INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION AND SCOPE OF RESEARCH

The medical model of health centers on the abolition of illness through diagnosis and effective cure. Improvement of animal health needs advancements in medical science through varieties of medicines. Veterinary medicines are fundamental part of the animal health care system. The various veterinary medicines are introducing into the market and these medicines are being either new entities or partial structural modification of the existing one. So, to evaluate quality and efficacy of these medicines is very important. Quantification means are used to check quality and efficacy of medicines right from the beginning. Quality and efficacy are checked by two ways. Firstly observing effect of medicine on various animal and second one is by analytical means. Practically it is not possible to opt animal models for every batch of medicine as it costs more, required long time and definitely more man power. In comparison to previous method analytical way is highly precise, more suitable and safe.

The analytical mean deals with quality standards and efficacy of the medicines. Sample representing any lot are analyzed for these standards and it is assumed that medicine which is having such standards are having desirable effect on usage. Concept of quality control strives to produce a perfect medicine by series of procedures designed to check and eliminate errors at different stage of manufacturing. The decision to release or reject a lot is based on control action.

Rapid growth of pharmaceutical industry results in numerous formulations in medical science. Developing analytical methods for new entities or partial structural modification is a matter of most significance because medicines may not be official in any pharmacopoeias and thus, no analytical method for
quantification is available. To assess the quality standards of the medicine various analytical methods are used. Modern analytical techniques are playing important role in measuring quality standards of medicine. Thus analytical techniques are required for setting standards of drug products and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality and efficacy of drug products is known as ‘CHROMATOGRAPHY’.

1.1.1 Impurity profiling in drug product development

Safety and efficacy of drug products are two crucial concerns in drug therapy. The quality and safety of formulated drug products are ensured by monitoring quantity of API and related substances present in dosage forms. The related substances present in drugs possess unwanted toxicological or pharmacological side effects. For that reason, it is recommended that any drug product intended for animal consumption should be completely identified and characterized. In drug products, the level of any impurity present beyond identification level should be qualified during clinical studies.

An extremely accurate, precise and specific validated analytical method should be developed for the estimation of the active pharmaceutical ingredient (API) and related components in the drug dosage forms to confirm drug efficacy. Related components are designated as impurities present in pharmaceutical products which are unwanted chemicals that remain in trace level quantity with the active pharmaceutical ingredients (APIs) during synthesis pathway, or form during stability study. The trace level quantity of impurities may influence the efficacy of formulated dosage form. Force degradation studies of the drug products provide valuable information to propose the degradation pathway and products’s inherent stability. A validated stability indicating analytical procedures should be used to evaluate the forced degradation studies and pharmaceutical product stability studies.
Regulatory guidance is available to establish the limit of impurities in drug products and API’s. The quality guidelines of VICH are commonly used to serve the purpose named “VICH GL10: Impurities in new veterinary drugs substances” and “VICH GL11: Impurities in new veterinary medicinal products.”

1.1.2 The role of regulatory guidelines in pharmaceutical industry

Today, the Food and Drug Administration (FDA) is more focussed on the quality, safety and efficacy of drug products. The technical requirements of VICH for registration of pharmaceuticals for animal use is an exclusive project that brings together the regulatory authorities of Japan, Europe and United States and professionals of the pharmaceutical industry from the three regions to discuss technical and scientific aspects of products registration.

Analytical procedures of active drug substances and products are developed and validated to ensure the accurate quantification of API’s and impurities in quantity control lab. Specification limits for impurities are concluded on the basis of stability studies data and product development study. The set limits of stability specification should not be greater than the toxicological level or less than the lowest quantification level of the method. The lowest quantitation level of the analyte peak should be less than or equal to the reporting threshold mentioned in VICH guidelines. The impurity present above identification level in the drug product should be isolated and characterized.

Stability studies during product development provide the information to method development scientists about unspecified degradants of drug. All unspecified and specified degradation products are quantified and monitored in these studies. Stress studies are useful to establish the specific stability indicating capability of test method to evaluate the impact on drug product stability due to any
accidental exposure to the unwanted environmental conditions except normal stage conditions (e.g. during transportation).

In addition to this, the stress studies are also useful.

