3. MATERIALS & METHOD

3.1 DRUG PROFILE

1. DARUNAVIR\textsuperscript{158,159}

Structure:

![Darunavir Structure](image)

IUPAC Name:

\[[(1R,5S,6R)-2,8-\text{Dioxabicyclo}[3.3.0]\text{oct-6-yl}]\text{-}N\text{-}[(2S,3R)-4\text{-}[(4\text{-}aminophenyl)sulfonyl}(2\text{-}methylpropyl)amino]\text{-}3\text{-}hydroxy\text{-}1\text{-}phenyl\text{-}butan-2\text{-}yl]\text{carbamate.}

**Molecular Formula:** C\textsubscript{27}H\textsubscript{37}N\textsubscript{3}O\textsubscript{7}S

**Molecular Weight:** 547.66

**Description:**

Darunavir is hygroscopic powder and white to off-white in colour. It contains 5 chiral centres, however the manufacturing process leads, in a consistent way, to the single enantiomer 3R, 3aS, 6aR, 1S, 2R. The absolute configuration has been confirmed by X-ray diffraction analysis.

**Solubility:** Its solubility in organic solvents varies significantly and it is very slightly soluble in aqueous solution (solubility directly proportional to pH). Therefore, the particle size is likely to be important to the rate and possibly to the extent of absorption of darunavir. Under commercial synthesis conditions, darunavir is isolated as a crystalline ethanolate (1:1 solvate). It can exist as a non-solvated amorphous form and as a hydrate form as well. Investigations of conditions under which
inter conversion between the different polymorphs occur showed that the hydrate form can be formed under conditions of high relative humidity and that both solvates can be converted into the amorphous form when subject to heat and/or extremely low relative humidity.

**Melting Point:** 74-76 °C

**Indications:** Prezista 75-, 150-, 300- and 600-mg tablets containing Darunavir, may be used to give applicable dose regimens:

1. for patients experience HIV-1 infection in antiretroviral-treatment (ART) even those of highly pre-treated.
2. for patients experience HIV-1 infection in ART of 3 years age & minimum 15 kg body weight.

Prezista 400-, 800-mg tablets may be used to give applicable dose regimens:

1. for ingenuous adults experience HIV-1 infection in ART.
2. for patients experience HIV-1 infection in ART with no darunavir-resistance-associated mutations (DRV- RAMs) and who have plasma HIV-1 RNA < 100,000 copies/ml and CD4+ cell count ≥ 100 cells x 10^6/l. In judging to begin treatment with Prezista in such patients experience ART, genotypic testing must guide for the use of Prezista.

Prezista 100-mg/ml oral suspension co-administration with low dose ritonavir is marked in combination with other antiretroviral medicinal products for the treatment of HIV-1 infection in adult patients as well as paediatric patients experiencing ART patients of 3 years age & minimum 15 kg body weight.

The recommended indication is: Darunavir co-administered with 100 mg ritonavir in combination with other antiretroviral medicament for treatment of HIV-1 infection in adult patients those highly pre-treated and also those who failed more than one regimen containing a protease inhibitor (PI).

**Contra-indications:**

Darunavir must not be taken those have hypersensitive to darunavir or any of the other ingredients. It should not be recommended those have acute problems with their liver, or who are administering the below medicines:

- rifampicin
- ritonavir-boosted lopinavir;
- St John’s wort (herbal preparation used for treatment depression);
medicines that are worn out in similar way as Darunavir and are dangerous at elevated levels in blood.

Adverse Effect:

In adults, generally side effects are diarrhoea, ‘immune reconstitution syndrome’, nausea, pyrexia and rash. Immune reconstitution syndrome happens when the patient’s immune system works again and fights existing infections, causing inflammation at the site of the infection. Side effects are same in children and also adolescents.

2. ROPIVACAINE

Structure:

![Ropivacain](image)

IUPAC Name: \((S)-N-(2,6-Dimethylphenyl)-1-propylpiperidine-2-carboxamide.\)

Molecular Formula: \(C_{17}H_{26}N_2O\)

Molecular Weight: 274.40

Description: It is a crystalline powder white in colour.

Solubility: Ropivacaine HCl is soluble in water of 53.8 mg/mL at 25°C, a distribution ratio between n-octanol and phosphate buffer at pH 7.4 of 14:1 and a pKa of 8.07 in 0.1 M KCl solution. Ropivacaine has an intermediate degree of lipid solubility as compared with bupivacaine and mepivacaine.

Melting Point: 144-146 °C

Indications:

Ropivacaine is a long-acting amide local anaesthetic agent that first formed as a pure enantiomer. It yields similar effects like other local anaesthetics via reversible inhibition of \(Na^+\) influx in nerve fibres. Ropivacaine has low lipophilicity than bupivacaine and is less likely to enter large myelinated motor fibre, in result decreased motor blockade occurs. Therefore, ropivacaine has a high degree of motor sensory variation that could be essential if motor blockade is not desirable. The decreased lipophilicity is related with decrease in potential for CNS toxicity and cardiotoxicity. The drug shows linear and dose proportional pharmacokinetics (up to
80 mg i.v.). It is metabolized in the liver & by urine it excreted.

Contra-indications:

Ropivacaine is contraindicated for intravenous regional anaesthesia (IVRA). However, as per latest information both ropivacaine (1.2-1.8mg/kg in 40ml) and levobupivacaine (40ml of 0.125% solution) may be used, due to their less CVS and CNS toxicity than racemic bupivacaine.

3. TELMISARTAN.\textsuperscript{162,163}

Structure:

IUPAC Name: 4'-(1',7'-Dimethyl-2'-propyl-1H,3'H-2,5'-bibenzod[\textit{d}]imidazol-3'-yl)methyl)biphenyl-2-carboxylic acid.

Molecular Formula: C\textsubscript{33}H\textsubscript{30}N\textsubscript{4}O\textsubscript{2}

Molecular Weight: 514.62

Description:

It is an angiotensin II receptor antagonist recommended for hypertension. Generally, angiotensin II receptor blockers (ARBs) such as telmisartan bind to the angiotensin II type 1 (AT1) receptors with bigger affinity, sush that the action of angiotensin II inhibited on vascular smooth muscle, resulting in reduction of arterial blood pressure. Recent learnig recommend that telmisartan may also have PPAR-gamma agonistic properties that could potentially deliberate useful metabolic effects.

Solubility:

It is a crystalline hygroscopic powder white or off white in colour. Practically insoluble in water, while solubility in DMSO: >5 mg/mL at 60 °C.

Melting Point: 261-263°C

Indications:

1. Hypertension.
3. Cardiovascular prevention.
4. Reduction of cardiovascular morbidity in patients with:
   a. manifest atherothrombotic cardiovascular disease
b. type 2-diabetes mellitus with documented target organ damage.

**Adverse effect:**

With Telmisartan side effects are not common. However, side effects are found in between 1 and 10 patients in 1,000 as: upper respiratory tract infection (colds) including inflammation of throat and sinuses, urinary tract infection including bladder infection, anaemia, hyperkalaemia, depression, insomnia, syncope, vertigo, bradycardia, hypotension, dyspnoea, cough, abdominal pain, diarrhoea, dyspepsia, flatulence, vomiting, hyperhidrosis, pruritus, rash, myalgia, back pain, muscle spasms, renal impairment including sudden kidney failure, chest pain, asthenia and increased blood levels of creatinine. Hypotension may be more general in patients taking Telmisartan to stop cardiovascular problems.

**4. PALIPERIDONE.**

**Structure:**

![Paliperidone molecule](image)

**IUPAC Name:**

3-(2-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)ethyl)-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one.

