerated stability studies of zone IV and according to registration rules in Sudan these formulation can be given a shelf life of two years.

- Tetracycline and Doxycycline are not stable when exposed to the conditions of the accelerated stability studies of zone IV, so milder conditions are needed for detection of their stability.

- High humidity and high temperature leads to degradation of tetracycline and Doxycycline and decrease their potencies and change of their colours.

- Aluminium foil laminated with thin layer of plastic does not protect the capsules of tetracycline from the high humidity of the accelerated stability. Aluminium foil laminated with thicker layer of the plastic can be used for the packaging of tetracycline capsules.

- The colouring material erythrosine is not stable in the conditions of zone VI so other colouring agents can be used in the formulation of erythromycin suspension.

- Biological assay is stability indicating method of analysis for the antibiotics used in the study.

- Microbial methods of analysis are time-consuming methods and expensive, also they need special types of instruments. They are affected with many factors and there is some difficulties in the control of test conditions. They cannot determine the degradation products of the tested substance so other chemical methods must be used for full detection of stability of drugs.
References

Abraham E. P., E. Chain, C. M. Fletcher, J. W. Florey, A.D. Gradner, N.G. Healthy, and M. A. Jennings, 1941, Lancet ii.; 177

Aulton, Michael E. 1991, Pharmaceutics: The Science of Dosage Form Design,

Banes , D 1968, J. Pharm. Sci. 57, 893


Bernard J; Sebastien F; DesvogmesA. 1977; J Ann Pharm Fr; 35 (9-12) 377-386; 475-488. Diffusion method applied to antiseptics: prospects and limits of the procedure

Boardman, L.H. 1949; J.Pharm. and Pharmacol., 5:934,


British Pharmacopoeia Volume II 1 December 1993, A164-69


Chen J.L., Cyr,G.N. and Langlykke, A.F. 1957; Drug & Cosmetics Ind., , 81:596.


Deimleym V 1953, Pharm and and phamacol, 5:969.


GieberB-A, Sokoloski TD, Mitscher LA, Malspeis L. 1974; J Pharm Sci 63(12):190


Groppo FC . Mattos- Filho TR, Del-Fiol FS. 2000 Sep, Biol Pharm Bull; 23(9); 1033-5. Bioassay of amoxicillin in rats.


Higuchi. R. Lachman 1955, Am. Pharm. Ass. (Sci Edn) 44, 521


Kavanagh Frederick, Analytical Microbiology Volume II 1972 Academic Press Inc. London, 4-9, 25, 47-49

Kavanagh F 1975 Aug; J Pharm Sci 64; 14011-1403. Automated system for analytical microbiology. 5.Calibration lines for antibiotics


Lightbrown Jw, Broadbridge RA IsaacsonP, Sharpe JE, Jones 1979 Mar, Analyst; 104; 201-207. Diffusion assay by an automated procedure.

Lin CC; Veals ; Korduba C; Hilbert NJ; Nomeir 1997 Dec,- A Ther Drug Monit; 19(6) 675-81. Analysis of isepamicin in plasma by radioimmunoassay, microbiological assay, and high performance liquid chromatography.


Nahata Mc, Hipple TF, Strausbaugh SD 1987, Hosp Pharm, 22:1131-3


Pascal c, Ramond B 1993 Sept, Pharmeuropa 5(3), 254-256


Stephenson, D.: 1953, J Pharm Nd Phrmacol. 5:999.


