1 INTRODUCTION

1.1 PREAMBLE

1.1.1 Antibiotic Definition:
An antibacterial word is defined as the agents or active chemical substance that can destroy or kill the bacteria or inhibits the growth of bacteria. The term can be used with the term antibiotic(s); nowadays, the knowledge of the curative agents of various diseases is increased so antibiotics has are denoted a broader spectrum of antibacterial agents, including antiprotozoal, fungicidal & other compounds.¹

The meaning of antibiotic comes from the Greek anti means 'against' & bios indicate 'life' (a bacterium is a life form). The word antibiotic was discovered by S. Waksman in 1942 to denote every compound generated by a micro-organism these may oppose to the growth of other micro-organisms in high dilution. The meaning separates compounds those destroy bacteria, but are not generated by miro-organisms (for example, juice of gastric secretion & H₂O₂). The antimicrobial compounds such as the sulfonamides are also excluded from the definition. Lot of antibacterial agents are exclusively small size molecule with a Mol. Wts.< 2000 amu.²

Inhibitors of cell wall synthesis

Penicillins:
- These are antibiotics having β-lactam ring.
- The antibiotics first bind to the Pencillin binding Proteins (PBP), & inhibit transpeptidase enzymes which inhibit transpeptidation reaction, after that binding these causes cross-linking of cell wall..
- Binding to PBP also results in release of autolytic enzymes.
- In presence of β-lactam antibiotics cell wall deficient forms are produced & lead to bacterial lysis.
- Gm +ve cell wall is entirely made up of peptidoglycans, while Gm-ve cell wall is having alternating layers of lipoprotein & peptidoglycan.

1.1.2 Chemistry:

The cephalosporins are obtained from the compound Cephalosporin C which is obtained by fermentation of the mould Cephalosporium. Cefalosporins are composed of three amino acids in biosynthesis that include valine, cysteine and amino adipic acid. These amino acid form a bicyclic ring composed of β-lactam and dihydrothiazine. This bicyclic ring is known as cefem. Chemically it is 1,4-thiazabicyclo [4.3.0] octane.

The ring can be hydrolyzed to 7-amino cefalosporinic acid and it is also liable to β-lactamase enzyme that destroys the ring and inactivate the cephalosporine antibiotics.

1.1.2 Classification:

1. According to spectrum of activity:

a) **Broad spectrum** have broad range of activity including both Gram-nagative& gram positive organisms e.g. tetracyclines, chloramphenical, 3rd generation cephalosporins.

b) **Narrow spectrum** have limited antimicrobial activity, e.g., penicillin G, streptomycin, erythromycin, nafcillin etc.

2. According to chemical structure:

a) Sulfonamides & related drugs – sulfadiazine, dapsone, PAS, sulfadoxine, sulfamethoxazole, sulfisoxazole, sulfasalazaline, sulfapyridine, sulfamerazine.
b) Diaminopyrimidines – trimethoprim, pyremethamine.

c) Quinolones – Nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin, sparfl oxacin.

d) \(\beta\)-lactam antibiotics – penicillins, cephalosporins, monobatams, carbapenams.

e) Tetracyclines – oxytetracyline, doxycycline, minocycline, demeclocycline.

f) Nitrobenzene derivatives - Chloramphenicol.

g) Aminoglycosides – streptomycin, gentamicin, neomycin, amikacin, kanamycin

h) Macrolides – erythromycin, azithromycin, roxithromycin, clarithromycin

i) Polypeptides – polymyxin B, bacitracin.

j) Glycopeptides – vancomycin

k) Oxazolidinone – Linezolid

l) Nitrofuran derivatives - nitrofurantoin, furazolidone.

m) Nitroimidazoles – metronidazole, tinidazole.

n) Nicotinic acid derivatives – INH, Pyrazinamide, ethionamide.

o) Polyene antibiotics – nystatin, amphotericin B.


q) Others – rifampin, clindamycin, lincomycin, ethambutol, etc.

3. According to type of action:

a) Primarily bacteriostatic, e.g., sulfonamides, tetracyclines, erythromycin, chloramphenicol, ethambutol.

b) Primarily bactericidal – penicillin, cephalosporins, aminoglycosides, vancomycin, quinolines, isoniazid (INH), rifampin, nalidixic acid.

4. According to mechanism of action:

a) Agents that inhibit the synthesis bacterial cell wall e.g. penicillin, cephalosporin, cycloserine, vancomycin, bacitracin, imidazole group of antifungal agents like ketoconazole. etc.

b) Agents that affect cell memberane permeability : amphotericin B

c) Agents that affects ribosomes, inhibit protein synthesis in a reversible way (bacteriostatics), e.g. chloramphenicol, clindamycin & erythromycin affects 50 S ribosomes whereas tetracyclines affects 30S ribosomes.

d) Agents that affect nucleic acid metabolism:
a. Those that affect DNA dependent RNA polymerase, e.g. rifampin.
b. Those that inhibit DNA supercoiling & DNA synthesis (inhibit DNA gyrase), e.g. quinolines.
e) Agents that binds 30S ribosomal subunit, after protein synthesis in such a way that leads to cell death (bactericidal), e.g. aminoglycosides.
f) Antimetabolites block specific metabolic steps that are essential to microorganisms e.g. trimethoprim & sulfonamides.
g) Nucleic acid analogues – agents bind to viral enzymes that are essential for DNA synthesis & thus stop their replication. e.g. acyclovir……..

5. According to their source:

a) Fungi – penicillin, cefalosporins, griseovofulvin.
b) Bacteria – polymyxin B, aztreonam, colistin, bacitracin.
c) Actinomycetes – aminoglycosides, tetracyclines, macrolides, chloramphenicol.
Some fifty different cephalosporins are in clinical use or at an advanced stage of development\(^6\),\(^7\),\(^8\),\(^9\) & many attempts have been made to classify these based upon stability to p-lactamases, potency, antibacterial spectrum & pharmacological properties. The most common approach has been to divide the group into various "generations" based primarily on their antibacterial spectrum.

**Figure 1.1**: Classification of antibiotics
Cephalosporins

Beta lactams

Penicillins  Cephalosporins

First generation
Second generation

Narrow spectrum  Wider spectrum  Third generation

β-lactamase Resistant  Fourth generation

Methicillin

Nafacillin  Broad Spectrum  Antipseudomonal  other β-lactams

Oxacilline  Ampicillin  Carboxy penicillin

Cloxacillin  Amoxicillin  Ticarcillin

Flucloxacillin  Bacampicillin  Uredniopenicillin

β-lactamase Susceptible

Penicillin G  Piperacillin

Penicillin V

Monobactam  Carbapenems  β-lactamase inhibitors

Aztreonam  Impenem  Clavulanic acid

Meropenem  Sulbactum

These are similar to penicillin in pharmacologically & chemically. Also have a beta lactam ring & do inhibit bacterial cell wall synthesis.
These are classified chronologically, spectrum varies with members of different groups.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Spectrum</th>
<th>Parenteral</th>
<th>Oral</th>
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<tbody>
<tr>
<td>First</td>
<td>Staphy (Except MRSA) Strepto (not enterococci), E.coli, Proteus, Klebsella</td>
<td>Cefazoline</td>
<td>Cephalexin</td>
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<td></td>
<td></td>
<td>Cefalothin</td>
<td>Cephradine.</td>
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<td></td>
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<td></td>
<td>Cefdroxil</td>
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<tr>
<td>Second</td>
<td>As 1&lt;sup&gt;st&lt;/sup&gt; generation plus Haemophilus (β-lactamase producers)</td>
<td>Cefam&amp;ole</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cefuroxime</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; (Cephamycenes)</td>
<td>As above + anaerobes(Bacteriosides)</td>
<td>Cefoxitin</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cefotetan</td>
<td></td>
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<tr>
<td>Third</td>
<td>As 2&lt;sup&gt;nd&lt;/sup&gt; generation plus most Gm-ve bacilli (except Pseudo), Gm-ve Cocci, Nesseriae, Morresilla</td>
<td>Cefoxatime (antipeneum)</td>
<td>Cefpodoxime</td>
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<td></td>
<td></td>
<td>Ceftriaxone (antipeneum)</td>
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<td>Cefaperazone</td>
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<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; /fourth</td>
<td>As above + Peudomonas</td>
<td>Ceftazidime</td>
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<td></td>
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<td>cefepime</td>
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**1<sup>st</sup> Generation Cephalosporins**

The first generation cephalosporins are specially active against gram positive bacteria and resistant to β-lactamase, so drugs are useful against penicillin resistant bacteria specially spectrococci and methicillin resistance staphylococcus aureus (MRSA)

- Cefdroxil
- Cepharadine
- Cefalothine
- Cephaloridine
- Cefazoline

**2<sup>nd</sup> Generation Cephalosporins**

2<sup>nd</sup> generation cephalosporin has extended spectrum of activity against gram negative bacteria and also effective against anerobes. The following antibiotics are 2<sup>nd</sup> generation

- Cefam&ole
- Cefuroxime
- Cefoxitin
- Cefotetan
- Cefpodoxime
- Cefixime
- Ceftazidime
- Cefepime
- Cefclor
- Cefuroxime
- Cefprozil
- Cefoxitome
- Loracaraf
- Cefam&ole

### 3rd Generation Cephalosporins

3rd generation cephalosporins have broad spectrum of activity against both gram +ve and gram -ve baterias. These antibiotics are specially effective against complicated infections like meningitis, typhoid in emergency cases.

