CHAPTER-III
MATERIALS AND METHODS
This chapter describes the materials and methods used in the present study. It is divided into two sections. Section-i describes the reagents, solvents, glass-ware, standards and test samples (dosage forms) used in the present study. Brief description of equipment and methods employed in the present study is given in section-ii.

Section-III. i: Reagents, chemicals, solvents, glassware, standards and test samples

III.1. Reagents and Chemicals

KH₂PO₄: Qualigens manufacture analytical grade (AR) of Potassium di-hydrogen phosphate is used for the analysis.

Ammonium acetate: Sd. Fine chemicals limited manufacture analytical grade (AR) of ammonium acetate is used for the analysis.

Sodium hydroxide (NaOH): Qualigens supplied Sodium hydroxide is used for this study.

Potassium hydroxide (KOH): Qualigens supplied Potassium hydroxide is used for this research work.

Sodium perchlorate (NaClO₄): Sd. Fine chemicals Ltd supplied sodium perchlorate is used for this study.

Ortho-phosphoric acid (H₃PO₄): Sd. Fine supplied analytical grade (AR) of ortho-phosphoric acid is used for the analysis.

Hydrochloric acid (HCl): Fisher scientific (Qualigens) supplied hydrochloric acid is used for the analysis.
Tri-ethyl amine: Sd. Fine chemicals limited supplied hydrochloric acid is used for the analysis.

Acetic acid (CH₃COOH): Sd. Fine chemicals limited supplied acetic acid is used for the analysis.

III.i.2. Solvents

Acetonitrile (CH₃CN): HPLC grade solvent is used for the analysis, manufactured by Merck India Ltd.

Methanol (CH₃OH): HPLC grade solvent is used for the analysis, manufactured by Merck India Ltd.

III.i.3. Glassware

Brand new of borosil, class-type glassware like pipette, volumetric flask, conical flask, glass bottle, test tubes, measuring cylinder and glass beaker were used for analysis.

III.i.4. Standards

Reference standards, glucosamine sulphate Potassium chloride, diacerein, donepezil hydrochloride, amiodarone hydrochloride, sildenafil citrate and its related compounds, beclomethsaone dipropionate, chloramphenicol, clotrimazole, lidocaine, chloroxylenol and terpineol were obtained from M/s. Vivimed Labs Ltd, Hyderabad.

III.i.5. Test sample (dosage forms)

Finished products, glucosamine and diacerein tablets-750mg/50mg, donepezil hydrochloride tablets-5mg and 10mg, amiodarone hydrochloride tablets-100 and 200mg tablets, sildenafil citrate tablets-25, 50 and 100mg
tablets, ear drops (composed of beclomethasone dipropionate-0.025%w/v, chloramphenicol-5.0%w/v, clotrimazole-1.0%w/v and lidocaine-1.73%w/v) and antiseptic solution (composed of chloroxylenol-4.8% w/v and terpineol-9.0% v/v) were obtained from M/s. Vivimed Labs Ltd, Hyderabad.

Section-III-ii: Equipment and methods

This section describes the equipment used in the present study and methods for dissolution and assay of glucosamine and diacerein, donepezil hydrochloride, amiodarone hydrochloride and sildenafil citrate tablets, ear drops (composed of beclomethasone dipropionate-0.025%w/v, chloramphenicol-5.0%w/v, clotrimazole-1.0%w/v and lidocaine-1.73%w/v) and antiseptic solution (composed of chloroxylenol 4.8% w/v and terpineol 9.0% v/v).

III.ii.1. Equipment

**HPLC:** Waters alliance 2695 module HPLC, equipped with dual nanometer absorbance detectors are used in the present study. A Shimadzu LC-2010 CHT HPLC used in the studies of sildenafil citrate tablets.

**UV Spectrophotometer:** Shimadzu make UV-1700 model is used in the analysis.

**Dissolution apparatus:** Lab India make DISSO-2000 is used in this study.

**Analytical balance:** Mettler Toledo make AB265-5/fact model is used for this research work.
Centrifuge: Remi limited make centrifuge instrument is used for samples preparation.

pH meter: Elico analytical instruments Ltd, Hyderabad make pH meter LI 614 model is used for this work.

Sonicator: Pharmtec scientific suppliers manufactured 25 liter sonicater was used for analysis.