United States Pharmacopoeia 23, National Formulary 18 1 January 1995, 1690-96

United States Pharmacopoeia 24, National Formulary 19 1 January 2000, 1837-47


WHO/Pharm/86.529 1986
WHO/Pharm/94.565/rev. 1 1994


### Table 9:
**Results of Accelerated Stability Study of Sample 1**

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Appearance</th>
<th>Disintegration time</th>
<th>Dissolution test</th>
<th>%Content of Doxycycline ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time Assay</td>
<td>Yellow biconvex rounded tablets.</td>
<td>4.0 minutes</td>
<td>101.0985% ± 0.1015</td>
<td>103.035 ± 4.665</td>
</tr>
<tr>
<td>1st Month Assay</td>
<td>Dark yellow biconvex rounded tablets.</td>
<td>4.0 minutes</td>
<td>99.8995% ± 0.4295</td>
<td>96.819 ± 2.0610</td>
</tr>
<tr>
<td>2nd Month Assay</td>
<td>Dark yellow biconvex rounded tablets.</td>
<td>4.0 minutes</td>
<td>99.9145% ± 1.7655</td>
<td>98.72 ± 8.19</td>
</tr>
<tr>
<td>3rd Month Assay</td>
<td>Very dark yellow biconvex rounded tablets.</td>
<td>4.5 minutes</td>
<td>95.5335% ± .0265</td>
<td>92.675 ± 2.795</td>
</tr>
<tr>
<td>6th Month Assay</td>
<td>Almost brown biconvex rounded tablets.</td>
<td>5.0 minutes</td>
<td>95.2705% ± 9.0595</td>
<td>92.582 ± 1.696</td>
</tr>
</tbody>
</table>

### Table 10:
**Statistical Analysis of the Results of the Stability Study of Sample 1**

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Time</td>
<td>103.0350</td>
<td>2</td>
<td>6.5973</td>
<td>4.665</td>
<td>0.655</td>
<td>Non significant</td>
</tr>
<tr>
<td>First Month</td>
<td>96.8190</td>
<td>2</td>
<td>2.9147</td>
<td>2.061</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Time</td>
<td>103.0350</td>
<td>2</td>
<td>6.5973</td>
<td>4.665</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Second Month</td>
<td>98.72</td>
<td>2</td>
<td>11.5824</td>
<td>8.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Time</td>
<td>103.0350</td>
<td>2</td>
<td>6.5973</td>
<td>4.665</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Third Month</td>
<td>92.675</td>
<td>2</td>
<td>3.9527</td>
<td>2.795</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Time</td>
<td>103.0350</td>
<td>2</td>
<td>6.5973</td>
<td>4.665</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Sixth Month</td>
<td>92.582</td>
<td>2</td>
<td>2.3984</td>
<td>1.6960</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability  
Confidence Interval 95%
P>0.05 non Significant
Table 11:  
Statistical Analysis of the Results of Dissolution Test of Sample 1

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Month</td>
<td>99.6097</td>
<td>3</td>
<td>0.6607</td>
<td>0.3814</td>
<td>0.593</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>99.9723</td>
<td>3</td>
<td>1.9979</td>
<td>1.1535</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Month</td>
<td>98.0820</td>
<td>3</td>
<td>3.6320</td>
<td>2.0969</td>
<td>0.593</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>99.9723</td>
<td>3</td>
<td>1.9979</td>
<td>1.1535</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third Month</td>
<td>93.8927</td>
<td>3</td>
<td>2.8231</td>
<td>1.6299</td>
<td>0.109</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>99.9723</td>
<td>3</td>
<td>1.9979</td>
<td>1.1535</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sixth Month</td>
<td>105.809</td>
<td>3</td>
<td>20.3778</td>
<td>11.7651</td>
<td>0.593</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>99.9723</td>
<td>3</td>
<td>1.9979</td>
<td>1.1535</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability  
Confidence Interval 95%  
P>0.05 non Significant

Table 12:  
The comparison between the Results of biological assay and dissolution test of sample 1

<table>
<thead>
<tr>
<th>Pairs</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Time B</td>
<td>0.655</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Month B</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>First Month D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair</td>
<td>Month</td>
<td>Value</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>3</td>
<td>Second</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>Month B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Third</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>Month B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sixth</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>Month B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td></td>
</tr>
</tbody>
</table>

P = Probability  
Confidence Interval 95%  
P>0.05 non Significant
Table 13: 
Results of Accelerated Stability Study of Sample 2

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Appearance</th>
<th>Dissolution test</th>
<th>%Content of Tetracycline ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time Analysis</td>
<td>Yellow capsule with red cover containing yellow powder</td>
<td>04.2867± 0.6846</td>
<td>02.72± 0. 1.6355</td>
</tr>
<tr>
<td>1st Month Assay</td>
<td>Yellow capsule with red cover containing brown powder</td>
<td>7.2633± 0.1732</td>
<td>9.99± 0.815</td>
</tr>
<tr>
<td>2nd Month Assay</td>
<td>Yellow capsule with red cover containing brown powder</td>
<td>9.065± 2.3247</td>
<td>00.895± 1.005</td>
</tr>
<tr>
<td>3rd Month Assay</td>
<td>Brittle yellow capsule with red cover containing dark brown powder</td>
<td>9.449± 1.5152</td>
<td>8.74± 1.2300</td>
</tr>
<tr>
<td>6th Month Assay</td>
<td>Completely damaged capsules containing almost black powder</td>
<td>Not analyzed</td>
<td>Not analyzed</td>
</tr>
</tbody>
</table>