- cefixime
- cefotaxime
- ceftriaxone
- cefdinir
- cefditoren
- cefpodoxime
- ceftazidime
- ceftibuten
- ceftizoxime
- cefoperazone

### 4th Generation Cephalosporins
4th generation ceflosporin antibiotics are most effective against hospital-acquired infections (Psedomonas aeruginosa). These antibiotics are very effective but cost factor is important.

The fourth generation cephalosporins are:

- cefipime
- cefozopran

1) Cephalexin:

- Oral 1st generation.
- Less active against penicillase producing staphylococcus aureus & H. influenza
- Less PPB & high concentration in bile.
- Can be combined with probenecid – Prolong duration of action.
- 0.25-1 gm 6-8 hourly.

2) Cefadroxil:

- Oral 1st generation
- More sustained action at site of infection.
- 12 hourly dosing despite short half life.
- Spectrum similar to cephalexin.
- 0.5 – 1 gm BD.
- Droxyl.

3) Cefuroxime:

- It is effective against Gram negative bacteria & resistant to beta lactmase.
- Parenteral 2nd generation, cefuroxime axetil – oral.
- Highly active against PPNG & ampicillin resistant H. influenza.
- Attains high level in CSF.
- Used in meningi, pneumo & h. influenza meningitis & single dose treatment for PPNG gonorrhea.
- 0.75-1.5 gm im/iv 8 hourly.
- 250-500 mg BD cef axetil – ceftum.
4) Cefotaxime:
- prototype 3rd generation cephalosporin – parenteral.
- It is effective against Aerobic Gm –ve & some GM +ve bacilli.
- It is not effective against anaerobes, staphylococcus & pseudomonas.
- Can cross BBB.
- Used for Gm –ve meningitis, HAI, & infections in immunity compromised host
- 1-2 gm im/iv, 6-12 hrly.

5) Ceftriaxone:
- longer duration of action
- wide spectrum – resistant cases of bacterial meningitis, typhoid, complicated UTI, septicemias.
- May cause hypothrombinemia & bleeding.

6) Ceftazidine:
- Antipseudomonal cephalosporin
- Febrile neutropenia, hematological malignancies, burns, etc.
- 0.5 -2 gm im/iv 8 hourly.

7) Cefixime:
- Orally active 3rd generation.
- Active against entrobactriaceae, H. influenza, streptococcal pyogenes, streptococcal pneumonia, resistance to many β lactamase.
- Not active against staphylococcus & pseudomonas.
- Used for respiratory, biliary & urinary infections.
- 200 -400 mg BD.

8) Cefepime:
- 4th generation parenteral.
- Highly resistant to beta lactamases.
- Reserved only for severe R cases of hospital acquired pneumonia, febrile neutropenia, bacteremia, septicemia.
- 1-2 gm iv 8-12 hrly.
Adverse effects:
- Hypersensitivity reactions –, 2% incidence.
  - Anaphylaxis, bronchospam, urticaria, mucopapular rash – more common.
  - Cross reactivity to acute penicillin allergy.
- Nephrotoxicity – cephaloridine – withdrawn.
- Pain on IM injection, thrombophlebitis on i.v. administration.
- Superinfections, neutropenia & thrombocytopenia – rare.
- Diarrhea – oral cephalosporins, cefoperazone, ceftriazone.
- Ceftriazone & cefoperazone may cause

Uses
1) These are very useful alternative for penicillin allergic persons.
2) Upper respiratory tract infections & otitis media (especially by Gm-ve):
   cefotaxime, cefuroxime axetil & ceftriazone.
3) Septicemia caused by gram negative bacteria
4) UTI – Cefuroxime, Cefixime
5) Prophlaxis in gynecological, urological, surgicals, etc., (S. aureus & S. epidermidis) eg. Cefazoline.
6) H. influenzae: Ceftazidime + gentamycin – Pseudomonas meningitis.
   Cefotaxime (preferentially in neonates
7) Gonococcal infections: ceftiazone
8) Multidrug R typhoid – alternative to FQs.
9) Infections by odd organisms & R HIAs, 3rd – 4th Generation.
10) Prophylaxis & treatment of neutropenic patients.

Monobactams:
- Effective only against aerobic gram-negative organisms: enteric bacilli & H. influenza at very low concentrations.
- Resistant to gram negative & beta lactmases.
- Have no activity against Gram-positive cocci or anaerobes.
- Chief indications are – HIA originating from Urinary, biliary, GI & female genital tract.
- Lacks cross sensitivity with other beta lactams.
- 0.5-2 gm im or iv 6-12 hrly.

**Carbapenems: (Imipenem, Meropenem)**

- have the broadest bacterial coverage of the $\beta$-lactam antibiotics.
- These are used against most infections with gram positive, gram negative enteronacteriaceae, Peudomonas auruginosa, listeria & anaerobic bacteria – closterdia & bacteria fragilis.
- Only carbepenam class of $\beta$-lactam antibiotics exhibits concentration-dependent killing-maximal activity occurs at 4-5 times the minimum inhibitory concentration (MIC) of the organism
- Of all $\beta$-lactam agents, only the carbapenams have postantibiotic effect.
- Is inactivated by an enzyme present on the renal brush border, dehydropeptidase I
- Cilatatin is dehydropeptidase inhibitor administered along with imipenem to prevent subtherapeutic levels of the antibiotic in urine.
- 0.5 gm iv 6 hrly – serious HIAs, in neutropenic, AIDS & cancer patients.
1.1.3 MECHANISM OF ACTION OF β-LACTAM CONTAINING ANTIBIOTICS:

Figure 1.2: Overview of Mechanism of β-lactam antibiotics

1.1.4 Need for analytical method

Nowadays there are so many drugs introduced in the market day by day. Every drug has to be analyzed by company before formulating in the dosage forms. The many developed methods are very complex, tedious, time consuming, very costly & not ecofriendly. Many formulations have combined dosage form. For testing the active ingredients in the different pharmaceutical dosage forms, we need very simple, easy, rapid, correct, reproducible, cheap & highly précised method is required by pharmaceutical chemists. First we have to go through proper literature survey, then
there should be a no interefareas of the excipients with active component of the dosage form. In this work we mainly emphasize to find a proper, optimum method in easy way to analyze the drug.

The following reason were found in brief to analyze different newer drugs from there pharmaceutical dosage forms.:  

- there is no proper analytical method for the drugs may due to patent regulations,
- Analysis of drugs in biological fluid system is very difficult because active component is available in mcg so very sofascitcated techniques are required to find out the amount of drug.,
- The analytical method may not be official in different pharmacopeias like IP, BP & USP.
- Analytical procedure are not found suitable for the active ingredients in the form of formulation because of the interference caused by the formulation additives,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents & solvents. It may also involve cumbersome extraction & separation methods & these may not be reliable.

Analytical techniques that are generally used for drug analysis are biological & microbiological methods, radioactive procedures, physical methods & miscellaneous techniques like conventional titrimetric, gravimetric & polarimetric methods.