**Molecular Formula:** C\textsubscript{23}H\textsubscript{27}FN\textsubscript{4}O\textsubscript{3}

**Molecular Weight:** 426.48

**Description:**

Paliperidone is a non-hygroscopic powder white to yellow in colour. There is one chiral centre in palliperidone which is synthesized as a racemic mixture. In addition to a hydrate two polymorphs were observed, polymorph I and II, and a solvate. In the final active substance only polymorph I is present, which is the thermodynamically stable crystal form. It has been observed that all examined active substance batches are of same crystalline form I.

**Solubility:**

Dissociation constants of Paliperidone are pKa1 = 8.2 (piperidine moiety) and pKa2 = 2.6 (pyrimidine moiety). Its solubility in water is 0.003 g/100 ml, increasing to 2.3 g/100 ml in 0.1 N HCl, and in ethanol is 0.076 g/100 ml. The logP
of substance as a neutral molecule in a 1-octanol/aqueous buffered solution (pH 11.9) is 2.39, where as logP of the substance regardless of its form in phosphate solution (pH 7.0) is 1.02.

**Melting Point:** 158-160 °C

**Indications:** Paliperidone is recommended for the treatment of schizophrenia. Paliperidone is recommended for the treatment of psychotic or manic symptoms of schizoaffective disorder. Effect on depressive symptoms has not been illustrated.

**Side Effects:**

Most frequently reported side effects are headache, insomnia (difficulty sleeping), sleepiness, parkinsonism (neurological symptoms including tremor and impaired muscular control), akathisia (restlessness), tachycardia (increased heart rate), dystonia (involuntary muscle contractions), tremor (shaking), upper respiratory tract infection (colds), anxiety, dizziness, increased weight, nausea, agitation, constipation, vomiting, fatigue (tiredness), depression, dyspepsia (heartburn), diarrhoea, dry mouth, toothache, muscle and bone pain, asthenia (weakness), high blood pressure, back pain, and prolonged electrocardiogram QT (an alteration of the electrical activity of the heart).

5. **ZALEPLON.**

**Structure:**

![Zaleplon Structure](image)

**IUPACName:** N-(3-(3-Cyanopyrazolo[1,5-a]pyrimidin-7-yl)phenyl)-N-thylacetamide.

**Molecular Formula:** C_{17}H_{15}N_{5}O

**Molecular Weight:** 305.33

**Description:** Pure zaleplon is solid white to off-white powder. Its partition coefficient in octanol/water t is constant (log PC = 1.23) in pH 1 - 7.

**Solubility:** It is sparingly soluble (Low solubility) in water, alcohol and propylene glycol.

**Melting Point:** 157-159 °C
Indications: Zaleplon is recommended for treatment of patients with insomnia who are difficult to falling asleep. It is recommended only when this disorder is serious, disabling or leading patient to utmost distress.

Side effects:

Usually most commonly side effects are amnesia, paraesthesia, somnolence and dysmenorrhoea. People should not use zaleplon who are hypersensitive (allergic) to zaleplon or any of other ingredients. Patients should not use it having acute liver or kidney problems sleep apnoea syndrome, myasthenia gravis or acute respiratory inadequacy or in patients below aged18 years.
3.2 MATERIALS:

The drugs used for the present investigation were donated as gift samples.

A. Bulk Drug

1. **Darunavir**:
   - Supplied by: Hetero drugs Ltd, Hyderabad
   - Quantity: 10.0 g
   - Purity (Assay): 99.82% w/w

2. **Ropivacain**:
   - Supplied by: Lupin Ltd, Pune
   - Quantity: 10.0 g
   - Purity (Assay): 99.8% w/w

3. **Telmisartan**:
   - Supplied by: Hetero drugs Ltd, Hyderabad
   - Quantity: 10.0 g
   - Purity (Assay): 99.83% w/w

4. **Paliperidone**:
   - Supplied by: Hetero drugs Ltd, Hyderabad
   - Quantity: 10.0 g
   - Purity (Assay): 99.87% w/w

5. **Zaleplon**:
   - Supplied by: Hetero drugs Ltd, Hyderabad
   - Quantity: 10.0 g
   - Purity (Assay): 99.21% w/w

B. Marketed Formulation:

The marketed preparation was purchased from the local market and is referred hereafter in this thesis by the name as such.

1. **Darunavir**:
   - Brand Name: Prezista
   - Manufactured by: Tibotec Company
   - Composition: Darunavir 300 mg

2. **Ropivacain**:
   - Brand Name: Naropin polyamp injectable solution
   - Manufactured by: AstraZeneca
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3. Telmisartan:
   - Brand Name: Telma
   - Manufactured by: Glenmark Pharma Ltd
   - Composition: Telmisartan 20 mg

4. Paliperidone:
   - Brand Name: Lamogin
   - Manufactured by: FDC Limited
   - Composition: Paliperidone 25 mg

5. Zaleplon:
   - Brand Name: Stilnite
   - Manufactured by: Zydus Cadila Healthcare Ltd
   - Composition: Zaleplon 5 mg

C. Reagents and chemicals
All reagents and chemicals used were of AR analytical and HPLC grade.

1. Methanol (AR grade)
2. Methanol (HPLC grade)
3. Sodium Dihydrogen Phosphate
4. Potassium Dihydrogen Phosphate
5. Water (HPLC grade)
6. Acetonitrile (HPLC grade)
7. 1, 10-Phenanthroline (Qualigens Fine Chemicals, Mumbai, India, assay 100%)
8. 2,2’-bipyridyl (Qualigens Fine Chemicals, Mumbai, India, assay 100%)
10. Acetone (S. D. Fine Chem., Mumbai, India)
11. p-Phosphoric acid (Merck, Mumbai, India, sp. gr. 1.75)
12. Whatman filter paper no 42
13. 1, 2-naphthoquinone-4-sulphonate (SD Fine Chem.)
14. Monobasic potassium phosphate (Aldrich)
15. Sodium hydroxide (Aldrich)
16. Boric acid (Aldrich)
17. Potassium chloride (Aldrich)
18. Ferric chloride hexahydrate (S. D. Fine Chem., Mumbai, India)
3.3 INSTRUMENTS:

A. Spectrophotometer

1) Double beam UV –visible spectrophotometer with 10 mm matched quartz cell
   i. Model             UV 2401 PC (Japan)
   ii. Make              Thermo

2) Double beam UV –visible spectrophotometer with 10 mm matched quartz cell
   i. Model            UV1601
   ii. Make            Shimadzu

3) HPLC
   Thermo Separation Product Quaternary Gradient HPLC System
   • Thermo Separation Product Quaternary Gradient
   • HPLC pump Spectra System P4000
   • Degasser – Membrane degasser
   • Rheodyne injector 9725 I with 20 µl loop
   • Variable UV-VIS detector of Spectra System U V 1000 with Data Ace Software.
   • Column Machenery .Nagel (M N) EC 250/4.6 Grace C18 column {4.6 x 250 mm (id)}.

B. Analytical balance:  Citizen Balance (C Y-104)

C. pH Meter:  Mettler Toledo

D. Sonicator:  Oscars

E. HPTLC assembly:  A Camag HPTLC system comprising of Camag Linomate V automatic sample applicator, Hamilton syringe (100 µl), densitometer (Model-CD60, Desega, Germany), Camag TLC scanner 3, Camag Wincats software.
3.4.1 VISIBLE SPECTROPHOTOMETRIC DETERMINATION OF DARUNAVIR & ROPIVACAINE BY REACTING WITH NQS.