Table 14: 
Statistical Analysis of the Results of the Stability Study of Sample 2

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
</table>

uuuu
<table>
<thead>
<tr>
<th>Pair</th>
<th>Month</th>
<th>Value</th>
<th>df</th>
<th>Mean</th>
<th>CI Low</th>
<th>CI High</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td>First Month</td>
<td>99.9850</td>
<td>2</td>
<td>1.1526</td>
<td>0.815</td>
<td></td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td></td>
<td>Zero Time</td>
<td>102.7245</td>
<td>2</td>
<td>2.3129</td>
<td>1.6355</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td>Second Month</td>
<td>100.8950</td>
<td>2</td>
<td>1.4213</td>
<td>1.005</td>
<td></td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td></td>
<td>Zero Time</td>
<td>102.7245</td>
<td>2</td>
<td>2.3129</td>
<td>1.6355</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 3</td>
<td>Third Month</td>
<td>101.865</td>
<td>2</td>
<td>1.7395</td>
<td>1.2300</td>
<td></td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td></td>
<td>Zero Time</td>
<td>102.7245</td>
<td>2</td>
<td>2.3129</td>
<td>1.6355</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability
Confidence Interval 95%
P>0.05 non Significant
### Table 15:
Statistical Analysis of the Results of Dissolution Test of Sample 2

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Month</td>
<td>97.2633</td>
<td>3</td>
<td>0.3001</td>
<td>0.1732</td>
<td>0.109</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>104.2867</td>
<td>3</td>
<td>1.1858</td>
<td>0.6846</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Month</td>
<td>99.0650</td>
<td>3</td>
<td>4.0265</td>
<td>2.3247</td>
<td>0.109</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>104.2867</td>
<td>3</td>
<td>1.1858</td>
<td>0.6846</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third Month</td>
<td>99.4491</td>
<td>3</td>
<td>2.6244</td>
<td>1.5152</td>
<td>0.109</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>104.2867</td>
<td>3</td>
<td>1.1858</td>
<td>0.6846</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability
Confidence Interval 95%
P>0.05 non Significant

### Table 16:
The comparison between the Results of biological assay and dissolution test of sample 2
B = Biological Assay
D = Dissolution Test

<table>
<thead>
<tr>
<th>Pairs</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td></td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time B</td>
<td>0.655</td>
<td></td>
</tr>
<tr>
<td>Zero Time D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td>Non significant</td>
</tr>
<tr>
<td>First Month B</td>
<td>0.180</td>
<td></td>
</tr>
<tr>
<td>First Month D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 3</td>
<td></td>
<td>Non significant</td>
</tr>
<tr>
<td>Second Month B</td>
<td>0.655</td>
<td></td>
</tr>
<tr>
<td>Second Month D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 4</td>
<td></td>
<td>Non significant</td>
</tr>
<tr>
<td>Third Month B</td>
<td>0.180</td>
<td></td>
</tr>
<tr>
<td>Third Month D</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability
Confidence Interval 95%
P>0.05 non Significant
### Table 17: Results of Accelerated Stability Study of Sample 3

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Appearance</th>
<th>% Content of Tetracycline ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time Analysis</td>
<td>Yellow capsule with red cover containing yellow powder</td>
<td>%121.02 ± 1.896</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; Month Assay</td>
<td>Yellow capsule with red cover containing brown powder</td>
<td>%100.57 ± 1.896</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; Month Assay</td>
<td>Yellow capsule with red cover containing brown powder</td>
<td>%101.865 ± 1.8960</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt; Month Assay</td>
<td>Yellow capsule with red cover containing almost black powder</td>
<td>%92.3 ± 1.4227</td>
</tr>
</tbody>
</table>