- To determine the stability of drug products and drug substances in the test solution exposed to different environmental conditions.
- To determine structural changes and conversions.
- To estimate trace level quantity of potential degradation products.
- To isolate the degradation products formed due to drug and excipients interaction.
- To identify potential degradation substances that may be impulsively generated during the storage of drug products.

The degradation products formed during stress testing are known as “potential” degradants. Six major categories are classified for the forced degradation studies.

- Thermolytic degradation
- Oxidative degradation
- Photolytic degradation
- Humidity degradation
- Acidic degradation
- Basic degradation

1.1.3 Mechanism of excipients-drug interaction

Definition of excipients as per International Pharmaceutical Excipients Council Europe and IPEC America is that “These are the components apart from active drug substance which has been added in a dosage form to either support manufacturing process, administration or absorption or increase shelf life,
enhance bioavailability and enhance any other characteristic of overall effectiveness drug product during storage or use.”

Although excipients are pharmacologically inactive but can induce, initiate or catalyze the chemical or physical reactions with drug substance, which may influence the efficacy and safety of drug product. Physical interactions can influence dissolution rate, hardness, disintegration time, uniformity of dosage unit.

Degradation products formed due to chemical interactions between excipients and drug could be detrimental to the patient. Preformulation study need to be performed to understand the drug excipient interaction which is the initial step to finalize active and placebo ratio. It is recommended evaluating the characteristic of API alone and in blend with excipients at different stability conditions.

1.2 INSTRUMENTATION

1.2.1 History of Chromatography and HPLC

In 1903, Russian botanist Mikhail Tswett produced a colorful separation of Plant pigments through calcium carbonate column. Chromatography word came from Greek language chroma = color and graphein = to write i.e. color writing or Chromatography¹.

Chromatography is defined as, “Chromatography is a technique for the separation of components of mixture distributed between two phases, a stationary phase and a mobile phase. Those components strongly retained by the stationary phase move slowly with the flow of mobile phase and elutes later. In contrast, components that are weakly held by the stationary phase travel rapidly down the column and elutes first. This difference in migration rates make the sample components to separate and can be analyzed quantitatively and qualitatively¹”. 
High Pressure Liquid Chromatography (HPLC) was developed in the mid-1970s and quickly improved with the development of column packing materials and the additional convenience of online detectors. New techniques improved separation, identification, purification and quantification. Computers and automation added to the convenience of HPLC.

By 2000, very fast development was undertaken in the area of column material with small particle size technology and other specialized columns.

1.2.2 Liquid chromatography

Liquid chromatography is a separation technique based on a solid stationary phase and liquid mobile phase. Separations are achieved by partition, adsorption or ion exchange processes depending on the type of stationary phase used. The most commonly used stationary phases are modified silica or polymeric beads. The beads are modified by the addition of long-chain hydrocarbons. The mobile phase is a solvent or mixtures of solvents.

There are mainly two modes of separation in HPLC viz. normal phase and reverse phase.

1.2.2.1 Normal phase chromatography

In liquid chromatography, if the stationary phase is more polar than the mobile phase, it is termed as normal phase liquid chromatography. In normal phase, polar bonded phases that have a diol, cyano, diethylamino, amino, or diamino functional groups are used as stationary phase. Due to lower affinity of non-polar compounds to the stationary phases used, non-polar compounds are eluted first while polar compounds are retained for longer time. Normal-phase chromatography is widely applied for chiral separations.
1.2.2.2 Reversed-phase chromatography

In liquid chromatography, if the stationary phase is less polar than the mobile phase, it is termed as reverse phase liquid chromatography. In this mode, C18, C8, Phenyl, and cyano-propyl functional groups that chemically bonded to micro porous silica particles are used as stationary phase. It is estimated that over 90% of all HPLC separations are executed in the reversed-phase mode.

1.2.3 Apparatus

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the column, a chromatographic column, a detector, and a data collection device. 

![Schematic representations of typical HPLC Instrument components](image-url)
1.2.4 Detector

Commonly used detectors in HPLC are UV (Ultraviolet) detector and PDA (Photodiode Array) detector.

HPLC UV detector works on the principle of ultraviolet spectroscopy. Ultraviolet spectroscopy refers to reflectance spectroscopy or absorption spectroscopy in the ultraviolet spectral region which ranges 200nm to 400nm. In both UV and visible spectroscopy, only valence electrons absorb energy, thereby the molecule undergoes transition from ground state to excited state. This absorption is characteristic and depends on the nature of electrons present.
The ultraviolet region is subdivided into three spectral regions.