Degasser: Borosil manufactured glass degasser apparatus is used and Millipore 0.45micron filter paper is used for degassing.

Milli-Q water purification system: Milli pore manufactured water purification system is used for analysis.

III.ii. 2. Methods

In the present study analytical methods are developed for glucosamine and diacerein, donepezil hydrochloride, amiodarone hydrochloride and sildenafil citrate tablets, ear drops (composed beclomethasone dipropionate-0.025%w/v, chloramphenicol-5.0%w/v, clotrimazole-1.0%w/v and lidocaine1.73%w/v) and antiseptic solution (composed chloroxylenol 4.8% w/v and terpineol 9.0% v/v) are described.

III.ii.2.1. Glucosamine and diacerein tablets:

Chromatographic Conditions:

The mobile phase was composed of a mixture of buffer (add 1.0mL of \( \text{H}_3\text{PO}_4 \) in to 2000mL of HPLC water and adjust pH of solution to 3.0± 0.1 with dilute Potassium hydroxide solution, degas it with 0.45µ filter) and acetonitrile
(55:45 %v/v) and degassed, reversed phase C8 column (4.6mm x 250mm, 5.0μ), 0.6mL per min flow rate and the injection volume is 20μL and wavelength of 195nm.

**Dissolution conditions:**

The conditions for dissolution were: 900 mL of Sodium phosphate buffer pH 7.0 with 0.75 % of Sodium lauryl sulphate as dissolution medium, using a USP-II (paddle) apparatus at a stirring rate of 50 rpm for 45min.

**Preparation of Standards:**

Diacerein stock solution was prepared by taking 28mg of diacerein in to 250 mL volumetric flask add 150mL of diluent (mixed HPLC water and acetonitrile in the ratio of 25:75 (%v/v) and degassed) and sonicate to dissolve the content, then made up to the volume with mobile phase, mix. Blank is prepared by mixing diluent and mobile phase in the ratio of 50:50 (%v/v). Glucosamine stock solution was prepared by adding 84mg of glucosamine sulphate Potassium chloride in to 50mL volumetric flask add 15mL of diluent and 20 mL of mobile phase and sonicate to dissolve the content and make up to the volume with mobile phase. Pipette out each 5.0mL of diacerein standard stock solution and glucosamine standard stock solution in to a 25 mL volumetric flask make up to volume with mobile phase, mix. For dissolution analysis prepared the standard solution equivalent to test sample concentration.

**Test solution preparation:**

Weighed 20 tablets and crushed. Weighed the crushed sample accurately equivalent to 168mg of glucosamine sulphate Potassium chloride and
transferred in to a 100mL of volumetric flask and added 40mL of diluent and
30mL of mobile phase, sonicated for 10min and diluted to volume with mobile
phase and further diluted 5 mL of the centrifuged supernatant solution in to
25mL with mobile phase.

Procedure:
Separately injected equal volume of about 20μL of blank, standard
preparation in replicate, sample preparation once in to the HPLC system and
recorded the chromatograms and calculated the results using below formula.

System Suitability solution:
The tailing factor of both ingredients peaks in standard solution is not
more than 2.0 and the percent (%) RSD of five replicate injections area is not
more than 2.0 percent (%).

Calculation:

Percentage (%) of glucosamine sulphate Potassium chloride=

\[
\frac{At \times WS \times 5 \times 100 \times 25 \times P \times \text{Avg. Wt of tablet} \times 100}{As \times 50 \times 25 \times WT \times 5 \times 100 \times \text{Label Claim}}
\]

Where in As is the area of glucosamine sulphate Potassium chloride
standard solution, At is the area of glucosamine sulphate Potassium chloride
test sample solution, WT is the weight taken for test solution preparation in mg,
WS is the glucosamine standard weight taken for standard solution preparation
in mg and P is the potency of glucosamine sulphate Potassium chloride on as
such basis.

Percentage (%) diacerein =

\[
\frac{At \times WS \times 5 \times 100 \times 25 \times P \times \text{Avg wt of tablet} \times 100}{As \times 250 \times 25 \times WT \times 5 \times 100 \times \text{Label Claim}}
\]
Where in $A_s$ is the area of diacerein standard solution, $A_t$ is the area of diacerein test sample solution, $W_s$ is the diacerein standard weight taken for standard solution preparation in mg, $W_t$ is the weight taken for test solution preparation in mg and $P$ is the potency of diacerein standard.