1.1.5CLASSIFICATION OF ANALYTICAL METHODS

1) Chemical Methods : -

a) Titrimetric Methods
   i. Involving acid base reactions
   ii. Involving precipitations
   iii. Involving redox reactions
iv. Involving complex reactions
v. Involving large cation reagents

b) **Gravimetric Methods**
i. Weighing the active ingredients after separation
ii. Weighing of the residue after ignition of the sample
iii. Precipitation & weighing of the derivatives of the active ingredients

2) **Instrumental methods**

a) **Spectroscopic techniques**
   i. UV & visible
   ii. Fluorescence & phosphorescence
   iii. Atomic emission & absorption
   iv. Infrared
   v. Raman
   vi. X-ray spectroscopy
   vii. Radiochemical techniques including activation analysis
   viii. Nuclear magnetic resonance spectroscopy
   ix. Electron spin resonance spectroscopy

b) **Electrochemical techniques**
   i. Potentiometry (pH & ion selective electrodes)
   ii. Voltammetry
   iii. Voltammetric techniques
   iv. Stripping techniques
   v. Coulometry
   vi. Electrogravimetry
   vii. Conductance techniques

c) **Chromatographic techniques**
   i. Gas chromatography (GC)
   ii. High pressure liquid chromatographic techniques

d) **Miscellaneous techniques**
i. Thermal analysis  
ii. Mass spectrometry  
iii. Kinetic techniques  
e) **Hyphenated techniques**  
   i. GC-MS (gas chromatography – mass spectrophotometry)  
   ii. ICP – MS (Inductive coupled plasma – mass spectrophotometry)  
   iii. GC-IR (gas chromatography – infrared spectroscopy)  
   iv. MS-Ms (mass – mass spectroscopy)  

1.1.6 TITRIMETRIC ANALYSIS

There are many areas in which titrimetric methods are very important. Following advantage are given below:

1) The precision (0.1%) is in very acceptable limits as compare to others.  
2) Superior analytical procedures are available for maximum drugs as compare with other drugs.  
3) When the sample throughout is small e.g. for one-off analysis, simple titrations are often preferable.  
4) There is no need of calibration of instrument as required in instrumental methods by time.  
5) The procedures are very cost effective they have unit cost for analytical methods  
6) Routine analysis of many chemical can be done with this type of methods using calibration techniques  
7) Automatic methods are availabe.  

There are, however, several disadvantages of classical titrimetric procedures. In these types of methods sensitivity is very poor for active components & these are less selective as compared with instrumental methods. Furthermore, when many determinations are carried out by analyst then instrumental methods are very much faster & often low cost than the more hardwork time cosuming-intensive titrimetric methods. Nevertheless, despite the widespread popularity of instrumental
techniques, it can be seen from the above that there is considerable scope for the use of titrimetric procedures, especially for practicing laboratory skills.

1.1.7 SPECTROPHOTOMETRIC ANALYSIS

The selective absorption of electromagnetic radiation as it passes through a solution causes the emerging beam to differ from the incident one. Spectrophotometry is based upon the measurement of decrease in the absorbance of the light by absorbing species. This decrease is due to electronic transitions within the molecule. Spectrophotometry mainly covers with the following areas of the spectrum:

1) Ultraviolet (UV) region : 200 nm to 400 nm
2) Visible region : 400 nm to 780 nm
3) Infrared region : 0.8 µm to 2.5 µm

Colourimetry covers the visible region of the spectrum.

Figure1.3: Labtronic Double Beam Spectrophotometer
1.1.8 Theory of Spectrophotometry & Colourimetry

**Beer & Lambert Law:** When electromagnetic light passes through the medium decrease in intensity of emergent light (emitted) with respect to thickness is proportional to initial intensity or we can say that intensity of electromagnetic light decrease exponentially as the thickness of medium increase arithmetically.

Mathematical expression

\[-\frac{dl}{dl} \propto I \] \hspace{1cm} \text{.................(1)} \] Where I is intensity & l is thickness

Integrate the equation (1)

\[
\log_e I = -Kl + C \] \hspace{1cm} \text{...........(2)} \] Where C is integral constant & K is absorption coefficient

At starting time I = I₀ so will be l = 0 \hspace{1cm} C = \log_e I₀

\[
\log_e I = -Kl + \log_e I₀
\]

\[
\log_e \left(\frac{I}{I₀}\right) = -Kl \text{ or } \frac{I}{I₀} = e^{-Kl} \] \hspace{1cm} [A = \log_e \left(\frac{I₀}{I}\right)]

I = I₀ e^{-Kl} \hspace{1cm} \text{.................(3) this is known as Lambert Law}

Beer applied this on solutions & gives

\[
I = I₀ e^{-Kc} \] \hspace{1cm} \text{.............(4) c is concentration of solution}

When we combine Equation (3) & (4) this known as Beer Lambert Law

\[
I = I₀ e^{-Kc} \text{ or } I = I₀ e^{-acl} \] \hspace{1cm} \text{.........(5) a is molar or specifies absorption constant}

So we can write \( A = acl \) where \( A = \log_e \left(\frac{I₀}{I}\right) \) or absorbance & \( T = I/ I₀ \) is transmittance
\[ A = \log_e \left( \frac{I_0}{I} \right) = \log_e \left( \frac{1}{T} \right) = -\log_e T = acl \quad ...........(6) \]

Relationship between molar absorption & specific absorption coefficient 
\[ \varepsilon = E^{1\% \, 1cm} \times M/10 \quad ....................(7) \]

where \( \varepsilon \) is molar absorption coefficient & \( E^{1\% \, 1cm} \) is Extinction or specific absorption coefficient or formerly optical density.

Limitation of Beer Lambert Law

(1) Solutions should be diluted (\( \mu g/ml \)).

(2) There should not be association or dissociation of ions that form complexes.

(3) The graph should be passed through the origin.

Some Important Definitions

(1) **Chromophore**: The moiety or group of atoms in the molecule that is responsible for complete absorption of electromagnetic radiation is known as Chromophore.

(2) **Auxochrome**: The group that does not absorb radiation but support the chromophore for absorption of radiations like methyl, amino, hydroxyl, alkoxy & halogen.

(3) **Bathochromic Shift** (Red Shift):- Shift to lower energy or longer wavelength.

(4) **Hypsochromic Shift** (Blue Shift):- Shift to higher energy or shorter wavelength.

(5) **Hyperchromic Shift**:- higher growth in intensity.

(6) **Hypochromic Shift**:- a go to lower in intensity.

**Transition state**: the electron absorb electromagnetic radiation & goes HOMO (highest occupied molecular orbital) to LUMO (lowest unoccupied molecular orbital). The transitions that take place are
Electronic Transitions

**K – Band**: this b& is formed when electron jumps $\pi$ to $\pi^*$ transition. The type of transition is mainly found in conjugated dienes that contain $\pi$ electrons.

**R – Band**: this b& is formed when nonbonding electron moves to antibonding $\pi$ orbital. These b&s form in carbonyl compounds. It is forbidden transition b&.

**E – Band**: this occurs due to $\pi - \pi^*$ transition in aromatic & heteroaromatic compounds due to ethylenic bond.

**B – Band**: this b& forms in benzanoid & quinoids structures.

**Source**: In ultraviolet range deuterium lamp is used as source of radiation for 200 – 400 nm. In visible region tungsten lamp is used as source of radiation for 400 – 800 nm. Xenon Arc lamp may be used as source of radiation in the range of 250 – 500 nm range.

**Tungsten lamp**: functioning similar to electric bulb. It contains tungsten filament that is heated electrically to white light.
**Hydrogen discharge lamp**: the gas is kept under high pressure. After that electric bulb is passed through the gas, nascent hydrogen molecule will be produced which emit ultraviolet light.

**Deuterium lamp**: Hydrogen gas is replaced by deuterium gas & the intensity of radiation is increased 3-5 times as compare to H$_2$ discharge lamp

**Xenon arc lamp**: Xenon gas is kept under pressure. The xenon pump passes to tungsten electrodes separately by about 8 mm when intense arc is formed the ultraviolet light is produced

**Monochromator**: -It consist of three parts 1) Entrance slit: It blocks the stray radiations or unwanted radiations 2) Dispersing element: It splits the radiations according to wavelength. It can be made of prism or grating. The prism generally made of glass, quartz or fused silica. The advantage is that wavelength do not overlap & disadvantage is that produce nonlinear dispersion

Grating: It is made of glass, quartz, aluminum or alkyl halide. These make them more efficient then prisms & produce linear dispersion. 3) Exit slit: it allows only light of required wavelength & falls on the sample.

**Detectors:**

**Photomultiplier tube**: The photomultiplier tube consists of an evacuated tube which contains photocathode & 9-16 electrodes known as dynode. When radiation falls on the metal surface of photo cathode, it emits electrons. Electron goes to 1st dynode due to attraction and plus charge. When the electrons strike the first dynode many electrons are emitted by the surface of dynode, these emitted electrons are then attracted by second dynode where similar type of electron emission takes place. This process is repeated over all the dynodes present in tube until a shower of electrons reach the collector. The number of electron reaching the collector is the measure of intensity of light falling on detector.

Instrumental effects may also cause deviations from Beer’s Law; for example photomultiplier tube is faulty, a straight line will be obtained for absorbance versus
concentration but the line will be obtained for absorbance versus concentration axis at a value other than zero. Similarly, if the cuvettes (cells) are dirty, the line will intersect the absorbance axis at a value greater than zero. Cuvettes are always matched for use in spectrophotometry.