Far or Vacuum UV region (Below 200nm)
Middle UV region (Between 200-300 nm)
Near UV region (Between 300-400nm)

Different molecule absorbs radiation of different wavelength. Absorption of UV and visible radiation in organic molecules is restricted to certain functional groups (chromophores) that have n, π or σ or a combination of these electrons. These bonding (σ and π) and non-bonding (n) electrons absorb the characteristic energy and undergoes transition from ground state to excited state. By this characteristic energy absorption elucidates the nature of the electrons presents and hence the molecule structure. The various transitions are n→π*, π→π*, n→σ* and σ→σ*. The energy required for excitation for different transitions are n→π* < π→π* < n→σ* < σ→σ*.

HPLC PDA (photo diode array) or DAD (diode array detector) works on the principle of photovoltaic detectors. HPLC PDA detector is used for recording the full UV-Vis absorption spectra of chromatographically separated samples.

**1.2.4.1 Beer-Lambert’s law**

Beer’s law states that “the intensity of a beam of monochromatic light deceases exponentially with increase the concentration of absorbing species arithmetically***”.

Lambert’s law states that “the rate of decrease of intensity (monochromatic light) with the thickness of the medium is directly proportionally to the intensity of the incident light”.

Mathematical derived equation of combine Beer-lambert law.

\[ A = \log_{10} \left( \frac{I_0}{I} \right) = \varepsilon \cdot c \cdot l \]
Where,

A is the measured absorbance,
Io is the intensity of the incident light at a given wavelength,
I is the transmitted intensity,
l is the path length through the sample,
and c is the concentration of the absorbing species.

For each species and wavelength, $\varepsilon$ is a constant known as the molar absorptivity or extinction coefficient.

We can say from above equation
1) Absorbance A is directly proportional to l path length of medium
2) Absorbance A is directly proportional to c concentration of sample

**1.2.5 Definitions and interpretation of chromatograms**

**1.2.5.1 Chromatogram**

The plot of detector response as a function of time is called chromatogram. It gives qualitative and quantitative information of analyte. The position of peak shows the identity of analyte and area under peak shows concentration of analyte.

**Figure 1.3** represents a typical chromatographic separation of two substances, 1 and 2. $t_{R1}$ and $t_{R2}$ are the respective retention times; h is the height, $h/2$ is the half-height, and $W_{1/2}$ is the width at half-height, for peak 1. $W_1$ and $W_2$ are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in LC. The retention time of these air peaks, or unretained components, is designated as $t_M$. 
**1.2.5.2 Number of Theoretical Plates (N)**

N is a measure of column efficiency. For Gaussian peaks, it is calculated by

$$N = 16 \left( \frac{t_R}{W} \right)^2,$$

Where $t_R$ is the retention time of the substance and $W$ is the peak width at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

Where electronic integrators are used, it may be convenient to determine the number of theoretical plates, by the equation.

Where, $W_{h/2}$ is the peak width at half-height.

$$N = 5.54 \left( \frac{t_R}{W_{h/2}} \right)^2$$

**1.2.5.3 Peak**

“The peak is the portion of the chromatographic recording of the detector response when a single component is eluted from the column”. Incomplete separation elutes one unresolved peak.
1.2.5.4 **Relative Retention Time (RRT)**

Also known as unadjusted relative retention\(^5\).

\[
RRT = \frac{t_{R2}}{t_{R1}}
\]

1.2.5.5 **Relative Standard Deviation in Percentage**

\[
\%RSD = \frac{100}{x} \left( \frac{1}{N} \sum_{i=1}^{N} \left( x_i - x \right)^2 \right)^{1/2}
\]

1.2.5.6 **Retention Time (t\(_R\))**

In liquid chromatography, the retention time, t\(_R\), is defined as “the time elapsed between the injection of the sample and the appearance of the maximum peak response of the eluted sample zone”. t\(_R\) may be used as a parameter for identification\(^5\).

1.2.5.7 **Resolution (R\(_S\))**

The resolution is the separation of two components in a mixture, calculated by:

\[
R_S = 2 \times \frac{(t_{R2} - t_{R1})}{(W_1 + W_2)}
\]

Where t\(_{R2}\) and t\(_{R1}\) are the retention times of the two components; and W\(_2\) and W\(_1\) are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline\(^5\).