Preparation of Reagent and Standard Stock Solution:

Reagents:

250 mg 1, 2-naphthoquinone-4-sulphonate (NQS) was dissolved in 50mL distilled water to get 0.5% (w/v) solution of NQS. This solution was freshly prepared and kept away from light during its use. In 200 mL standard flask 50 mL of 0.2M aqueous solution of boric acid (Content of boric acid - 12.368 g / litre ) and potassium chloride (Content of potassium chloride-14.90g / litre) was mixed with 21.3 mL of 0.2M sodium hydroxide and calibrated by the pH meter & the prepared solution was Clark and Lubs buffer solution pH 9

Darunavir (DN) & Ropivacain (RP) Standard Solution.

An accurately weighed amount (50 mg) of DN & RP were quantitatively transferred into two different 25 mL calibrated flasks, dissolved in 20 mL distilled water, completed to volume with the same solvent to obtain a stock solution of 2 mgmL$^{-1}$. Then these stock solutions were further appropriately diluted with water to get working solutions in range of 10–80 µgmL$^{-1}$.

Tablets Sample Solution.

An accurately weighed quantity of the powdered tablets/injectable equivalent to 100 mg of DN & RP was transferred into a 100mL calibrated flask and in about 40mL of distilled water was dissolved. Then the flasks content were thoroughly swirled and sonicated for about 5 minutes, and then with water made to volume. The contents were mixed thoroughly and then filtered, removing the first portion of filtrate. Then that solution was appropriately diluted quantitatively with distilled water to get a suitable concentration for analysis.

General Recommended Procedure:

For NQS:

Accurately measured aliquots of DN & RP solutions containing 10–50 µgmL$^{-1}$ were transferred into separate 10mL calibrated flasks. Then added 1mL Clark and Lubs buffer solution (pH 9) followed by 1mL of 0.5%, w/v NQS solution. The reaction solution was allowed to proceed by keeping in room temperature (25 ± 5° C) for about 10 minutes. The reaction mixture was made completed to volume with methanol,
and the resulting solution was measured at 488 nm for DN & 565 nm for RP against reagent blank treated similarly. The absorption spectra are presented in Fig. 4.1.1 & 4.1.2.

**Influence of pH:**

Job’s method of continuous variation was employed. Master equimolar (5 × 10^{-3} M) aqueous solutions of DN/RP and NQS were prepared. Series of 10mL portions of the master solutions of DN/RP and NQS were made up comprising different complementary proportions (0 : 10, 1 : 9, . . . , 9 : 1, 10 : 0, inclusive) in 10mL calibrated flasks which is containing 1mL of pH 9 buffer solution. The solution was manipulated as described under the general recommended procedures. The results are in figure 4.1.3 & 4.1.4.

**Influence of amount of 1, 2-naphthoquinone-4-sulfonic acid sodium:**

In limiting logarithmic method, 02 sets of experiments were carried out making use of general recommended procedures described above. The first set of experiments was carried out using increasing NQS concentrations (1.9 × 10^{-3} – 9.6 × 10^{-3} M) at fixed DN/RP concentration (1.37 × 10^{-5} M). The second set of experiments was carried out using increasing DN/RP concentrations (0.3 × 10^{-5} – 2.04 × 10^{-5} M) at fixed concentration (1.92 × 10^{-2} M) of NQS. The logarithms of the obtained absorbances were plotted as function of the logarithms of the NQS and DN/RP concentration in the first and second sets of experiments, respectively. The slopes of the fitting lines in these sets of experiments were calculated. The results are in figure 4.1.5 & 4.1.6.

**Influence of surfactant**

Accurately measured aliquots of DN & RP solutions containing 10–50 µgmL^{-1} were transferred into separate 10mL calibrated flasks. One milliliter of Clark and Lubs buffer solution (pH 9) and then one of various surfactant was added followed by 1mL of NQS solution (0.5%, w/v). Then the reaction solution was allowed to proceed by keeping at room temperature (20 ± 5° C) for 10 minutes. The reaction mixture was make completed to volume with methanol, and the resulting solution was measured at 488 nm for DN & 565 nm for RP against reagent blank treated similarly. The results are in figure 4.1.7 & 4.1.8.
**Effect of sequence of reagents added**

Accurately measured aliquots of DN & RP solutions containing 10–50 µgmL$^{-1}$ were transferred into separate 10mL calibrated flasks. Then added 1mL of Clark and Lubs buffer solution (pH 9) followed by 1mL 0.5%, w/v NQS solution. The reaction solution was allowed to proceed by keeping at room temperature (25 ± 5° C) for 10 minutes. The reaction mixture was made completed to volume with methanol, and the resulting solution was measured at 488 nm for DN & 565 nm for RP against the reagent blank treated similarly. The sequence of reagents to be added was varied in the above procedure and the observations were made. The results are listed in tabulated form in Table 4.1.1.

**Influence of standing time**

Accurately measured aliquots of DN & RP solutions containing 10–50 µgmL$^{-1}$ were transferred into separate 10mL calibrated flasks. Then added 1mL of Clark and Lubs buffer solution (pH 9) followed by 1mL 0.5%, w/v NQS solution. The reaction solution was allowed to proceed by keeping at room temperature (20 ± 5° C) for 5mins, 10mins, 15mins, 20mins, 25mins & 30 minutes. The reaction mixture was made completed to volume with methanol, and the resulting solution was measured at 488 nm for DN & 565 nm for RP against reagent blank treated similarly. The results are in tabulated form in Table 4.1.2.

**Influence of organic solvent**

Accurately measured aliquots of DN & RP solutions in one of the different organic solvent mentioned containing 10–50 µgmL$^{-1}$ were transferred into separate 10mL calibrated flasks. Then added 1mL of Clark and Lubs buffer solution (pH 9) followed by 1mL 0.5%, w/v NQS solution. The reaction solution was allowed to proceed by keeping at room temperature (25 ± 5° C) for 10 minutes. The reaction mixture was made completed to volume with methanol, and the resulting solution was measured at 488 nm for DN & 565 nm for RP against reagent blank treated similarly. The results are in figure 4.1.9 & 4.1.10.

**Recovery Studies:**

On view of the recovery studies carried out by the standard addition method the accuracy of proposed method was confirmed. The results of the recovery studies and the statistical data are recorded in Table 4.1.3.
**MATERIALS & METHODS**

**Procedure:** Same as described above in procedural part. The percent recovery was then determined by the formula;

\[
\text{% Recovery} = \frac{A}{B+C} \times 100
\]

Where-

- \( A \) = Total amount of drug estimated.
- \( B \) = Amount of drug obtain on preanalysed basis.
- \( C \) = Amount of pure drug added.

**Analysis of marketed formulation by proposed method:**

1 g/mL of formulation is weighed then the solution is taken in volumetric flask sonicated for 5 min, diluted to make concentration of 50 \( \mu \)g/ml of drug. Then solution was filtered using Whatman filter paper no. 41. Absorbance of the sample solution was determined at their respective wavelength in 1 cm cell against the blank. Drug content in tablet was calculated using the formula;

\[
\text{% Label Claim} = \frac{C_x \text{ or } C_y}{W} \times 100 \quad \text{(a)}
\]

Where,

- \( C_x \) or \( C_y \) = Conc. of drug in gm/100ml.
- \( W \) = weight of sample taken.

Results are reported in the table 4.1.4.

**3.4.2 VISIBLE SPECTROPHOTOMETRIC DETERMINATION OF TELMISARTAN & PALIPERIDONE BY REACTING WITH FeCl₃:**

**Preparation of Reagent and Standard Stock Solution:**

**Reagents:**

1.35 g of the chemical dissolved in 100 ml of water for preparation of 0.05 M ferric chloride hexahydrate aqueous solution and it was stored in a dark 200 mL standard flask. Then the stock solution was made dilution with water to obtain working concentration of 0.0033 M, 1, 10-phenanthroline (0.01 M) which was prepared by dissolving 198 mg of the chemical in water and volume made up to 100 ml with water. O-phosphoric acid (0.02 M) Concentrated acid was appropriately
diluted with water to get the required concentration. The solution was freshly prepared earlier to its use in the experiment.