1.1.9 Methods for One Component Analysis:

1. Use of Standard Absorptivity Value:

The absorbance of a sample solution in definite solvent is measured at selected wavelength (usually $\lambda_{\text{max}}$) & concentration of the analyte is calculated by using pre-established specific absorptivity $A_{(1\%,1\text{cm})}$ or molar absorptivity values.

$$A_{(1\%,1\text{cm})} = \frac{\text{Absorbance}}{\text{Concentration of analyte (g/100 mL)}} \quad \text{………… (L)}$$

2. Use of Calibration Graph:

A standard calibration curve is plotted using a series of standard solutions, as concentration against absorbance & concentration of sample is determined from graph.

3. Single- or Double-Point Standardization:

The single-point procedure involves the measurement of the absorbance of a sample & of a standard solution of the reference sample. Concentration of the substance in sample $s$ calculated from the proportional relationship that exists between absorbance & concentration.

$$C_{\text{test}} = \frac{A_{\text{test}} \times C_{\text{std}}}{A_{\text{std}}} \quad \text{……………………………………………… (M)}$$

$C_{\text{test}}$ & $C_{\text{std}}$ are the concentrations & $A_{\text{test}}$ & $A_{\text{std}}$ are the absorbances of the sample & the standard solutions respectively. This procedure is preferred method of assay of the substances that obey Beer's Law.
1.2 CHROMATOGRAPHY

1.2.1 Introduction

Chromatography is word derived from chromatograph which means column. Analysis of sample in column is known as chromatography. The technique is useful for the analysis of mixtures for identification purpose & to determine quantity of sample by measure through UV detector. The technique is useful as qualitative & quantitative. The chromatography came from classical column chromatography in which sample is put on the top of the packed column with silica gel or cotton & then m. phase is run through the column as gravitational process. M. Phase’ passes through stationary phase by
percolation method & it separates the mixture in different components by using gradual increase in polarity from non polar to polar solvent system.

1.2.2 History

Russian botanist Mikhail S. Tswet invented the chromatography technique first time in 1900. It is mainly used to separate the constituents of chlorophile using calcium carbonate column.

1.2.3 Chromatography Theory

Chromatography is based on two principal adsorption & partition when stationary phase is solid then adsorption & partition both principles work to separate the mixture, if both the phase M. Phase’ & stationary phase are liquid then separation is totally on partition basis. The chromatography works on plate theory & rate theory.

1.2.4 Thin Layer Chromatography (TLC)

Thin layer chromatography is based on the adsorption & partition principle. In this technique we spread a sample on thin layered glass or aluminium plate. The plate is prepared by using silica gel sultry spreading on the plate by spreader or h&. Then plates are made dried over night or in oven by heating, aluminum plates are readymade available. The sample is applied on the plate by capillary or rubber dropper. Then the plates are kept in the tank containing M. Phase’ of different solvents. The spot travels on the plate & fixed at a place. That is identified by spraying agent & \( R_f \) factor is measured by using formula. This is useful for monitoring reaction & identifying amino acids.

1.2.5 Liquid chromatography (LC)
Liquid chromatography (LC) is a separation method in which the mobile phase is liquid. Liquid chromatography can be carried out either in a column or a plane. Traditionally it is column chromatography. In this method both phases are liquid and separation is achieved by partitioning the solute sample between two immiscible liquid phases. One phase is generally hydrophilic and another is lipophilic in nature. For normal liquid chromatography, the stationary phase is polar like polyethylene glycols, while the mobile phase is non-polar like n-heptane, n-hexane & cyclohexane. In reversed-phase LC (RP-LC), the stationary phase is nonpolar like zipax & squalene while the mobile phase is polar like water, acetonitrile & methanol. Today, chromatography commonly uses very tiny packing particles and a mainly high-pressure system referred to as high pressure liquid chromatography (HPLC). To run the mobile phase, high pressure is used for HPLC. The mobile phase is run in isocratic or gradient mode, which may be binary or ternary or quaternary. The sample is injected through a micro volume rotating valve system which rotates by connecting itself to the mobile phase & sample holder. The detector is an UV Photodiode array type in HPLC.

1.3 Introduction of HPLC & Method validation

(Background, History & Criticality)

Chromatography is a useful, separation method that has utility to all science branches. Chromatography was invented by the Russian botanist at the beginning of 20th century & the name of the scientist was Mikhail Tswett. In this technique the name he choose for the method (Greek Chrome meaning “color” & graphein meaning “write”) the application of the chromatography have grown explosively in the last fifty years, owing not only to the development of several new type of chromatography techniques but also to growing needs by the scientists for better methods for characterizing complex mixtures. In all chromatography separation the sample is transported in a mobile phase it is then passed through stationary phase, which is fixed in a column or on solid surface. High pressure liquid chromatography is capable to separation of macromolecules & ionic species & labil natural products, polymeric materials & a selection of another high molecular weight polyfunctional group. The obvious way to increase the F. R.& become efficient separation is to the liquid force by positive displacement pump. This flexibility
can be completed by creating certain variations in column & by using smaller diameter & smaller surface area of column particles & by using other suitable packing structure. That is High performance liquid chromatogram. HPLC is a high speed & high resolution liquid chromatography.

& other word defines the chromatography “A method used primarily for the separation of the constituents of a sample, in which the components are disseminated amongst two phase, one of is stationary whereas other moves. The stationary phase possibly will be a solid or a liquid maintained on a solid or a gel, & filled in a column, extent as a layer or dispersed as the flicks. The M. Phase’ can be gaseous or liquid. According to mechanisms of separation, chromatographic methods are divided into two main types ; they are adsorption & partition chromatography 2.

**Adsorption chromatography**, the analytes interact with solid stationary surface & are displaced with the eluent for active site on surface.

**Partition chromatography**, results from a thermodynamic distribution between two liquid (or liquid like) phase.

### 1.3.1 Basic opinion of HPLC

High pressur liquide chromatography is essentially a highly developed form of column chromatography. In its place of a solvent being allowed to fall through a column under gravity, it is compulsory forced through a column under highest pressures of up to 6000 Psi. That was makes it much faster. High pressure liquid chromatography is a separation system using difference in distribute of the compound in to 2 phase, known as stetionary phase & mobile phase. The stetionary phase labels a thin layer produced on the surface of small subdivisions & M. phase describes the liquid flow in excess of the elements. Below a sure dynamic condition, each composition in a sample has not the same dissemination equilibrium depending on M. Phase’ is frequently provided for in to the column inlet at unbroken speed by a liquid pump. The column is a resin or stainles stel cylinder that is packed with round compact particles.
A sample was inject through a sample injector, situated close to the column inlet. The inject sample by injector moves in the column through the M. Phase’& the constituents in the sample travel during, pasing between the stationery phase &M. Phase’s. Compound shift through column first, as soon as is in M. Phase’, Compoundes that have a tendency to be dispersed in the stationary phase travel more rapidly within the column whereas compounds that tend to be dispersed in the stationary phase travel with low speed. in this technique,

1.3.2 Isocratic elution

The M. Phase’ might to be involved of a one line of solvent. In isocratic elution a sample is inserted into a known column & the mobil phas is unaffected concluded the time necessary sample modules to elute through column. The isocratic division of sample extensively varying with K’ (partetion ratio) values lengthy elution period. To directly grip samples that have together weakli recollected & powerfully recollected substance, the rates of different b& relocation have to be change for the duration of elution.

1.3.3 Gradient elution

Constant changes of the mobil phase combination for the duration of the chromatographic run are called gradient elution. The main thinks of determination of gradient elution is to motive powerfully retain componants of the combination more rapidly, but consuming the least retain components well determined.

Opening through the small content of the organic components will eluent. We agree to the least recollected components to be separated. Strongly recollected constituents will sit on the adsorbent seeming on the top of the column, or will moving very gradually.

As soon as we start to intensification an expanse of organic constituent in the eluent acetonitrile then powerfully retained components will travel faster & more rapidly, for the reason that of the constant raise of the competition for the adsarption places.
Gradient elution correspondingly increases effectiveness of the column. These determinations tend to relates zone & thin the resultant peak.

1.3.4 HPLC instrumentation

HPLC is widely held technique of analysis for the reason that it is easy to learn & expenditure & is not restricted by the volatility or stability of the sample compound.

The general instrumentation for High pressure liquid chromatography includes the follow components:

A. Solvent storage is intended for the mobile phase.
B. The mobile phase necessity is delivering to column through means of different kind of pump. To achieve separation one or the other base on small analysis time or beneath most favorable pressure, an extensive range of pressure & flow is necessary. The pump scheme need to be pulse-free or different have obstruction to keep away from generate baseline uncertainty in the detector.
C. Into the future of the partition column present may possibly, to inhibit contamination of the core column by minor particulate.
D. The separation column contain space filler wanted to achieve the wanted HPLC separation. These might to be silica’s for adsorption chromatography, merged phases for liquid-liquid chromatography, ion-exchange chromatography, gels of particular absorbency in prohibition chromatography, or some other irreplaceable protective material for a specific separation technique.
E. Detector using some category of data treatment device finishes the basic instrumentation.
1.3.5 Mobile – phase supply system

The M. Phase’ essential to be provided to the column over an extensive range of F. R. & pressure. A degasser is needed to remove to melt in solution air. Additional desirable characteristics in solvent distribution technique are the capability for producing a solvent gradient.