III.ii.2.2. Donepezil hydrochloride tablets:

The chromatographic method was specifically developed for the determination of dissolution profile and assay content estimation of donepezil hydrochloride tablets.

**Chromatographic conditions:**

Chromatographic separations were performed using isocratic elution at ambient temperature, absorbance measured at 230nm, mobile phase was composed of a mixture of buffer (1.0gm of ammonium acetate in to 1000mL of HPLC water, degas it with 0.45μ filter) and acetonitrile (50:50 %v/v), degassed. A reversed phase C18 column (3.9x150mm, 5.0μm). The flow rate was set at 1.0mL per min and the injection volume was 10μL. Diluent is a mixture of 1:1 %v/v ratio of water and acetonitrile.

**Dissolution conditions:**

900mL of 0.1N HCl as dissolution medium, using a USP-II (paddle) apparatus at a stirring rate of 50rpm for 40min.

**Standard solution preparation:**

Weighed accurately 50mg of donepezil hydrochloride, transferred in to a 100mL of volumetric flask, added 40mL of diluent and sonicate for 10min and
diluted to volume with diluent and further diluted 5 mL of the centrifuged supernatant solution in to 50mL with diluent. For dissolution analysis prepared the standard solution equivalent to 11.1ppm.

**Test solution preparation:**

Weighed the crushed tablets powder accurately equivalent to 50mg of donepezil hydrochloride and transferred in to a 100mL of volumetric flask and 40mL of diluent has added, sonicated for 10min and diluted to volume with diluents. Further diluted 5mL of the centrifuged supernatant solution into 50mL with diluent.

**Procedure:**

Separately injected equal volume of blank, standard preparation in replicate, sample preparation once in to the HPLC system and record the chromatograms and calculated the results.

**System Suitability solution:**

The tailing factor of donepezil peak in standard solution is not more than 2.0 and the percent (%) RSD of five replicate injections area is not more than 2.0 percent (%).

**Calculation:**

\[
\text{Percentage} \,(\%) \text{ of donepezil hydrochloride} = \frac{A_X \cdot W_S \cdot \text{Avg. wt of tablet} \cdot P}{A_s \cdot W_T \cdot \text{Label Claim}}
\]

Where in As is the area of donepezil hydrochloride standard solution; At is the area of donepezil hydrochloride test sample solution, WS is the donepezil hydrochloride standard weight taken for standard solution preparation in mg,
WT is the weight taken for test solution preparation in mg and P is the potency of donepezil hydrochloride standard.

**III.ii.2.3. Amiodarone hydrochloride tablets:**

**Chromatographic conditions:**

Chromatographic separations were performed using isocratic elution at ambient temperature, UV detector set at 240nm. The mobile phase was composed of a mixture of buffer (2.0gm of ammonium acetate in 1000mL of HPLC water, degas it with 0.45µ filter) and acetonitrile (15:85 %v/v), degassed and run into a reversed phase C2 column (4.6 X 250mm, 5.0µm). The flow rate was set at 1.0mL per min and the injection volume was 20µL. Diluent is a mixture of 1:1 %v/v ratio of water and acetonitrile.

**Dissolution conditions:**

1000mL of 1.0% SLS in water as dissolution medium, using a USP-II (paddle) apparatus at a stirring rate of 100rpm for 90min.

**Preparation of standards:**

Weighed accurately 50mg of amiodarone hydrochloride standard and transferred in to a 100mL volumetric flask, added 75mL of diluent, sonicated for 15min and diluted upto mark and further diluted 5mL of resoluting solution in to 50mL with diluent. For dissolution analysis, prepare the standard solution equivalent to sample concentration.
**Test solution preparation:**

Weighed accurately equivalent to 50mg of amiodarone hydrochloride and transferred in to a 100mL of volumetric flask and add 40mL of diluent and sonicated for 10min and diluted to volume with diluents. Further diluted 5mL of the centrifuged supernatant solution in to 50mL with diluent.

**Procedure:**

Separately injected equal volumes of blank, standard preparation in replicate, sample preparation once in to the HPLC system and record the chromatograms and calculated the results.

**System Suitability solution:**

The tailing factor of amiodarone peak in standard solution is not more than 2.0 and the percent (%) RSD of five replicate injections area is not more than 2.0 percent (%).