**Telmisartan (TR) & Paliperidone (PP) Standard Solution.**

An accurately weighed 50 mg of TR & PP were quantitatively transferred into two different 25 mL calibrated flasks, then dissolved in 20 mL distilled water, completed to volume with the same solvent to obtain a stock solution of 2 mgmL\(^{-1}\). Then these stock solutions were further appropriately diluted with water to get a working solution in the range of 10–80 µgmL\(^{-1}\).

**Tablet Sample Solution.**

An accurately weighed the powdered tablet equivalent to 50 mg of TR & PP were transferred into a 100 ml calibrated flask, then added 40 ml acetone and to extract the drug the content shaken intensely for about 15-20 min, and then filtered by Whatman No. 42 filter paper. After then filtrate and washings both were evaporated till to dryness. Then the residue was dissolved in water and diluted to the mark with water. First 10 ml of the filtrate was discarded and a suitable aliquot of the filtrate (500 µg ml\(^{-1}\) TR or PP) was diluted stepwise with water to get 10 µg ml\(^{-1}\) concentrations. The assay of TR or PP was completed by following the recommended procedures.

**Placebo blank analysis.**

A placebo blank of the composition: talc (10 mg), starch (10 mg), acacia (10 mg), methyl cellulose (10 mg), sodium citrate (10 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) was extracted with acetone and its solution was prepared as described under ‘procedure for tablets’, and then subjected to analysis.

**General Recommended Procedure:**

For FeCl\(_3\):

Separate aliquots (0.25 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml) of the standard10 µg ml\(^{-1}\) TR & PP solutions were exactly measured and then transferred into an order of 10 ml calibrated flasks and the total volume was made to 5 ml by water. Into each flask, first 1.5 ml of ferric chloride (0.0033 M) and then 2.5 ml of 1, 10-phenanthroline (0.01 M) added then after 0.5 ml of o-phosphoric acid (0.02 M) added and then volume was completed to 10 ml by distilled water. Then flasks were closed by stopper, the content sonicated thoroughly and flasks kept stand for 30 min with an occasional shaking. Then after absorbance of all the solution was determined.
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at 488 nm for TR & 565 nm for PP against reagent blank treated similarly. The absorption spectra are reported in Fig. 4.1.11 & 4.1.12.

Influence of amount of 1, 10-Phenanthroline:

The aliquot 2.0ml of the standard 10 µg ml\(^{-1}\) TR & PP solution were exactly measured then transferred into an order of 10 ml calibrated flasks and then with water total volume was made to 5 ml. To every flask, first 1.5 ml of ferric chloride (0.0033 M) and then 0.5, 1.0, 1.5, 2.0, 2.5 & 3.0 ml of 1, 10-phenanthroline (0.01 M) added succeeded by 0.5 ml of \(\alpha\)-phosphoric acid (0.02 M), and volume was completed 10 ml by distilled water. The flasks were closed by stopper, the content sonicated thoroughly and flasks kept stand for 30 min with a periodic shaking. Then absorbance of all the solutions was determined at 488 nm for TR & 565 nm for PP against the reagent blank treated similarly. The results are reported in figure 4.1.13 & 4.1.14.

Influence of amount of FeCl\(_3\):

The aliquot 2.0ml of the standard 10 µg ml\(^{-1}\) TR & PP solution were exactly measured and transferred into an order of 10 ml calibrated flasks and then with water total volume was made to 5 ml. To every flask, first 0.5, 1.0, 1.5, 2.0, 2.5 & 3.0 ml of ferric chloride (0.0033 M) and then 2.5 ml of 1, 10-phenanthroline (0.01 M) consecutively added succeeded by 0.5 ml of \(\alpha\)-phosphoric acid (0.02 M), and volume was completed to 10 ml by distilled water. The flasks were closed by stopper, the content sonicated thoroughly and the flasks kept stand for 30 min with a periodic shaking. Then, the absorbance of each solution was determined at 488 nm for TR & 565 nm for PP against the reagent blank treated similarly. The results are reported in figure 4.1.15 & 4.1.16.

Influence of surfactant

An aliquot 1.0 mL of the standard 10 µg ml\(^{-1}\) TR & PP solution were precisely measured and taken into a series of 10 ml calibrated flasks and then with water total volume was made to 5 ml. To every flask, 1.5 ml of ferric chloride (0.0033 M) and 2.5 ml of 1, 10-phenanthroline (0.01 M) were consecutively added succeeded by 0.5 ml of \(\alpha\)-phosphoric acid (0.02 M), and one of the different surfactant added then volume was completed to 10 ml by distilled water. The flasks were closed by stopper, the content mixed thoroughly and the flasks were kept to stand for 30 mins with a periodic shaking. Then, the absorbance of all solution was determined at 488 nm for TR & 565
Influence of organic solvent
An aliquot 1 mL of the standard 10 µg ml\(^{-1}\) TR & PP solution in different organic solvents were exactly measured and then transferred into an order of 10 ml calibrated flasks and then with water total volume was made to 5 ml. To every flask, first 1.5 ml of ferric chloride (0.0033 M) and then 2.5 ml of 1, 10-phenanthroline (0.01 M) added succeeded by 0.5 ml of orthophosphoric acid (0.02 M), and then volume was completed to 10 ml by distilled water. The flasks were closed by stopper, the content sonicated thoroughly and flasks kept to stand for 30 mins with an occasional shaking. Then, the absorbance of all solutions was determined at 488 nm for TR & 565 nm for PP against the reagent blank treated similarly. The results are reported in figure 4.1.19 & 4.1.20.

Influence of standing time
An aliquot 1.0 mL of the standard 10 µg ml\(^{-1}\) TR & PP solution were exactly measured and then transferred into an order of 10 ml calibrated flasks and then with water total volume was made to 5 ml. To every flask, first 1.5 ml of ferric chloride (0.0033 M) and then 2.5 ml of 1, 10-phenanthroline (0.01 M) added succeeded by 0.5 ml of orthophosphoric acid (0.02 M), and volume was completed to 10 ml by distilled water. The flasks were closed by stopper and content sonicated thoroughly and flasks kept to stand for 0min, 10mins, 20mins, 30mins, 40mins, 50mins & 60mins respectively, with an occasional shaking. Then, the absorbance of all solution was determined at 488 nm for TR & 565 nm for PP against the blank treated similarly. The results are tabulated in table 4.1.8.

Linearity and Sensitivity:
According to USP tablet powder was taken equal to 80, 90, 100, 110, and 120 percent of label claim and then dissolved & diluted thoroughly with mobile phase to get a concentration in range of 80-120 percent of test concentration. The resulting solutions chromatograms were recorded. Drugs in marketed formulation were got to be linear in a range ± 20% of test concentration of the individual drug.

Procedure: As per the optimized parameters the chromatographic conditions were adjusted and with stationary phase mobile phase was equilibrated as was marked by the steady baseline. Standard solutions of the different concentration were injected separately and the chromatograms were recorded. Peak areas were noted for each
injected concentration of drugs and the calibration curves, concentration against peak area were determined for the drugs. The results are noted in table 4.1.5.

Recovery Studies:
Accuracy of proposed method was evaluated on the basis of recovery studies carried out by standard addition method. The results of recovery studies and statistical data are recorded in Table 6.