F. R. is usually expressed in milliliter per minute. High pressure capability is needed to overcome the resistance to fluid flow arising from the tightly packed particle (stationary phase) in the column. Therefore pumping system should be able to deliver a wide range of reproducible F. R. against back pressure in the system of up to 6000 psi (408 atm.).
However, 6000 psi (408 atm.) is a more required pressure limit. On behalf of many analytical columns only reasonable movement rates of 0.5-2 ml per min essential to be created.

1.3.6 Separation columns

Column is the more essential portion of a HPLC instrument. Columns are assembled of heavy-wall, glass lined metallic tubing or stainless steel piping to weather high pressures increase (up to 680 atm.) & the chemical phase. Maximum column lengths range from 10 to 30 cm; & fast columns are 3 to 8 cm lengthy. For exception, chromatography, columns are 50 to 100 cm long.

1.3.6.1 Quality of columns

Various HPLC separations are completed on columns through an internal diameter of 3 to 5 mm. this types of columns arrange for a good settlement between efficacy, illustration capability & the quantity of packging & solvant compulsory. Column protective material characterizes particles that is homogeneously sized & mechanically unchanged. Particle diameters remain in lying position in the range 3-5µm, irregularly up to 10µm or higher designed for preparative chromatography. The columns are categorized into various classifications depending on their carbon packing.

1.3.7 Detectors

The understanding of universal detector for HPLC has not been developed until now. Consequently it is compulsory to select a detector on the basis of the problem. Visual detectors based on UV- Visible concentration are the work hours of HPLC, establishing over 70% of the all detection technique in precede. Essentially three types of absorbance detector are obtainable: a variable wavelength detector; a fixed wavelength detector; & a scanning wavelength.
1.3.7.1 Fixed wavelength detectors

A fixed wavelength detector procedures a light cause that produces maximum light strength at one or quite a lot of discrete wavelength that is lonely by suitable filters.

1.3.7.2 Variable wavelength detector

A variable or adjustable wavelength detector is a comparatively wide-pass. Its proposals a wide choice of UV & Visible wavelength, but at an improved price.

1.3.7.3 Photo diode array (PDA) detector

On the way to achieve a real time spectrum used for each solute as it elutes, solid-state diode arrays are necessary. The diode arrays operation in comparable, at the same time observing all wavelengths.

1.3.7.4 Reflective Index detector (RID)

The reflective index (RI) detector is the only universal detector in HPLC. In this detector principle involve measuring of the change in reflective index of the column effluent passing through the flow-cell. Greater the Reflective index difference between sample &M. Phase’, the larger the imbalance will become. Thus, the sensitive will be higher for the higher difference in Reflective Index between sample &M. Phase’.

On the basis of relative polarities of stationary &M. Phase’s partition chromatography can be divided into normal-phase & reverse-phase chromatography.

1.4 Types of HPLC

1) Normal Phase Chromatography (NPC)
2) Reverse Phase Chromatography (RPC)
3) Size Exclusion Chromatography (SEC)
4) Ion Exchange Chromatography (IEC)
1.4.1 **Normal phase chromatography**

Normal-phase liquid-liquid chromatography uses a polar stationary phases and less polar mobile phases. To select an optimum mobile phase, it is best to start with a pure hydrocarbon mobile phase such as heptanes. If the sample is strongly retained, the polarity of the mobile phase should be increased, perhaps by adding small amounts of methanol or dioxane. In the normal phase mode, separation of oil-soluble vitamins, essential oils, nitrophenols, or more polar homologous series have been performed using alcohol/heptanes as the mobile phase. Column used in normal phase chromatography for chiral separation: Chiral OJ & Chiral OD.

1.4.2 **Reverse phase chromatography**

Octadecyl or octyl efficient group & a polare (mobile) movable phase, often a partially or fully aqueous mobile phase. Polare materials choose the mobile phase & elut principal. As the hydrophobic character of the solutes rises, retention rises. Generally, the lesser the polarity of the mobile (moveable) phase, the higher is its eluent strength. The elution order of the classes of compounds is reversed (thus the name reverse-phase chromatography). Hydrocarbons are retained more strongly than alcohols. Thus water is the weakest eluent. Methanol & acetonitrile are popular solvents because they have low viscosity & are readily available with excellent purity.
M. Phase' selection

The M. Phase' is generally polar in reverse phase & non polar in normal phase HPLC. The order of solvent system from polarity to non polarity is given below.

1. Heptane
2. Hexane
3. Isooctane
4. Cyclo-hexane
5. Carbon-tetrachloride
6. Toluene
7. Benzene
8. Ethyl ether
9. Chloroform
10. Methylene chloride
11. Tetra Hydro Furan (THF)
12. Acetone
13. Dioxane
14. Ethyl acetate
15. Acetonitrile
16. Pyridine
17. 1-Propanol
18. Ethanol
19. Methanol
20. Acetic acid
21. Water

Non polar solvents are aliphatic hydrocarbons (n-heptane, iso-octane, n-hexane), aromatic hydrocarbon (benzene, toluene, xylene) & carbon tetrachloride, chloroform, Dichloromethane (DMF) & petroleum ethers are used.
1. Information on sample, define separation goals

2. Need of procedure, guard column

3. Choose detector & detector setement

4. Choose Liquid Chrom. method; primary run; find optimum separation cond

5. To make best separation conditions

6. Determine for problem of special method

7a. Get purified material

7b. Quantitative calibration

7c. Quantitative method

8. Validate method for release to routine laboratory
Factors affecting RP-Chromatography retention of a compound

i. M. Phase’ effects

ii. Column & temperature effects

i. M. Phase’ effects

Select of M. Phase’ is based on properties of the solvents:

- Viscosity
- UV-Transparency
- Purity
- Inert with respect of sample compounds
- Corrosion resistance
- Toxicity

R. T. is if possible adjusted by varying mobile phase concentration or ratio or solvent strength. Retention is inversely proportional to less polar mobile phases & solvent strength. Solvent strength depends on both organic solvent & concentration of organic solvent.

Literature data suggest that RP-Chromatography solvent strength decreasing order as:

weakst to strong,

Water < Me OH < ACN < ethanol < tetrahydrofuran < propanol < methyl chloride

In RP-chromatography, acetonitrile, methanol, tetrahydrofuran are generally used solvents to controller selectivity & separation.

ii. Column & temperature effects:

The RP-Chromatography separations are generally accepted with silica-based, bonded phase columns. The illustration retentions are determined by in three characteristic of the column; type & concentration of bonded phase & column surface area.
Retention differs with countryside of bonded phas & commonly increases as the chain length or hydrophobiciti of bonde phase group rises.

Retention is also proportional to column surface area. Very hydrophobic sample area powerfully recollected & in certain cases their elutione beginning column may not be possible, even with NARP (Non-aqueous reverse phase) conditions.

An increase in temerature 1.0°C will regularly decrease R. T. 1 to 2 % for a non-ionic compound. Thus, modification in temperature can be used to controller sample retention. RP-Chromatography retention for non-polar, non-ionic compounds generally as follow;

(Weak) unbonded silica << cyano< C₁ (TMS) < C₃ C₄< phenyl < C₈ ≈ C₁₈ (strong).

The modification of R. T. in RP-Chromatography is performed by using the following techniques; Table 1.1.

**Table 1.1: Effect of column & temperature on retention**

<table>
<thead>
<tr>
<th>Decrease Retention</th>
<th>Increase Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Greater polar column (Cyano &amp; C₄)</td>
<td>• Low polar column (C₈, C₁₈)</td>
</tr>
<tr>
<td>• Low polar M. Phase’ (increase the quantity of organic phase)</td>
<td>• Greater polar M. Phase’ (decrease the quantity of organic phase)</td>
</tr>
<tr>
<td>• High temperature</td>
<td>• Lower temperature</td>
</tr>
</tbody>
</table>

**Chromatographic rule of conduct**

- *To give away of analytes between phases*

The delivery of analytic among phas can regularly to be defined reasonably basically. An analytic is in balance between the 2 phases;
$K$, is as a equilibrium constant, is labeled the partition coefficent; distinct by means of the molar concentration of analites in the mobile phas distributed by the molare absorption of the analite in the, stationary phas.