### Table 18: Statistical Analysis of the Results of the Stability Study of Sample 3
<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Month</td>
<td>100.57</td>
<td>2</td>
<td>4.9497</td>
<td>0.500</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>121.02</td>
<td>2</td>
<td>0.311</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third Month</td>
<td>101.865</td>
<td>3</td>
<td>2.6375</td>
<td>1.896</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>121.020</td>
<td>2</td>
<td>0.311</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sixth Month</td>
<td>92.300</td>
<td>3</td>
<td>2.4419</td>
<td>1.4227</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>121.020</td>
<td>2</td>
<td>2.6813</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability  
Confidence Interval 95%  
P>0.05 non Significant
Table 19: Results of Accelerated Stability Study of Sample 4

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Appearance</th>
<th>%Content of Tetracycline ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time Analysis</td>
<td>Yellow capsule with red cover containing yellow powder</td>
<td>105.24 ± 1.2465</td>
</tr>
<tr>
<td>1st Month Assay</td>
<td>Yellow capsule with red cover containing dark yellow powder</td>
<td>101.83 ± 4.6735</td>
</tr>
<tr>
<td>2nd Month Assay</td>
<td>Yellow capsule with red cover containing</td>
<td>111.82 ± 3.1150</td>
</tr>
<tr>
<td>Pairs</td>
<td>Mean</td>
<td>N</td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
<td>----</td>
</tr>
<tr>
<td>Pair 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Month</td>
<td>101.83</td>
<td>2</td>
</tr>
<tr>
<td>Zero Time</td>
<td>105.25</td>
<td>2</td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Month</td>
<td>111.82</td>
<td>2</td>
</tr>
<tr>
<td>Zero Time</td>
<td>105.25</td>
<td>2</td>
</tr>
<tr>
<td>Pair 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third Month</td>
<td>96.96</td>
<td>2</td>
</tr>
<tr>
<td>Zero Time</td>
<td>105.25</td>
<td>2</td>
</tr>
<tr>
<td>Pair 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sixth Month</td>
<td>88.80</td>
<td>2</td>
</tr>
<tr>
<td>Zero Time</td>
<td>105.25</td>
<td>2</td>
</tr>
</tbody>
</table>

P = Probability
Confidence Interval 95%
P>0.05 non Significant
Table 21: Results of Accelerated Stability Study of Sample 5
<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Appearance</th>
<th>%Content of Gentamicin ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time</td>
<td>Colourless homogenous solution</td>
<td>%02.28± 2.4212</td>
</tr>
<tr>
<td>1st Month</td>
<td>Colourless homogenous solution.</td>
<td>%00.99± 3.3045</td>
</tr>
<tr>
<td>2nd Month Assay</td>
<td>Colourless homogenous solution.</td>
<td>%8.41±5.5199</td>
</tr>
<tr>
<td>6th Month Assay</td>
<td>Colourless homogenous solution.</td>
<td>%8.95±1.4017</td>
</tr>
</tbody>
</table>

Table 22: Statistical Analysis of the Results of the Stability Study of Sample 5

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>First Month</td>
<td>100.99</td>
<td>2</td>
<td>5.7235</td>
<td>3.3045</td>
<td>0.593</td>
</tr>
<tr>
<td></td>
<td>Zero Time</td>
<td>102.28</td>
<td>2</td>
<td>4.1936</td>
<td>2.4212</td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Second Month</td>
<td>98.41</td>
<td>2</td>
<td>9.5607</td>
<td>5.5199</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>Zero Time</td>
<td>102.28</td>
<td>2</td>
<td>4.1936</td>
<td>2.4212</td>
<td></td>
</tr>
<tr>
<td>Pair 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sixth Month</td>
<td>98.95</td>
<td>2</td>
<td>2.4279</td>
<td>1.4017</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>Zero Time</td>
<td>102.28</td>
<td>2</td>
<td>4.1936</td>
<td>2.4212</td>
<td></td>
</tr>
</tbody>
</table>

P = Probability
Confidence Interval 95%
P>0.05 non Significant
### Table 23: Results of Accelerated Stability Study of Sample 6

<table>
<thead>
<tr>
<th>Time</th>
<th>Appearance</th>
<th>%Content of Gentamicin ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time Analysis</td>
<td>Colourless homogenous solution</td>
<td>100.226 ± 1.896</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; Month Assay</td>
<td>Colourless homogenous solution</td>
<td>95.74 ± 1.79</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; Month Assay</td>
<td>Colourless homogenous solution.</td>
<td>102.44 ± 0.500</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; Month Assay</td>
<td>Colourless homogenous solution.</td>
<td>99.50 ± 2.97</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt; Month Assay</td>
<td>Colourless homogenous solution.</td>
<td>106.79 ± 1.9750</td>
</tr>
</tbody>
</table>