Procedure: Same as described above in procedural part. The percent recovery was then determined by the formula;

\[
\% \text{ Recovery} = \frac{A}{B+C} \times 100
\]

Where-
A = Total amount of drug estimated.
B = Amount of drug found on preanalysed basis.
C = Amount of pure drug added.

Analysis of marketed formulation by proposed method:
1g/mL of formulation is weighed then the solution is taken in volumetric flask sonicated for 5min, diluted to make concentration of 50 µg/ml of drug. The solution was filtered using Whatman filter paper no. 41. Absorbance of the sample solution was determined at their respective wavelength in 1 cm cell against blank.

The drug content in tablet was calculated by the formula;

\[
\% \text{ Label Claim} = \frac{C_x \text{ or } C_y}{W} \times 100 \quad \text{(a)}
\]

Where,

\[ C_x \text{ or } C_y = \text{Conc. of drug in gm/100ml.} \]

\[ W = \text{weight of sample taken.} \]

Results are reported in the table 4.1.9.

Optimization of the reaction conditions:
The optimization parameters were perfected by carrying out proposed method by varying different units individually and observing response. The results are tabulated in table 4.1.7 & 4.1.8.
3.4.3 VISIBLE SPECTROPHOTOMETRIC DETERMINATION OF ZALEPLON BY REACTING WITH FeCl₃:

Preparation of Reagent and Standard Stock Solution:

Reagents:

1. 1.35 g ferric chloride hexahydrate dissolved in 100 ml water to get aqueous solution of 0.05 M ferric chloride hexahydrate then stored in a dark 200 mL standard flask. The stock solution was then diluted appropriately with water to get a working concentration of 0.0033 M. 1, 10-phenanthroline (0.01 M), prepared by dissolving 198 mg 1, 10-phenanthroline in water and diluted to 100 ml with water. O-phosphoric acid (0.02 M) was appropriately diluted with water to get the required concentration.

2. 2,2’-bipyridyl (0.01 M) Prepared by dissolving 156 mg 2, 2’-bipyridyl in water and diluted to volume in a 100 ml calibrated flask. The solution was prepared afresh just before using in the experiment.

Zaleplon (Zpl) Standard Solution.

An accurately weighed amount (50 mg) of Zpl were quantitatively transferred into two different 25 mL calibrated flasks, dissolved in 20 mL distilled water, completed to volume with the same solvent to obtain a stock solution of 2 mgmL⁻¹. Then these stock solutions were further appropriately diluted with water to get working solutions in the range of 10–80 µgmL⁻¹.

Capsule Sample Solution.

An accurately weighed quantity of the powder from capsule equivalent to 50 mg of ZPL was transferred into a 100 ml calibrated flask, 40 ml of acetone were added and the content shaken thoroughly for about 15-20 min to extract the drug, and filtered by Whatman No. 42 filter paper. The filtrate and washings both were evaporated till to dryness. The residue was dissolved in water and diluted to the mark with water. First 10 ml of the filtrate was discarded and a suitable aliquot of the filtrate (500 µg ml⁻¹ Zpl) was diluted stepwise with water to get 10-100 µg ml⁻¹ concentrations. The assay of Zpl was done by following the recommended procedures.

Placebo blank analysis.

A placebo blank of the composition: talc (10 mg), starch (10 mg), acacia (10 mg), methyl cellulose (10 mg), sodium citrate (10 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) was extracted with acetone and its solution was prepared as described under ‘procedure for capsules’, and then subjected to analysis.
General Recommended Procedure:

Method A:

Different aliquots (0.25 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml) of the standard 10 µg ml\(^{-1}\) ZPI solution were accurately measured and transferred into a series of 10 ml calibrated flasks by a micro burette and with water total volume was made to 5 ml. To every flask, 1.5 ml of ferric chloride (0.0033 M) and 2.5 ml of 1, 10-phenanthroline (0.01 M) were successively added succeeded by 0.5 ml of \(o\)-phosphoric acid (0.02 M), and the volume was brought to 10 ml with distilled water. The flasks were closed by stopper, the content mixed thoroughly and the flasks were allowed to stand for 30 min with an occasional shaking. Then, the absorbance of each solution was measured at 525 nm for Zpl against the reagent blank treated similarly. The absorption spectrum is reported as Fig. 4.1.21.

Method B:

Different aliquots (0.25 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml) of the standard 10 µg ml\(^{-1}\) ZPL solution were accurately measured and transferred into a series of 10 ml calibrated flasks by a micro burette and the total volume was adjusted to 5 ml by adding water. To each flask, 1.5 ml of ferric chloride (0.0033 M) and 2.5 ml 2,2' -bipyridyl (0.01 M) were successively added succeeded by 0.5 ml \(o\)-phosphoric acid (0.02 M), and the volume was brought to 10 ml with distilled water. The flasks were closed with stopper, the content mixed thoroughly and the flasks were allowed to stand for 30 min with occasional shaking. Then, the absorbance of each solution was measured at 535 nm for Zpl against the reagent blank treated similarly. The absorption spectrum is reported as Fig. 4.1.22.

Linearity and Sensitivity:

According to USP tablet powder was taken equal to 80, 90, 100, 110, and 120 percent of label claim and then dissolved & diluted thoroughly with mobile phase to get a concentration in range of 80-120 percent of test concentration. The resulting solutions chromatograms were recorded. Zpl in marketed formulation were got to be linear in a range ± 20% of test concentration of the drug.

Procedure: As per the optimized parameters the chromatographic conditions were adjusted and mobile phase was equilibrated with stationary phase as was indicated by the steady baseline. Standard solutions of different concentration were injected separately and the chromatograms were recorded. Peak areas were recorded for each
injected concentration of drugs and the calibration curves, concentration against peak area were constructed for the drugs. The results are noted in table 4.1.10.

**Recovery Studies:**

Accuracy of proposed method was ascertained on the basis of recovery studies performed by standard addition method. The results of recovery studies and statistical data are recorded in Table 4.1.11.

**Procedure:** Same as described above in procedural part. The percent recovery was then calculated by using formula;

\[
\% \text{ Recovery} = \frac{A}{B + C} \times 100
\]

Where-

- \(A\) = Total amount of drug estimated.
- \(B\) = Amount of drug found on preanalysed basis.
- \(C\) = Amount of pure drug added.

**Analysis of marketed formulation by proposed method:**

1g/mL of formulation is weighed then the solution is taken in volumetric flask sonicated for 5min, diluted to make concentration of 50 µg/ml of drug. The solution was filtered by Whatman filter paper no. 41. The absorbance of sample solution was determined at their respective wavelength in 1 cm cell against blank. The drug content in tablet was calculated by following formula;

\[
\% \text{ Label Claim} = \frac{C_x \text{ or } C_v}{W} \times 100 \quad (a)
\]

Where,

- \(C_x\) or \(C_v\) = Conc. of drug in gm/100ml.
- \(W\) = weight of sample taken.

Results are reported in the table 4.1.12.
3.5 HPTLC METHOD:

**Standard Solution:**
Standard bulk drug (10mg) was weighed exactly and dissolved in methanol to get a concentration of 100µg/mL of the drug.

**Formulation Solution:**
The content of 20 tablets/capsules/ampoules was obtained. Approximately 5mg of drug was taken into the volumetric flask and dissolved/diluted with methanol. Then solution was ultrasonicated for 15 mins. Filtered out extracts by Whatmann filter paper no 41 and the residue were washed with methanol. The extract and washings were mixed and then taken into a 250 mL volumetric flask and the volume was completed with methanol. Desired dilutions were made to get 100 µg/mL of drug. TLC plates were pre washed with methanol. Activation of the plates was done at 50°C for 5 mins.