Time period in the middle of sample adding & analyt peak achievement a detactor known retention time $(t_R)$. Every one analite in a sampl determination need a changed retention time. The time occupied used for the M. Phase’ to passing by the column is termed known as $t_M$.

\[ K' = \frac{t_R - t_M}{t_M} \]

$A k'$, is as a retention factor, is regularly used to define the immigration amount of an anelyte on a column. It is also known as capacitii factor. The retaining factor meant for anelyt A is distinguished as a following formul a;

\[ K' A = \frac{t_R - t_M}{t_M} \]

$t_R$ & $t_M$ are definitely found from a chrometogram. While an anelytes retentione factor is a smaller amount of one, elutione is so fast that exact detarmination of the retention period is very challenging. In elevation retention factor (>20) resources that elution proceeds a exact extensive period. Preferably, the retantion factor for an analite is among one & five.

We describe a quantiti entitled the $\alpha$, is a selectivity factor, which defines the distributed of two specis (A & B) on the colon;

\[ \alpha = \frac{k' B}{k' A} \]

As soon as scheming the selectiviti factor, speces A elutes quicker than specis B. the selecteitivity factor is constantly bigger than one.
B& enlargement & column efficiency

To achieve ideal separations, sharp peaks essential to be gained. This funds that band broadening must be confined. It is as well valuable to quantity the proficiency of column.

The Theoretical Plate Exemplary of Chromatography

Fig. 1.9: The Theoretical Plate Exemplary of Chromatography

The plat says that chromatograph column surrounds great numbr of unconnected layeres, known as theoretical plates. Isolated equilibretions of the illustration among the stationery phase & mobie phase to happen in thers “plats”. It is essential to recollect that the plates do not actually occur; they are a fabrication of the thoughts that supports know, or through maintaining the plate heigeht; the height corresponding to a theoratical plate (HETP).

If the size of the column be present L, at that time HETP is

\[ \text{HETP} = \frac{L}{N} \]

The quantits of theoratical plats that a actual column retains consist to be establish by investigative a chromatogrephic peek subsequently elution;
\[ N = 5.55 t_{R}^2 \]

Where \( W_{1/2} \) is the present a peak (size) width on half-height.

- **The Rate Concept of Chromatography**
  
  A more faithful explanation of the procedures intimates

- **A-Eddy distribution**
  
  The molecules in the column distribute according to eddy equation to be equilibrate with mobile phase

- **B Longitudinal dispersion**
  
  The m. phase has longitudinal distribution according to the column and found longitudinal dispersion of molecule between M. Phase’ and stationary phase of the system..

- **C-Resistance to mass transmission**
  
  The analyte precede a certain quantity of time to equilibrate between the stationari phase & mobilee phase. If the speed of the M. Phase’e is lofty (High), & the analite consumes a robust attraction used for the stationary phase, formerly the analyte in the mobilee phase determination travel fast of the analyte in the stationary phase. Therefore the b& of analyte is enlarged.

- **Van Deemter plot**
  
  A plot diagram of height plate vs. average linear of M. Phase’ velocity.
Fig. 1.10: A plot of plate height vs. average linear of mobile phase velocity
Such plots are of significant use in influential the ideal M. Phase’F. R..

➢ Resolution

Even though the selection factor, $\alpha$, defines the separation of b&e insides, it
does not takings in to account peak measurements. Additional quantity of in what way
will species have been disconnected is providing by capacity of the resaolution. The
resolution determination of 2 types, A & B is decide as;

$$R = \frac{2[(t^R)_B - (t^R)_A]}{W_A + W_B}$$

Starting point resolution is accomplished while $R = 1.5$.

It is beneficial to communicate the resolution to the total quantity of plates in
column, the selectivity factor, & retention factor of the two solutes;

$$R = \frac{\sqrt{N}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{1 + k'_A}{k'_B} \right)$$

To achieved high resolution, the 3 expressions essentially to be maximized. An
intensification in N, these number of theoretical plates, by enlargement the column
indications to an intensification in R. T.& enlarged b& broadening- which may not be
desired. It is frequently initiate that by adjusting the capacety factor, $k'$, separation can
be significantly better-quality. The selection factor, $\alpha$, can similarly to be influenced to
increase separation. When `$\alpha'$ is close to unanimity, optimizing $k'$ & cumulative N is not
enough to give good parting in a useful time. In these cashs, $k'$ is optimized 1st, & then
`$\alpha'$ is improved by one of the subsequent processes:

• Varying mobyle phase composition or ratio.
• Varying column temperature
• Varying configuration of stationary phase
• Exhausting unusual chemical effect (such as integrating a types which developments with one of the solutes addicted to the stationery phase)

1.5 PROBLEMS ON HAND

There are so many cephalosporin antibiotics in the market & very complicated methods are available. There are many problems with the methods.

➢ These are very costly methods which are not afforded by simple chemists so some simple easy, cost effective methods are required for daily routine analysis of drugs.

➢ As compared with gas chromatography HPLC is versatile method & spectrophotometric methods are more specific to maximum absorption method. Maximum drugs are analyzed by these two techniques.
1.6 RESEARCH OBJECTIVE

- The aim of the present work is to evolve certain simple & reliable analytical methods that cater to the requirements of even small scale industries & academic institutions.

- There are many methods available on the analysis of these Cephalosporins. They include both classical & instrumental methods. The methods have been developed keeping in view the requirements. Consequently, certain methods are also focused on the analysis of the drugs.

- The objective of the study is to develop & validate analytical methods for third generation cephalosporin by applying suitable validation parameters.
1.7 SCOPE OF RESEARCH WORK

The research work is mainly concerned with analytical methods for Quantification of 3rd generation cefalosporins using high pressure liquid chromatographic techniques. 3rd generation cefalosporins are very useful in emergency cases in hospitals. These are very costly drugs so very correct, reproducible, precise, cost effective methods are developed for the different drugs. Third generation cefalosporins are mainly available as parenteral form because of stability problems for example cefotaxime, cefotriaxone, ceftazidime, cefpodoxime, cefototetan, cefdozixime, cefoperazone etc. so in this research stability indicating methods of cefotaxime sodium, cefotriaxone sodium, cefpodoxime were developed. The method may used in daily routine testing and analysis of drugs in daily life. The mainly used instrument is H.P.L.C. that is very sensitive and fast method for assaying of drugs nowadays. The method is versatile as compared to other analytical method because in this technique there is no limitation of volatility of compounds and unstability of compounds. The second thing that is very important in the research there is wide choice of selection of M. Phase’ in gradient elution techniques. Maximum 3rd
generation cefalosporins are lipophilic in nature so these are easily analyzed by using C-18 column with ODS bonded stationary phase. The M. Phase’ is polar for these antibiotics so RP-HPLC is most suitable analytical method for these drugs. M. Phase’ generally contain acetonitrile, methanol and different ratio of buffer system as the suitability of the drug solubility. The main problem for newer drugs that maximum methods are patented by different pharmaceutical companies and pharmacopoeial methods for newly released 3rd generation cefalosporins are not available. The major scope of research is development of simple, accurate, reproducible and fast cost effective methods for new cefalosporins. The methods are validated with recovery studies using bulk drug of 80%, 120% and 40%. Specific method is confirmed by checking interference of expients and assay method. The interday and intraday assay are also performed for checking robustness of the system. The minimum detection limit is checked by using formula LOD = 3.3 \sigma \text{(Stand)} / \text{Slop}, where \sigma indicate standard deviation and S denotes slop of the regression straight line. The quantification limit is determind by using LOQ = 10 \sigma \text{(Stand. dev.)}/ S that is minimum concentration of drug can be quantified. Linearity is found in the limit of Beer’s law, straight line was constructed within the given range of the conc. of the drugs. So can develop and validate a new, reproducible, correct, and easy, less time consuming, cheap and ecofriendly method for daily analysis of drug in our general life.
1.8 ORGANISATION

1.8.1 Where work is carried out: The work is carried out at the Institute of Pharmacy, Shri Jagdishprasad Jhabarmal Tibrewala University under the guidance of Dr Rakesh K Jat. The whole work is done in the central instrumentation Laboratory. The instruments are mainly used in the research work is HPLC (High Pressures Liquid Chromatography & Double Beam Spectrophotometer (Company Labtronics). The Solvents were used HPLC & analytical grade in the analytical method development & validation; drug samples were taken from different pharmaceutical companies like Zydus Cadila, Sun Pharma & Ranbaxy Laboratories. The different pharmaceutical dosage forms like tablet, capsules & injections were procured from the market. The sample & mobiles solvents are filtered from 0.45 µm filter & drug was sonicated for ten minutes before sample injection in the HPLC. The column was saturated with mobile phase before injection.

1.8.2 Introduction to drug CEFOTAXIM SODIUM

DRUG PROFILE
CEFOTAXIME SODIUM

**IUPAC Name:** Cefotaxime is designated by IUPAC rules as (7)-3-acetoximethyl-7-[(Z)-2-(2-aminothiaizol-yl)-2-(methoxiimino)acetemido]-3-cefam-4-carboxilate.