### Table 24: Statistical Analysis of the Results of the Stability Study of Sample 6

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1 First Month</td>
<td>95.740</td>
<td>2</td>
<td>2.5314</td>
<td>1.790</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>100.226</td>
<td>2</td>
<td>2.6813</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2 Second Month</td>
<td>102.440</td>
<td>2</td>
<td>7071.</td>
<td>0.500</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>100.226</td>
<td>2</td>
<td>2.6813</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 3 Third Month</td>
<td>99.5</td>
<td>2</td>
<td>4.2002</td>
<td>2.97</td>
<td>0.655</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>100.226</td>
<td>2</td>
<td>2.6813</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 4 Sixth Month</td>
<td>106.785</td>
<td>2</td>
<td>2.7931</td>
<td>1.975</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>100.226</td>
<td>2</td>
<td>2.6813</td>
<td>1.896</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability  
Confidence Interval 95%

---

fff
P > 0.05 non Significant
### Results of Accelerated Stability Study of Sample 7

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Appearance</th>
<th>% Content of Erythromycin ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time Analysis</td>
<td>White flavoured powder form pink homogenous suspension on addition of water.</td>
<td>106.14 ± 0.940</td>
</tr>
<tr>
<td>1st Month Assay</td>
<td>White flavoured powder form light pink homogenous suspension on addition of water.</td>
<td>105.22 ± 3.2085</td>
</tr>
<tr>
<td>2nd Month Assay</td>
<td>White flavoured powder form light pink homogenous suspension on addition of water.</td>
<td>97.62 ± 1.4252</td>
</tr>
<tr>
<td>3rd Month Assay</td>
<td>White flavoured powder form light pink homogenous suspension on addition of water.</td>
<td>103.64 ± 2.7012</td>
</tr>
<tr>
<td>6th Month Assay</td>
<td>White flavoured powder form light almost white homogenous suspension on addition of water.</td>
<td>99.58 ± 0.2805</td>
</tr>
</tbody>
</table>
Table 26: Statistical Analysis of the Results of the Stability Study of Sample 7

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Mean</th>
<th>N</th>
<th>Std Deviation</th>
<th>Std. Error Mean</th>
<th>P</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Month</td>
<td>105.2155</td>
<td>2</td>
<td>4.5375</td>
<td>3.2085</td>
<td>0.655</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>106.1400</td>
<td>2</td>
<td>1.3294</td>
<td>0.9400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Month</td>
<td>97.6187</td>
<td>2</td>
<td>2.0156</td>
<td>1.4252</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>106.1400</td>
<td>2</td>
<td>1.3294</td>
<td>0.9400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third Month</td>
<td>103.6374</td>
<td>2</td>
<td>3.8201</td>
<td>2.7012</td>
<td>0.655</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>106.1400</td>
<td>2</td>
<td>1.3294</td>
<td>0.9400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sixth Month</td>
<td>99.5805</td>
<td>2</td>
<td>0.3967</td>
<td>0.2805</td>
<td>0.180</td>
<td>Non significant</td>
</tr>
<tr>
<td>Zero Time</td>
<td>106.1400</td>
<td>2</td>
<td>1.3294</td>
<td>0.9400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = Probability
Confidence Interval 95%
P>0.05 non Significant
### Table 27:
Results of 1st month assay of sample 4

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>U1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Transmittance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>18.67</td>
<td>30</td>
<td>45.67</td>
<td>56.8</td>
<td>71.2</td>
<td>44.37</td>
</tr>
<tr>
<td>Range</td>
<td>1.5</td>
<td>2.5</td>
<td>3.4</td>
<td>5.5</td>
<td>2.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Observed Range</td>
<td>0.2957 (USP 2000 Limit &lt; 0.539)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Concentration (+ve Control)</td>
<td>13.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 28:
Results of zero time assay of sample 7