**Chromatographic conditions:**
- **Stationary Phase:** Precoated silica gel 60F\textsubscript{254} aluminium sheets
- **Mobile phase:** Methanol: Chloroform: Triethylamine (1:8.8:0.2)
- **Migration Distance:** 65mm
- **Scanning wavelength:** 270nm
- **Slit Dimension:** 5 X 0.45mm
- **Radiation Source:** Deuterium Lamp
- **Sample volume:** 1µl-10 µl

**Procedure:**
Aliquots of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 µl of standard solution of drug were applied on the TLC plates at constant temperature. The TLC plates were dried, developed and analysed photometrically as mentioned previous. The standard calibration curve was generated using regression analysis with MS excel.

**Validation:**
A solvent system which gives dense and compact spot with marked Rf value was desired for quantification of drugs in bulk as well as the formulation. The intraday precision was evaluated by analyzing standard solutions in the range of 10-70ng/spot three times on the same day, while the interday precision was ascertained by analyzing corresponding standards for three days over a period of one week. Repeatability of
sample application was evaluated by spotting 1µl (10ng/spot) of the drug solution 5 times on a TLC plate, then followed by developing the plate then recording the peak area for five spots. Repeatability of measurement of peak was determined by spotting 1µl (10ng/spot) of drug (API or formulation mixture) on the TLC plate and developing the plate. The three level recovery studies were carried out for accuracy parameters. The results are tabulated in table 4.2.1 while the chromatograms are recorded in figure 4.2.1 & 4.2.2

1. **Linearity and Range:**
   
   In accordance with USP tablet powder was taken equal to 80, 90, 100, 110, and 120 percent of label claim and then dissolved & diluted thoroughly with mobile phase to get a concentration in range of 80-120 percent of test concentration. The resulting solutions chromatograms were recorded. Drugs in marketed formulation were got to be linear in a range ± 20% of test concentration of the individual drug.

   **Linearity of Response:** Aliquot portions of the standard stock solution A (2, 4, 6, 8, 10 ml) were diluted to 10.0 ml with mobile phase to get conc. of 10-100 µg/ml for drug.

   **Procedure:** As per the optimized parameters the chromatographic conditions were adjusted and with stationary phase mobile phase was equilibrated as was marked by the steady baseline. Standard solutions of different concentration were injected separately and the chromatograms were recorded. Peak areas were recorded/noted for each injected concentration of drugs and the calibration curves, concentration against peak area were determined for the drugs. The results are tabulated in table 4.2.1.

2. **Precision:**
   
   Precision of analytical method is expressed as SD and RSD of series of replicate measurements. Precision was evaluated by replicate analysis of homogeneous samples of drug powder. The results are reported in table 4.2.1.

3. **Accuracy:**
   
   Accuracy of proposed method was determined on basis of recovery studies carried out by standard addition method. The results of recovery studies and statistical data are recorded in Table No. 4.2.5 – 4.2.9.

   **Standard solution:** Standard solution C (conc. 100 µg/ml) was prepared. From that taken 2ml, 4ml, 6ml in 3 different 10 ml volumetric flask, completed the volume with mobile phase to get 20,40 and 60 µg/ml solution respectively.
Sample solution: Accurately weighed quantities of pre-analyzed tablet powder equivalent to about 10 mg of drug were transferred to 10.0 ml volumetric flask and adjust volume with mobile phase. In 1 ml of the above solution in three 10ml volumetric flask (100µg/ml) added the 1ml of 20, 40, 60 µg/ml standard solution were added in three different flasks. The flasks were sonicated for 15 minute and volumes were made up to the mark with mobile phase.

Procedure: Same as described under estimation of drug in tablet. The percent recovery was calculated by formula;

\[
\% \text{ Recovery} = \frac{A}{B + C} \times 100
\]

Where-

\( A \) = Total amount of the drug estimated.
\( B \) = Amount of the drug found on preanalysed basis.
\( C \) = Amount of the pure drug added.

4. Ruggedness:
The studies were brought about for various parameters i.e. different times (Intraday and Interday) and different analysts.

Ruggedness studies were brought about accordingly

I. Days
II. Analyst.

a. Interday (Different days):
Here procedure carried out was same as in marketed formulation analysis on different days. The % label claim was calculated. Data obtained for day 1, day 2, and day 3 is shown in Table 4.2.2

b. Intraday:
Here procedure was carried out same as in marketed formulation analysis and absorbances were recorded at interval of 3 hrs. in a day. The percent label claim was calculated. Result and statistical data are shown in Table 4.2.3.

c. Different analyst:
Two different analysts prepared sample solution and similar procedure was carried out as mentioned previous. The percent label claim was calculated like calculated in estimation of marketed formulation. Data are shown in Table 4.2.4
3.6 ESTIMATION OF DARUNAVIR, ROPIVACAINE, TELMISARTAN, PALIPERIDONE, AND ZALEPLON IN THEIR BULK AND DOSAGE FORM BY UV-VISIBLE SPECTROSCOPY.

A. Absorption Ratio Method:

a. **Drug stock standard solution:**

   An accurately weighed quantity of drug (10g) was transferred to the 100 ml volumetric flask, add 25 ml water and sonicate for 5 min. Then the volume was adjusted to the mark with water (100 mg/ml).

1. **Study of spectra and selection of wavelength:**

   The aliquot portions of stock standard solutions of drug were diluted properly with solvent to obtain concentration 20 mg/ml of each drug. Then the solutions were scanned in the range of 400 – 200 nm in 1 cm cell against the blank.

   Injection volume: 20µl

   Column: Machereny Nagel (MN) EC 250/4.6 Grace 100-5 C 18

A. The overlain UV absorbance spectrum of Darunavir and Ropivacaine is shown in Fig.

![Overlaid UV spectrum](image)

**Fig. 3.2.1: Overlain spectra of DN and RP**

From this spectrum the wavelengths picked for estimation of drugs were:

- $\lambda_{\text{max}}$ of DN = 282 nm
- $\lambda_{\text{max}}$ of RP = 322 nm
- Isobestic point = 305 nm
B. The overlain UV absorbance spectrum of Telmisartan and Paliperidone is shown in Fig. 3.2.2.

![Overlaid UV spectra of TR and PP](image)

**Fig. 3.2.2: Overlain spectra of TR and PP.**

From this spectrum the wavelengths picked for estimation of drugs were;

\[ \lambda_{\text{max}} \text{ of TR} = 286 \text{ nm} \]
\[ \lambda_{\text{max}} \text{ of PP} = 262 \text{ nm} \]

Isobestic point = 282 nm

C. The UV absorbance spectrum of Zaleplon is shown in Fig. 3.2.3.

![UV spectra of Zpl](image)

**Fig. 3.2.3: UV spectra of Zpl.**

From this spectrum the wavelength picked for estimation of drugs was;

\[ \lambda_{\text{max}} \text{ of Zpl} = 242 \text{ nm} \]

2. **Study of Beer-Lambert law:**

The aliquot portions of stock standard solutions of drug were diluted properly with solvent to get a series of concentration between 2-20 (µg/ml) of drug. Similarly aliquot portions of stock standard solutions were mixed and diluted to get series of concentration between 2-20 (µg/ml).
MATERIALS & METHODS

The absorbance of each solution was measured at 282nm, 322nm, 286nm, 262nm and 242nm for DN, RP, TR, PP & Zpl respectively, in 1 cm cell against solvent blank and tabulated in table 4.3.1. The graphs plotted as concentration Vs absorbance at set wavelengths are plotted in Fig 4.3.1-4.3.3.