![Chemical Structure of Cefotaxime Sodium]

**Formula:** \( \text{C}_{16}\text{H}_{16}\text{N}_{5}\text{NaO}_{7}\text{S}_{2} \)

Cefotaxime is a semisynthetic agent obtained from various species of the mold *Cephalosporium* & *Streptomyces*. Cephosporins inhibit bacteria CW synthesis& peptidoglycane cross linking is inhibited [Charles, R. C. *et al*].

**Molecular Weight:** 455.47 g/mol

**Category:** Antimicrobial against *S. aureus*, Coagulase negative Staphylococci, *S. pneumonia*, *Streptococcus* sp., *H. influinzae*, *M. catarhalis*, *N. menigitides*, *N. gonorhoeae*, *Enterobacteriaceae*, *E. coli*

**Description:** Off-white crystalline powder [I. P.2007].

**Solubility:** Freely soluble in water.

**Storage:** Stored in an air-tight light resistant amber coloured containers.
Standards: Cefotaxime Na contains the equivalent of NLT 85.5 % & NMT 96.4 % of cefotaxime, $C_{16}H_{17}N_{5}O_{7}S_{2}$.

Antibacterial activity: Cefotaxime is highly stable to many of the bacterial $\beta$-lactamase & active against gram-positive & gram-negative aerobic bacteria [Goodman & Gilman’s (2006)].

Mechanism of action: Cephosporins mainly inhabit bacterial CW synthesis & inhibiting cross linking of peptidoglycan. The cefalosporins are also considered in the augmentation of bacterial cell autolysiins that put in to bacterial lysis. Cefotaxime is bactericidal for both intracellular & extracellular microorganism.

Pharmacokinetic data:

- Urinary excretion : 60 % as unchanged drug & metabolites [THE MERK MANUAL (2013)].
- Plasma protein binding : 13-38 %
- Clearance : $3.5 \pm 1.6$ ml/min/kg
- Volume of distribution : 14 L
- Half life : 1 - 1.7 hrs
- Peak time, serum: I.M. : 30 minutes
- Distribution : Crosses the placenta & distributed into milk [MedlinePlus].

Therapeutic uses: Bacterial meningitis, lyme disease, bacterial arthritis, bacterial meningitis, peritonitis, septicaemia, bone & soft tissue infections, pneumoneae, gonorrhea.
**Dose:** 250 mg, 500 mg reconstitute powder are available with distill water

**Adult Dose:**
- Menningitis: 1-2 g intra venous
- Infection of Bone: 1-2 g iv/im every 8 h
- Gonococcus urethritis: 0.5 g Intra Muscular x 1 dose
- R T I infection: 1-2 g iv/im every 8 h
- Bacter. Peritontis: 1-2 g every 8-12 h

**Dosing in pediatrics:** 100-150 mili gram divided every 4-6 h

### 1.9 PROBLEMS

#### 1.9.1. History of Antibiotics:

History of the antibiotic is very interesting and it can be divided into two segements. In the ancient times drug were obtained from different plant extracts like ephedra was isolated from chineese herb Ma huang and quinine from cinchona, atropine from belladonna, reserpine from Raulfia serpentine. One era is before chris and second era is after chris. The second era is very important in the field of development of antibiotics. The plant extract are modified by different reactions to modify there pharmacological action and reduce toxicity profile of different drugs.

#### 1.9.2 Early History:

In the ancient time the mold and fungi are used for treatment of different infections. The drugs extract were applied to wound for treatment. For example the leaves of erythroxylon coca was used to prevent hunger and the saliva of mouth was used on the
wound to relieve pain because of local anesthetic activity. In different country different type of bark, root, fruit, entire plant, stem, rhizomes, flowerbuds were used as liquid extract or powder for treatment of different disease.

### 1.6.3 Modern History:

The modern history of antibiotics was started from 1930 to 1940. The few arsenicals are used to treat syphilis by Poul Ehrlich in this decades. He was known as father of chemotherapy. The arsenicals are called magic bullets to treat syphilis. But due to their toxic effects these agents were not used further but these agents attracted the scientist to synthesize more chemical synthetic agents. The chemist Domagd found the new synthetic dye protosil rubrum to treat infection caused by streptococcal and staphylococcal species of bacteria. The protosil was converted to an active metabolite sulphanilamide from prodrug. These agent became very popular in treatment of many infections. These agents were very cheap and remain popular until the discovery of penicillin antibiotics.

**TABLE 1.2:** Mechanism of Antibiotics.

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Antimicrobial agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of bacterial cell wall synthesis</td>
<td>Penicillins, Cephalosporins, Impenem/meropenem, azetreonam, vancomycin</td>
</tr>
<tr>
<td>Inhibition of bacterial protein synthesis</td>
<td>Aminoglycosides, Chloramphenicol, Macrolides, tetracyclines, streptogramins, linezolid,</td>
</tr>
<tr>
<td>Inhibition of nucleic acid synthesis</td>
<td>Fluoroquinolones, rifampin</td>
</tr>
<tr>
<td>Inhibition of folic acid synthesis</td>
<td>Sulfonamides, trimethoprim, pyremethamine</td>
</tr>
<tr>
<td>Disruption of cell membrane function</td>
<td>Azoles &amp; antifungal polyene antibiotics</td>
</tr>
</tbody>
</table>
1.9.4 The Discovery of Antibiotics

The antibiotic discovery starts with penicillin that is effective against gram positive bacteria inhibiting cell wall synthesis of gram positive bacteria. Sir Alexander Fleming was working on the development of streptococcal culture in his laboratory situated in the basement. He went to spend his summer vacation in other place when returned back from his summer vacation he noticed the thing that was very surprising. One of petri dish has developed a mold inside and the mold secreted a liquid around itself that liquid or extract inhibited the growth of the streptococcal bacteria around itself. Alexander Fleming isolated this mold and gave name Penicillium notatum. One of friend flowry helped him and grown the mold in large amount with suitable medium further the extract was collected and used against many bacterial infections in mice. They found that one mice infected with streptococcal infection recovered with the injection of liquid extract. This was very interesting. Further chain joined them and produced large amount of pencillin using mold penicillium chrysogenm. Fleming, Flowry and Chain received
Noble prize for discovery and production of penicillin antibiotics. After discovery of pencillin Waksman declared bacillus anthrax can be treated with a chemical agent that is produced from streptococcus gresius. Waksman discovery aminoglycoside antibiotic streptomycin. After that there are so many antibiotics are discovered by different scientists. The cephalosporins were discovered from fungus cefalosporinium from the different cultures. Other antibiotics like macrolide, tetracycline, chloramphenicol were discovered one by one. These all are produced by fermentation techniques using slant then developing in suitable growth medium. The slant is grown upto optimum growth then leave for growth and release of antibiotics. The antibiotics are collected on commercial scale. Thus biotechnology grown very fastly with the advent of antibiotics.
Core structure of cephalosporin

HISTORY
The fungus Cephalosporium acremonium was discovered by the scientist Brotzu G in 1945. The C. acremonium was used against many gram + ve and gram -ve bacteria’s culture and found to be very active. Thus cefalosporins were discovered.

Figure: Sir Abraham discovered cephalosporin antibiotics
1.9.5 GENERAL FORMULA OF β-LACTUM ANTIBIOTICS:

**Penicillins:**

- Benzylpenicillin  \( R = \text{phenyl-CH}_2^- \)
- Penicillin G  \( R = \text{phenyl} \)
- Phenoxymethylpenicillin  \( R = \text{phenyl-O-CH}_2^- \)

**Penicillinase resistant Parenteral Penicillin**

- Methicillin  \( R = \text{phenyl} \)

**Penicillinase sensitive (Broad-Spectrum) Parenteral Penicillin**

- Carbenicillin
- Carindacillin
- Ticarcillin

**Beta-Lactamase Inhibitors:**

- Clavulanic acid  \( X = H \)
- Sulbactum  \( X = \text{OH} \)
1.9.6 ANTIBIOTIC RESISTANCE:

Bacterial resistance to antimicrobial agents:

Acquired resistance

Bacteria, fungi, protozoa tini organism produces enzymes that destroy the antibiotic and the cause of resistance:

- The bacteria streptococci, staphylococci and others produces β-lactamase or cefalosporinase or penicillinase that destroy the β-lactam ring of pencillin and cefalosporine antibiotics.
- The drugs that are effective against gram negative bacteria resistance is produced by enzymes adenylase, acetylase and phophorylase from the bacterial species.
- Resistance can be produce by mutation of bacterial species that alter the structure of the proteins.
- Alteration in transport system also casues development of resistance in some antibiotics.
- Genotype and phenotype resistance may be developed
1.9.7 Classification of β-lactam antibiotics:

1) Natural penicillin – penicillin G

2) Semisynthetic penicillins:
   a. Acid resistant alternative to penicillin G – penicillin V
   b. Penicillinase resistant penicillins – methicillin, oxacillin, cloxacillin.
   c. Extended spectrum penicillins –
      i. Aminopenicillins – ampicillin, bacampicillin, amoxicillin
      ii. Carboxypenicillins – Carbenicillin, ticarcillin.
      iii. Ureidopenicillins – piperacillin, mezlocillin, azlocillin.