**Calculation:**

\[
\text{Percentage (\%)} \text{ of amiodarone hydrochloride} = \frac{At \times WS \times \text{Avg. wt of tablet} \times P}{As \times WT \times \text{Label Claim}}
\]

Where in As is the Area of amiodarone standard solution, At is the Area of amiodarone test sample solution, WS is the amiodarone standard weight taken for standard solution preparation in mg, WT is the Weight taken for test solution preparation in mg and P is the potency of amiodarone standard.
III.ii.2.4. Sildenafil citrate tablets:

Chromatographic conditions:

Chromatographic separations were performed using isocratic elution at ambient temperature. The mobile phase was composed of a mixture of buffer (8.70g of K$_2$HPO$_4$ in to 1000ml of HPLC water) and acetonitrile (40:60 %v/v) and degassed. Sildenafil citrate and three related impurities have separated on a reversed phase C18 column (4.6 x 250mm, 5.0µ). The flow rate was set at 1.0mL per min and the injection volume was 20µL. UV measurements were made at a wavelength of 225nm.

Dissolution conditions:

Dissolution was performed using by dropping six tablets in six unit Lab India Disso 2000 instrument. The dissolution medium was 900mL 0.01NHC1 with USP apparatus-I (basket) with 100rpm for 45 minutes and water bath temperature of 37°C ± 0.5°C.

Standard Preparation:

Weighed accurately 55.5mg of sildenafil citrate and transferred in to a 100mL of volumetric flask and added 40mL of diluent and sonicated for 10min and diluted to volume with diluents. Further diluted 5 mL of the centrifuged supernatant solution in to 50mL and diluted with diluent.

Test solution preparation for Assay:

Weighed the test sample accurately equivalent to 55.5mg of sildenafil citrate in to 100mL of volumetric flask and added 40mL of diluent and
sonicated for 10 min and diluted to volume with diluents. Further diluted 5 mL of the centrifuged supernatant solution into 50 mL with diluent.

Test solution preparation for dissolution:

Selected six tablets and introduced one each tablet into six different dissolution flasks containing 900 mL of dissolution medium (previously equilibrated at 37°C ± 0.5°C). Stirred it for 45 minutes then withdrawn 10 mL sample from dissolution vessel and further diluted 5 mL into 10 mL with medium.

Procedure:

Separately injected equal volume of blank, standard preparation in replicate, sample preparation once into the HPLC system and record the chromatograms and calculated the results.

System Suitability solution:

The tailing factor of sildenafil citrate peak in standard solution is not more than 2.0 and the percent (%) RSD of five replicate injections area is not more than 2.0 percent (%).

Calculation:

\[
\text{Percentage of sildenafil citrate} = \frac{At \times DS \times P}{As \times DT}
\]

Where in \(As\) is the area of standard solution, \(At\) is the area of test sample solution, \(DT\) is the dilution factor for test solution preparation, \(DS\) is the sildenafil citrate standard dilution factor for standard solution preparation and \(P\) is the potency of sildenafil citrate.
III.ii.2.5. Ear drops (composed of beclomethasone dipropionate-0.025%w/v, chloramphenicol-5.0%w/v, clotrimazole-1.0%w/v and lidocaine-1.73%w/v):

A single RP-HPLC method was developed for the estimation of beclomethasone dipropionate, chloramphenicol, clotrimazole and lidocaine in ear drops simultaneously.

**Chromatographic conditions:**

Chromatographic separations were performed using isocratic elution at ambient temperature and UV absorbance at 254nm. The mobile phase was gradient program, buffer (1.6gm of ammonium acetate in 1000 mL of HPLC water, add 10mL of tri-ethylamine and adjust the pH 6.4± 0.1 with diluted acetic acid and degas it with 0.45μ filter) and acetonitrile. The flow rate was set at 1.0 mL per min. Gradient program (0-4 min 35% acetonitrile; 4-8 min linear from 35% CH₃CN to 65% CH₃CN; 8-15min 65% CH₃CN; 15-20min linear from 65% CH₃CN to 45% CH₃CN; 20-22min linear from 45% CH₃CN to 35% CH₃CN; 22-25min linear from 35% CH₃CN) with a reversed phase C18 column (4.6 x 250mm, 5.0μm), the injection volume was 20μL. Diluent is a mixture of buffer and Acetonitrile 1:1 %v/v ratio.