3. Study of Additivity on absorbance for drugs:

The data obtained in the study of Beer – Lambert’s law was also again used to study additivity on absorbance of the drugs at their respective wavelength. Mixture of drugs shows additivity of absorbance at set wavelengths as mentioned in table 4.3.2.

4. Determination of Absorptivity values of drugs at selected wavelengths:

Aliquot portions of the drug stock standard solutions were diluted with the solvent to get the different concentration of each drug. Then absorbance of each solution was measured at their respective absorbance wavelength.

\[ A \ (1\%, \ 1\text{cm}) = \frac{\text{Absorbance}}{\text{Conc. (g/100ml)}} \]

Results of absorptivity values of drugs are shown in Table 4.3.3.

5. Analysis of marketed formulation by proposed method:

1g/mL of formulation is weighed/ calculated with the help of density bottle then the solution is taken in volumetric flask sonicated for 5min, diluted to make concentration of 50 µg/ml of drug. The solution was filtered by Whatman filter paper no. 41. The absorbance of sample solution was measured at their respective wavelength in 1 cm cell against blank. The drug content in tablet was calculated by using the the formula;

\[ \% \text{ Label Claim} = \frac{C_x \ or \ C_v \times 100}{W} \]  

Where,

\[ C_x \ or \ C_v \] = Conc. Of drug in gm/100ml.

\[ W \] = weight of sample taken.

Results are reported in the table 4.3.4.
VALIDATION OF PROPOSED METHOD:

1. **Accuracy:**
   
   It is the closeness of test results, got by that method to the true value. It was determined based on recovery studies carried out by standard addition method. Results are tabulated in Table 4.3.5-4.3.9.

2. **Precision:**
   
   It is the degree of agreement among individual test results when the method is employed repeatedly to multiple sampling of homogenous sample. It is expressed as Standard Deviation (S.D.), or Relative Standard Deviation (R.S.D.) of series of measurements and was determined by replicate estimation of the drugs by proposed method. Results are in Table 4.3.10.

3. **Ruggedness:**
   
   Ruggedness studies were brought about in two type conditions:
   
   - Days and Analyst
     
     a. **Intraday:** Here similar procedure as in marketed formulation analysis was performed and absorbance recorded at interval 3 hrs. in a day. The % label claim was calculated by formula as for marketed formulation analysis. Results are noted in Table 4.3.11.
     
     b. **Interday:** Here also same procedure as in marketed formulation analysis was performed and absorbances of same sample were recorded on different days. The % label claim was calculated by formula as in marketed analysis. Results and statistical data are shown in Table 4.3.12.
     
     c. **Different analyst:** Two different analysts prepared sample solutions and same procedure brought out as mentioned previous. The percentage label claim was calculated as calculated for estimation of marketed formulation. Results and statistical data are shown in Table 4.3.13.

4. **Linearity and range:**
   
   Tablet powder was taken equal to 80, 90, 100, 110, and 120 percent of label claim and then dissolved & diluted thoroughly with mobile phase to get a concentration in range of 80-120 percent of test concentration. The resulting solutions chromatograms were recorded at respective wavelength. Drugs were found to be linear in 80 – 120 percent of the test concentration.
3.7 ESTIMATION OF DARUNAVIR, ROPIVACAINE, TEMLISARTAN, PALIPERIDONE AND ZALEPLON BY RP-HPLC METHOD.

1. Selection of mobile phase:
   a. Preparation of standard solutions:

   Drug standard solution:

   Exactly weighed 5g of drug taken then dissolved in methanol and made volume up to 25 ml mark (200 mg/ml). And then diluted further with methanol to obtain the final concentration of about 10 mg/ml of drug.

   b. Procedure:

   The methanol was made allow equilibrating with stationary phase till steady baseline was attained. The standard solution containing drug was run and then different individual solvents and also combinations of solvents have been tried to obtain a good chromatogram. Each mobile phase was filtered by Whatman filter paper No. 42. Various mobile phase compositions were evaluated to achieve acceptable peak symmetry and other parameters using selected chromatographic conditions.

   From various mobile phases tried, different mobile phases were selected for DN, TR, PP, RP & Zpl respectively, as it gave sharp, reproducible peak with suitable retention time for drugs which are mentioned below. The chromatogram for each of them is reported in results section.

CHROMATOGRAPHIC CONDITIONS:

The following chromatographic conditions were established by trial and error and were kept constant throughout method.

Column : Grace 4.6 (id) x 250 mm
Particle size packing : 5 µm
Stationary phases : C18 Grace
Mobile phase : MEOH: KH2PO4 Buffer (10 mM):( 90:10 pH 6.0) (for DN)
               MEOH: KH2PO4 Buffer (10 mM):( 80:20 pH 5.5) (for RP)
               MEOH: KH2PO4 Buffer (10 mM):( 70:30 pH 5.0) (for TR)
               MEOH: KH2PO4 Buffer (10 mM):( 65: 35 pH 4.0) (for PP)
               MEOH: KH2PO4 Buffer (10 mM):( 60:40 pH 4.4) (for Zpl)
Detection wavelength :282nm(DN),286nm (TR),262nm(PP),322nm(RP), 242nm (Zpl)
Flow rate : 1 ml/min.
Temperature                :  Ambient
Sample size                 :  20 µL

2. **Preparation of calibration curve:**
   a. **Preparation of standard solutions:**
      Drug standard stock solution:

      Accurately weighed quantity 10g of drug was dissolved in methanol and the volume was completed to 100 ml mark (100mg/ml). The stock standard solution was then diluted further with mobile phase to get various concentrations.

      **Procedure:**
      The mobile phase was made equilibrating with the stationary phase till steady baseline was attained. A series of concentration from 2-20 µg/ml of both drug solutions were injected and recorded the peak area and tabulated in table 4.4.1. The graph plotted as the concentration of the drug Vs peak area depicted in fig 4.4.1-4.4.5.

3. **System suitability test:**

      It is a pharmacopoeial need which employed to check whether resolution and reproducibility of chromatographic system are sufficient for analysis to be performed. The tests were carried out by collecting data from five replicate injections of standard solutions.

   a. **Preparation of standard drug solution.**

      Appropriately weighed quantity 10g of drug was dissolved in mobile phase and the volume was completed to 100ml mark. The stock standard solution was diluted more with mobile phase to obtain the final concentration of about 50 mg/ml of drug.

   b. **Procedure:**

      Filtered mobile phase was allowed to equilibrate with the stationary phase until steady baseline was obtained. A 20 µL std. drug solution was injected which was made in five replicates and the system suitability parameters were recorded as shown in table 4.4.2.
4. Application of proposed method for estimation of drug in formulation:

a. Standard stock solution:

Appropriately weighed 10g of drug was taken into 100 ml volumetric flask, with vigorous shaking for five minutes, then made volume up to mark with mobile phase. The standard solution of drug were mixed and then diluted with mobile phase carefully to get laboratory mixtures containing a concentration 10µg/ml of drug.

b. Sample solution preparation:

Different sample of drugs were prepared by accurately weighing the quantities of drug samples so as to obtain the concentration of 10µg/ml of each drug. The peak area of sample solution was compared to obtain the concentration.

c. Procedure:

Equal volumes (20 µL) of standard and sample solution were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response, that is the peak area of major peaks were measured. The drug content was calculated by comparing a sample peak with that of standard.

Amount of drug in tablet was calculated by following formula;

\[
\text{Assay (mg/ml)} = \frac{A_t}{A_s} \times \frac{D_s}{D_t} \times \frac{W_s}{W_t} \times \frac{P}{100} \times W_t \text{ (mg/ml) of test sample}
\]

\[
\% \text{ Label claim} = \frac{\text{Assay (mg/ml)} \times 100}{\text{Label claim in mg/ml}}
\]

Where -

At = Area count for sample solution.