β-lactam inhibitors: - Clavulanic acid, sulbactam.

Penicillin G:

- Benzyl penicillin.
- Narrow spectrum primarily for Gm +ve bacteria

Spectrum:

- Cocci – streptococci, pneumonia, staphylococci, Gm –ve – Neisseria gonorrhea & meningitdis. (R)
- Bacilli – Gm +ve – B. anthracis, C. diphtheriae, clostridia, listeria, spirochetes (treponema),
- Actinomyces – israeli

Resistance:

- Produce penicillinase(β-lactamase) – staphylococcus aureus
- Include structural change in PBPs – MRSA, Pn R pneumococci
- Change in porin structure – pseudomonas.
Pharmacokinetics:

- Acid labile, destroyed by gastric acid.
- Sodium Penicillin G Intra Muscular
- Widely distributed, penetration in CSF is poor.
- In presence of inflammation – adequate amount reaches the CSF.
- Renal excretion – active tubular secretion – blocked by probenecid.

Preparation & Dose:
- Sodium Penicillin G – crystalline Pn – 0.5-5 MU im/iv 6-12 hourly.
- Repository salts of Pn G in plasma. Procaine penicillin – 0.5-1 MU in 12-24 hrly.
- Fortified procaines Penicillin G – 3 lac U procaine penicilline + 1 lac U sodium pencillin G, benzathine penicillin G – 0.6-2.4 MU, every 2-4 weeks. Long acting, penicillin G.

Uses:

1) Streptococcal infections: pharyngitis, OM, RF, SABE.
2) Pneumococcal infections: Lobar pneumonia & meningitis.
3) Meningococcal infections: Rifampin is used for prophylaxis of MM.
4) Gonorrhea: unreliable due to resistance. Fluoroquinolones & cefriaxone is used
5) Syphilis: Treponoma Pallidum still sensitive to penicillin G & is the drug of choice (DOC). 2-4 MU 1-3 weekly doses for 4 weeks.
6) Diphtheria: procaine penicillin 1-2 MU im + antitoxin therapy.
7) Tetanus & Gas gangrene:
8) Drug of choice for anthrax, actinomycyes, trench mouth, rat bite fever, & listeria & pasturella infections.
9) Prophylaxis in
- Rheumatic fever – benzathine penicilline1-2 Mu every 4 weeks till 18 years of age or 5 years after an attack, whichever more
- Gonorrhea or syphilis
- Bacterial endocarditis – dental extractions, endoscopies, catheterization, etc.
- Agranulocytosis patients
- Surgical infections – wound infections.
Semisynthetic Penicillins:
- Better orally efficacy,
- Some resistance to penicillinase.
- Broad spectrum
- $\beta$-lactamase inhibitors – not antibiotics augment activity of penicillin against $\beta$-lactamase producing organisms.

Penicillin V:
- Acid stable – used orally.
- Used for streptococcus pharyngitis, sinusitis, otitis media, RF, etc.
- 250-500 mg 6 hourly.

Penicillinase resistant penicillins:
- Additional side chains protect inner $\beta$-lactam ring by penicillinase.
- Only used for penicillinase producing staphylococcus
- Not resistant to Gm-ve $\beta$-lactamase.

Methicillin:
- Penicillinase resistance, not acid resistance – given IV.
- MRSA developed rapidly.
- DOC – vancomycin, linezolid & ciprofloxacin.
- ADR – hematuria, albuminiuria & interstitial nephritis.

Cloxacillin:
- Active against penicillinase producing staphylococcus aureus, but not against MRSA.
- Can used orally 6 hourly.

Extended Spectrum penicillin:
- Broader spectrum of activity, not resistance to penicillinase.
Aminopenicillins: (ampicillin, amoxicillin, bacampicillin)

Ampicillin:
- All penicillin sensitive organisms + many Gm –ve – H. influenza, E. coli, proteus, salmonella, shigella.
- More active against – streptococcal viridans & enterococci, equally active against – pneumo, gonorrhea, & meningococci.
- Penicillinase producing staph are not affected.

Pharmacokinetic:
- Not degraded by gastric acid, food interferes with absorption.
- 0.5-2gm oral/iv/im. Every 6 hours.
- 250-500 mg caps.

Adverse effects
- Alteration of bacterial flora in gut – diarrheas.
- Hypersensitivity.
- May cause failure of conception with OCP because of interference with enterohepatic cycling.

Uses:
1) UTI – rapid resistance, fluoroquinolones & cotrimoxazole is used now.
2) RTI – bronchitis, sinusitis, otitis media.
3) Meningitis- usually combined with 3rd generation cephalosporin/chloramphenicol
4) Gonorrhea – drug of choice 3.5 gm ampicillin + 1 gm probenecid single dose
5) Typhoid fever – R
6) Bacillary dysentery – quinolones now.
7) Cholecystitis – because attains high concentration in bile.
8) SABE
9) Septicemia.

Bacampicillin:
- Prodrug to ampicillin,
- Better tissue penetration, high plasma concentration.
- Does not markedly interfere with GI flora.
- 400-800 mg BD

**Amoxicillin:**

- Similar to ampicillin except
- Oral absorption is better, food does not interfere.
- Incidence diarrhea is less
- Less active against shegella & H. influenza.
- Used for typhoid, bronchitis, UTI, SABE & gonorrhea.
- 0.25 – 1 g TDS oral.

**Carboxypenicillin: (Carbenicillin):**

- Active against Peudomonas aeruginosa & proteus.
- Penicillin G & aminopenicillin does not act on these.
- Neither penicillinase resistance nor acid resistance
- Only used for serious pseudomonas or proteus infections – burns, UTI, septicemias.

**Ticarcillin:** more potent than carbecillin.

**Ureidopenicillin: (Piperacillin)**

- More active than carbecillin.
- Has antipseudomonal activity
- Also active against kleibesella.
- Used mainly in neutropenic or immunocompromised patients with serious Gm +Ve infections & in burns.
- 100-150 mg /kg/day.

**β-lactamase Inhibitors: (Clavulinic acid)**

- Obtained from clavuliferus, has β-lactam ring but no antibacterial activity.
- Inhibits all β-lactamase except cephalosporinase.
- It is a progressive or suicide inhibitor – binding with β-lactamase is reversible initially & becomes covalent later, gets inactivated after binding to the enzyme.
- Also inhibits β-lactamase of Gm-ve bacilli.

**Pharmacokinetics:** rapid oral absorption, combined with amoxicillin. (Co-amoxiclav)

**Uses:**
- Addition of CA to mox, reestablishes its activity against - β-lactamase producing staph aureus – but not MRSA, also against H. influenza, proteus, E.coli, klebsella, salmonella, shigella & B. fraglis.
- Co-Amoxiclav is indicated for:
  - Skin & soft tissue infections, intraabdominal & gynec sepsis, UTI, RTI & bialary infection.
  - Gonorrhea – single dose
  - Augmentin 250 mg mox + 125 ng clavulanic acid tab.

**Sulbactum:**
- Also a progressive or suicidal inhibitor.
- Less potent than clavulanic acid.
- Given parenterally.
- Used for
  - PPNG gonorrhea
  - Mixed aerobic, anerobic infections.
    - Sublain – ampicillin 1 gm + sulbactum 0.5 gm per vial.
- May cause pain & thrombophlebitis of injected vein.

**Tazobactam:**
- Also β-lactamase inhibitor, combined with piperacillin.
1.9.8 ALLERGIC REACTION TO ANTIBIOTICS:

Adverse effects: (most nontoxic antibiotics)

- May cause local irritation & direct toxicity – pain at IM site. Thrombophlebitis at injection site.
- Accidental iv injection of procaine penicillin – hallucinations & convulsions.
- Hypersensitivity – 1-10% incidence. Most common drug causing drug allergy.
- Manifested as fever, rash, itching & urticaria. May cause anaphylaxis in severe cases.
- Partial cross sensitivity exists
- H/O allergy must be elicited & intradermal test should be done before its use.
- Also very toxic if used topically – banned topically.
- Jarisch – hexheimer reaction – in secondary syphilis. Due to sudden release of spirochetal lytic products, 12-72 hrs.
- Does not recur & aspirin & sedation suffice.
Figure 1.11: Allergic reactions of beta lactum antibiotics