1.2.5.8 **Symmetry Factor (A\(_S\))**

The symmetry factor, also known as the tailing factor, of a peak (Figure 1.4) is calculated by
Where \( W_{0.05} \) is the width of the peak at 5% height and \( f \) is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline\(^5\).

![Figure 1.4 Calculation of Symmetry Factor for a peak in a typical chromatogram](image)

### 1.2.6 Isocratic and gradient liquid chromatography system operation

Two basic elution modes are used in HPLC. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, remains the same throughout the run.

The second type is called gradient elution, wherein, as its name implies, the mobile phase composition changes during the separation. This mode is useful for samples that contain analyte having wide range of chromatographic polarity. As the separation proceeds, the elution strength of the mobile phase is increased to elute the more strongly retained sample components.
1.3 DRUGS SELECTED FOR RESEARCH STUDY

1.3.1 Marbofloxacin tablets

Marbofloxacin is a carboxylic acid derivative third generation fluoroquinolone antibiotic. It is a veterinary medicine used in treatment of dermal, respiratory & urinary tract infection borne from Gram positive bacteria, Gram negative bacteria and Mycoplasma in dogs and cats. Marbofloxacin’s mechanism of action is to inhibit the bacterial enzymes like DNA-gyrase & topoisomerase IV which eventually helps in bactericidal activity.

1.3.2 Deracoxib Chewable tablets

Deracoxib is non-narcotic, nonsteriodal anti-inflammatory drug (NSAID) of the coxib class. It is a veterinary medicine used in treatment of postoperative pain and inflammation associated with orthopaedic surgery and control of inflammation and pain associated with osteoarthritis in dogs. Deracoxib’s mechanism of action is to inhibit the synthesis of prostaglandins. The enzyme inhibited by NSAID is cyclo-oxygenase (COX) enzyme. The COX enzyme is in two isoforms COX-1 and COX-2. COX-1 is responsible for synthesis of prostaglandins important for maintaining healthy gastrointestinal tract, platelet function and renal function. COX-2 is responsible for synthesizing prostaglandins that are important mediators of fever, inflammation and pain.

1.4 REVIEW OF LITERATURE

A detail literature review reveals that no Assay and Related Substances HPLC method is available for Marbofloxacin tablets and Deracoxib chewable tablets. Only bioanalytical methods are available. Few of them are mentioned below.
1.4.1 **Sherry K. Cox, Jacob Roark, Adam Gassel and Karen Tobias** have developed a new method for the determination of deracoxib in feline plasma samples using high performance liquid chromatography.

1.4.2 **P. Marín, L.F. Alamo, E. Escudero, E. Fernandez-Varon, V. Hernandis and C.M. Cárceles** have studied pharmacokinetics of marbofloxacin in rabbit after intravenous, intramuscular, and subcutaneous administration.

1.4.3 **Adnan H. Mahmood, Gregory A. Medley, Jeffrey E. Grice, Xin Liu and Michael S. Roberts** have developed and validated a method for the determination of trovafloxacin and marbofloxacin in sheep plasma samples by HPLC using UV detection.

1.4.4 **Marino García-Montijano, Samanta Waxman, J. Julio de Lucas, I. Luaces, María Dolores de San Andrés and Casilda Rodríguez** have studied the disposition of marbofloxacin in vulture (Gyps fulvus) after intravenous administration of a single dose.

1.4.5 **Jose Julio de Lucas, Casilda Rodriguez, Samanta Waxman, Fernando Gonzalez, Isabel Uriarte and Manuel Ignacio San Andres** have studied the pharmacokinetics of marbofloxacin after intravenous and intramuscular administration to ostriches.

1.4.6 **M. Ismail and Y.A. El-Kattan** have studied the comparative pharmacokinetics of marbofloxacin in healthy and Mannheimiahaemolytica infected calves.
1.5 REFERENCES


3. https://www.google.co.in/search?q=serviceagilent_1100_hplc.gif&es_sm=122&bm=isch&tbo=u&source=univ&sa=X&ei=E74iVY6_F8uKuAS1tYDwBw&ved=0CB8QsAQ&biw=1366&bih=643.


5. USP 40-NF 35. Chromatography. (621), 508, 8071.