As = Area count for standard solution.

Ds = Dilution factor for standard.

Dt = Dilution factor for sample.

P = Potency of drug.

Results were tabulated in table 4.4.3-4.4.5.
VALIDATION OF PROPOSED METHOD:
Validation of proposed method was performed for linearity & range, precision, accuracy, ruggedness and robustness.

1. **Linearity and Range:**
In accordance with USP tablet powder was taken equal to 80, 90, 100, 110, and 120 percent of label claim and then dissolved & diluted thoroughly with mobile phase to get a concentration in range of 80-120 percent of test concentration. The resulting solutions chromatograms were recorded. Drugs in marketed formulation were got to be linear in a range ± 20% of test concentration of the individual drug.

**Linearity of Response:** Aliquot portions of the standard stock solution A (2, 4, 6, 8, 10 ml) were diluted to 10.0 ml with mobile phase to get conc. of 10-100 µg/ml for drug.

**Procedure:** As per the optimized parameters the chromatographic conditions were adjusted and with stationary phase mobile phase was equilibrated as was marked by the steady baseline. Standard solutions of different concentration were injected separately and the chromatograms were recorded and tabulated in table 4.5.1-4.5.3.

The peak areas were noted for each injected concentration of the drugs and the calibration curves, then the concentration against peak area were determined for the drugs and reported as figure 4.5.1-4.5.5.

2. **Precision:**
Precision of analytical method that is expressed as SD and RSD of series of replicate measurements. Precision of estimation of the drug by proposed method was determined by replicate analysis of homogeneous samples of tablet powder. The results are in tabular form in table 4.5.4-4.5.6.

3. **Accuracy:**
Accuracy of proposed method was determined on basis of recovery studies carried out by standard addition method. The results of recovery studies and statistical data are recorded in Table 4.5.7-4.5.9.

**Standard solution:** Standard solution C (conc. 100 µg/ml) was prepared. From that take 2ml, 4ml, 6ml in 3 different 10 ml volumetric flask, completed the volume with mobile phase to get 20, 40 and 60 µg/ml solution respectively.

**Sample solution:** Accurately weighed quantities of pre-analyzed tablet powder equivalent to about 10 mg of drug were transferred to 10.0 ml volumetric flask and
adjusted volume with mobile phase. Taken 1 ml of the above solution in three 10ml volumetric flask (100µg/ml) and the 1ml of 20, 40, 60 µg/ml standard solution were added in three different flasks. The flasks were sonicated for 15 min and volumes were made up to the mark with mobile phase.

**Procedure:** Same as described under estimation of drug in tablet. The percent recovery was then calculated by using formula;

\[
\% \text{ Recovery} = \frac{A}{B + C} \times 100
\]

Where-

- A = Total amount of drug estimated.
- B = Amount of drug found on preanalysed basis.
- C = Amount of pure drug added.

4. **Ruggedness:**

The studies were brought for various parameters i.e. different times (Intraday and Interday) and different analysts.

Ruggedness studies were brought about accordingly-

I. Days

II. Analyst.

a. **Interday (Different days):**

Here procedure carried out was same as in marketed formulation analysis on different days. The % label claim was calculated. Data obtained for day 1, day 2, and day 3 is shown in Table 4.5.10-4.5.12.

b. **Intraday:**

Here also same procedure was carried out as marketed formulation analysis and absorbance recorded at interval 3 hrs. in a day. The percent label claim was calculated. Result and statistical data are shown in Table 4.5.13-4.5.15.

c. **Different analyst:**

Two different analysts prepared sample solution and similar procedure was carried out as mentioned previous. The percent label claim was calculated like calculated in estimation of marketed formulation. Result and statistical data are shown in Table 4.5.16-4.5.18.
5. **Robustness:**

Change in Wavelength (±2 nm)

The tablet sample was analyzed using scheduled method after a careful change in detection wavelength for estimation by ±2 nm. Results are as shown in Table 4.5.19-4.5.21.

**3.8 ANALYSIS OF STRESSED SAMPLES OF DRUG BY HPLC**

All stressed samples were analysed by the proposed method and % drug to be remained was calculated from standard calibration curve.

1. **Hydrolysis studies:**

   a. **Acid hydrolysis:**

      It was performed in 0.1 N Hydrochloric acid heated for 4 h at 80 °C.

      **Procedure:** Appropriately weighed quantity of drug (10mg) was mixed with Hydrochloric acid (10mL, 0.1 N) in round bottomed flask (250mL). The reaction mixture was refluxed 4 hour at 80˚. The samples were withdrawn at 0, 2, 4 h intervals and further diluted with mobile phase to get concentration of 100 µg/mL. The chromatographic conditions were set as detailed under the study of linearity range and mobile phase allowed to equilibrate with stationary phase. The 20µL of sample solution were injected. The chromatograms were recorded. The % content of drug to be remained was calculated using calibration curve. The chromatograms are presented in Fig 4.6.1-4.6.20. The results shown in Table 4.6.1-4.6.5.

   b. **Alkali hydrolysis:**

      It was performed in 0.1 N sodium hydroxide solution heated for 4 h at 80°C.

      **Procedure:** Appropriately weighed quantity of drug (10 mg) was mixed with sodium hydroxide solution (10 mL, 0.1 N) in round bottomed flask (250mL). The reaction mixture was refluxed for 4 hour at 80˚. The samples were withdrawn at 0, 2, 4 h intervals and further diluted with mobile phase to get concentration of 100 µg/mL. The chromatographic conditions were set as detailed under the study of linearity range and mobile phase allowed to equilibrate with stationary phase. The 20µL of sample solution were injected. The % drug to be remained was calculated using calibration curve. The chromatograms are presented in Fig. 4.7.1-4.7.20. The results are presented in Table 4.7.1-4.7.5.
c. **Neutral hydrolysis:**

The hydrolysis of drug was carried out in water for 4 h reflux.

**Procedure:** Appropriately weighed quantity of drug (10 mg) was dissolved in water (10 mL) in round bottomed flask (250 mL). The reaction mixture was refluxed for 4 hour with the help of condenser and samples were withdrawn at 0, 2, 4 h intervals. Further dilutions were made in the mobile phase to get concentration 100 µg/mL. Each sample was injected separately after making steady state baseline. The chromatograms were recorded and presented in Fig. 4.8.1-4.8.20. The results are shown in Table 4.8.1-4.8.5.

2. **Oxidation Studies:**

   a. **Peroxide Test:**

   This test was performed on drug

   **Procedure:** 30 mg of drug was dissolved in 30 ml 3 % H2O2 (3mg/ml) and the solution is kept at room temperature with constant stirring. Withdraw the sample 1ml and dilute to 10ml with the mobile phase at the interval of 12 hr for 24 hr. The chromatograms were recorded and presented in Fig. 4.9.1-4.9.20. The results are shown in Table 4.9.1-4.9.5.

3. **Photochemical Studies:**

   **Procedure:** 10 mg samples of drug, evenly spread in thin layer in a covered Petri-dish were kept in sunlight and withdraw the sample at 14 day interval, for 28 days.

   The chromatograms were recorded and presented in Fig. 4.10.1-4.10.20. The results are shown in Table 4.10.1-4.10.5.

4. **Thermal Stability Studies:**

   **Procedure:** 1 gm samples of Drug in different weighing bottles were kept at 70 °C for different time (14 day ) intervals. The chromatograms were recorded and presented in Fig.4.11.1-4.11.15. The results are shown in Table 4.11.1-4.11.5.