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>U1</th>
<th>S2</th>
<th>U2</th>
<th>S3</th>
<th>U3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone Diamtre</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>13.125</td>
<td>13.25</td>
<td>14.75</td>
<td>15</td>
<td>16.5</td>
<td>16.625</td>
</tr>
<tr>
<td>Range</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Observed Range</td>
<td>0.25 (USP 2000 Limit &lt; 0.342)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 18  The Standard Curve of Tetracycline (Transmittance against Concentration by turbidimetric method Using Staphylococcus aureus

\[ r = 0.975 \]

\[ a = 11.469, \quad b = 117.352 \]
Fig. 19 The Standard Curve of Tetracycline by Turbidimetric Method
Using Staphylococcus aureus as Test Organism

\[ r = 0.999 \]
\[ a = -1.215 \quad b = 0.01336 \]
Curve of Sample and Standard of Erythromycin Assayed by Agar Diffusion Method Using B. pumilus

Fig. 20
### 1st Run

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Manufacturer</th>
<th>Mfg. Date</th>
<th>Exp. Date</th>
<th>Start of the study</th>
<th>End of the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline HCl 250mg/capsule</td>
<td>Local</td>
<td>1/1999</td>
<td>1/2002</td>
<td>21/2/1999</td>
<td>21/8/1999</td>
</tr>
</tbody>
</table>

Table 5: Samples used in the 1st Run of the study

### 2nd RUN

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Manufacturer</th>
<th>Mfg. Date</th>
<th>Exp. Date</th>
<th>Start of the study</th>
<th>End of the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline Hyclate 100mg/tablet</td>
<td>Local</td>
<td>9/1999</td>
<td>9/2001</td>
<td>15/12/1999</td>
<td>15/6/2000</td>
</tr>
</tbody>
</table>

Table 6: Samples used in the 2nd Run of the study
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Micro-organism</th>
<th>Media pH &amp;</th>
<th>Initial Diluent</th>
<th>Final Diluent</th>
<th>Serial Dilution (Standard)</th>
<th>Serial Dilution Sample</th>
<th>Incubation Temperature &amp; Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycline</td>
<td><em>Bacillus cereus</em> NCTC 10,320</td>
<td>A; 6.6 (BP) Antibiotic Medium No. 1 (USP)</td>
<td>0.1 HCl</td>
<td>KH₃PO₄ 1.36% in H₂O</td>
<td>0.5, 1.0, 2.0 µg/ml</td>
<td>0.5, 1.0, &amp; 2.0 µg/ml</td>
<td>30° to 37°, 18 hrs</td>
</tr>
<tr>
<td>Erythromycin</td>
<td><em>Bacillus pumilus</em> NCTC 2841</td>
<td>A; 7.9 (BP) Antibiotic Medium No. 11 (USP)</td>
<td>Methanol</td>
<td>Phosphate Buffer pH 8 (BP) Buffer No. 3 (USP)</td>
<td>5, 10, &amp; 20 µg/ml</td>
<td>5, 10, &amp; 20 µg/ml</td>
<td>30° to 37°, 18 hrs</td>
</tr>
<tr>
<td>Gentamicin</td>
<td><em>Bacillus pumilus</em> NCTC 2841</td>
<td>A; 7.9 (BP) Antibiotic Medium No. 11 (USP)</td>
<td>Phosphate Buffer pH 8 (Buffer No. 3)</td>
<td>Phosphate Buffer pH 8 (BP) Buffer No. 3 (USP)</td>
<td>5, 10, &amp; 20 µg/ml</td>
<td>5, 10, &amp; 20 µg/ml</td>
<td>35° to 39°, 18 hrs</td>
</tr>
<tr>
<td>Tetracycline</td>
<td><em>Staphylococcus aureus</em> ATCC No. 9144</td>
<td>Antibiotic No. 3 (USP)</td>
<td>0.1 HCl</td>
<td>Water</td>
<td>0.1067, 0.16.0, 0.24, 0.36, &amp; 0.54 µg/ml</td>
<td>0.24 µg/ml</td>
<td>36° to 37.5°, (2-4) hrs</td>
</tr>
</tbody>
</table>

Table 7: Antibiotics and their test conditions including test organisms, media, solvents, serial dilutions, and incubation conditions
Fig. 17 Mean of Content of erythromycin in Sample 7 assayed by Agar Diffusion Method using *Bacillus pumilus* as Test Organism

(USPXXIII Range (150 mg -112.5 mg))