**Beclomethasone dipropionate standard stock solution:**

Weighed accurately 40mg of beclomethasone dipropionate and transferred in to a 100mL volumetric flask, added 75mL of diluent and sonicated to dissolve and then diluted to volume with diluent.
Standard solution:

Weighed accurately 75mg of chloramphenicol, 26mg of lidocaine and 15mg of clotrimazole and transferred in to a 100mL volumetric flask added 75mL of diluent and 1.0mL of beclomethasone standard stock solution and sonicated for 10min, diluted to volume with diluent.

Test preparation:

Transferred 1.5mL of test solution in to a 100mL volumetric flask, added 75mL of diluent, sonicated for five minutes and dilute to volume with diluent.

System Suitability:

The %RSD of five replicate standard injections is NMT 2.0%. Tailing factor of standard peaks is NMT 2.0.

Calculations:

Percentage (%) of beclomethasone dipropionate =

\[
\frac{At \times CS \times P}{As \times CT}
\]

Where in As is the area of beclomethasone dipropionate standard solution; At is the area of beclomethasone dipropionate test sample solution, CS is the beclomethasone dipropionate concentration for standard solution preparation, CT is the concentration of beclomethasone dipropionate in test solution and P is the potency of beclomethasone dipropionate standard.

Percentage (%) of chloramphenicol =

\[
\frac{At \times CS \times P}{As \times CT}
\]
Where in As is the area of chloramphenicol standard solution, At is the Area of chloramphenicol test sample solution, CS is the chloramphenicol concentration for standard solution preparation, CT is the concentration of chloramphenicol in test solution and P is the potency of chloramphenicol standard.

**Percentage (%) of clotrimazole =**

\[
\frac{At \times CS \times P}{As \times CT}
\]

Where in As is the area of clotrimazole standard solution, At is the area of clotrimazole test sample solution, CS is the clotrimazole concentration for standard solution preparation, CT is the concentration of clotrimazole in test solution and P is the potency of clotrimazole standard.

**Percentage (%) of lidocaine =**

\[
\frac{At \times CS \times P}{As \times CT}
\]

Where in As is the area of lidocaine standard solution, At is the area of lidocaine test sample solution, CS is the lidocaine concentration for standard solution preparation, CT is the concentration of lidocaine in test solution and P is the potency of lidocaine standard.

**III.ii.2.6. Antiseptic solution (composed of chloroxylenol 4.8% w/v and terpineol 9.0% v/v):**

**Chromatographic conditions:**

Chromatographic separations were performed using isocratic elution at ambient temperature. Detector was at 195nm, the mobile phase was composed
of a mixture of buffer (4.0gm of Sodium perchlorate in to 1000mL of HPLC water, degas it with 0.45μ filter) and acetonitrile 62:38 %v/v. and degassed, C18 column (4.6mm x150mm, 3.5μM), flow rate was set at 1.0 mL per min and the injection volume was 20μL.

**Preparation of Standard:**

Standard solution was prepared with chloroxylenol and terpineol standards to get known concentration of 48ppm for chloroxylenol and 90ppm for terpineol with methanol.

**Preparation of test sample:**

Pipette the liquid test sample to get known concentration of chloroxylenol 48ppm and terpineol 90ppm.

**Procedure:**

Separately injected equal volumes of 20μL of blank, standard preparation in replicate, sample preparation once in to the HPLC system and record the chromatograms and calculated the results.

**System Suitability solution:**

The tailing factor of both ingredients peaks in standard solution is not more than 2.0 and the percent (%) RSD of five replicate injections area is not more than 2.0 percent (%).

**Calculations:**

Percentage (%) of chloroxylenol=

\[
\frac{At \times CS \times XP}{As \times CT}
\]
Where in As is the area of chloroxylenol standard solution, At is the Area of chloroxylenol test sample solution, CS is the chloroxylenol concentration for standard solution preparation, CT is the concentration of chloroxylenol in test solution and P is the potency of chloroxylenol standard.

\[
\text{Percentage (\%) of terpineol} = \frac{At \times CS \times P}{As \times CT}
\]

Where in As is the Area of terpineol standard solution, At is the Area of terpineol test sample solution, CS is the terpineol concentration for standard solution preparation, CT is the concentration of terpineol in test solution and P is the potency of terpineol standard.