Review and Summary of the study

The selection of topic was done by opportunities and challenges available in study, TB is the disease on which researchers are working since decades and studies are currently going on for new diagnosis and novel treatment to avoid deaths occurring due to TB over world-wide. The anti-TB drug classification is not updated from time to time as limited novel molecules are included, recently bedaquiline and delamanide were added to classification and were updated by WHO. Working on the topic was a great opportunity as novel molecule data was not available in literature so it was a privilege to publish data for the first time; while the data available for old drugs were not sufficient, therefore the data was completed and updated.

Three drugs were chosen from each category of anti-TB drug classification;

1. Terizidone (Approved in 1981 in India)
2. Rifabutin (Approved in 2007 in India)
3. Bedaquiline (Approved in 2015 in India)

The **Terizidone** is the anti-TB bactericidal drug which is used since more than 39years and is still effective against the *m.tuberculosis* bacteria. It is included in first line treatment for TB. The extensive literature survey showed that UV and HPLC methods for analytical method development were published. The stability study by HPLC was also published but degradation products were not separated in chromatogram and incomplete stability study was performed, therefore degradation products were well separated in this study. The degradation products were identified by HPTLC and LC/MS by two authors; comparing the data available literature and found in this study showed that the studies those are published identified different DPs while new DPs are identified and characterized in this study with supportive data.

The bulk drug sample was identified by Infrared spectroscopy, Ultraviolet spectroscopy, melting point and solubility data. The data obtained and available in literature was compared to confirm the identification of bulk drug. The drug sample was confirmed as Terizidone bulk drug.

The UV spectroscopic method was developed for TRZ and validation of UV method was done in range 0.01-0.07mg/ml. The chromatographic method was developed to separate the DP and bulk drug peaks in HPLC chromatogram without interference of any substance. The stability indicating method was developed in gradient scheme using 0.01M ammonium acetate (pH 4.7 using glacial acetic acid) and acetonitrile. The method was validated in range 0.05-0.3mg/ml, the precision, linearity and accuracy' relative standard deviations were <2.0%

indicated that method was precise, reproducible and accurate. The recovery in accuracy study was in range 98.4-100.0% which was in the range for 98-105% (recovery range for formulation).

The stability study was completed in different stressor and different stressor concentrations till the bulk drug degradation 5-100% degradation. The stability study was performed in hydrolytic solutions (acid, alkaline and water), oxidative solution, photolytic degradation, thermal degradation for different stressor concentration, temperatures and time till 5-100% bulk drug degradation occurs. TRZ degraded in acid, alkaline, neutral and oxidative medium, while it was stable in photo degradation and thermal degradation.

For acid degradation 0.5N HCl was used at RT (room temperature) for 8hrs, three new peaks appeared in chromatogram with 44.86% decrease in bulk drug intensity. The major peak with peak purity was obtained at Rt 15.3 minutes and this DP was isolated and characterized while other minor DPs were identified and structure was elucidated for DPs. The ESI/MS spectrum identified 7 new DPs among which one DP with *m/z 161* is reported in literature. While another 6 DPs are new including major DP. The major DP was isolated by preparative HPLC and identified using LC/ESI/MS (*m/z 237)* and LC/MS/MS (*m/z* 237 ↔132). The major DP was named as *(E)*-2-amino-*N*-(4-(((2-(hydroxyamino) ethyl)imino)methyl)benzyl)ethan-1-aminium with chemical formula $C_{12}H_{21}N_4O$. The degradation pathway was generated for acid degradation of TRZ.

For alkaline degradation 0.5N NaOH was used for 60minutes at RT but TRZ showed a pattern for degradation; initially no new peak observed but at certain point depending on concentration of NaOH, the molecule break down in fragments and showed a number of peaks without bulk drug peak. The ESI/MS spectrum showed a number of *m/z* for $[M+H]^+$ therefore reporting or identifying of DP was not possible in alkaline media. The same pattern was observed in lower NaoH concentration (0.01N-0.1N) but break down point was extended.

The degradation in water was done by keeping the solution at room temperature for 12hrs. The chromatogram showed five new peaks with bulk drug peak area 62.85%. The major peak was observed at Rt 16.24minutes. DPs were identified by ESI/MS and structures were elucidated for them. The ESI/MS spectrum showed 12 *m/z* values for DPs among which three DPs were identified based on area in UPLC chromatogram. The major DP was isolated using preparative HPLC (purity >98.0%) and identified using ESI/MS (*m/z* 347.3) and tandem MS (*m/z* 347.3↔174.09). The isolated DP was characterized by NMR (^1H , ^{13}C and APT NMR). The DP was named as is *(3S, 4R)* -3-ethoxy-5-oxo-*N*- *(((E)*-4-(((*S*)-5-oxoisoxazolidin-4-yl)

imino) methyl) benzylidene) isoxazolidin-4-aminium, and chemical formula is $C_{16}H_{19}N_4O_5$. The degradation pathway was generated for TRZ behavior in aqueous solution.

For oxidative degradation of TRZ, 6% hydrogen peroxide was used at room temperature for 24 hrs. Two new peaks were appeared other than hydrogen peroxide and TRZ bulk drug peak. The major peak with peak purity obtained at Rt 15.3 minutes, it was isolated using preparative HPLC and characterized by Tandem MS. The ESI/MS spectrum showed 11 *m/z* values for DPs among which one major DP were identified based on area in UPLC chromatogram. The major DP ESI/MS (*m/z 323.3*) and tandem MS (*m/z* 323.3 ↔238.13) data was used for structure elucidation and NMR (^{1}H NMR) data was used for characterization of structure. The major DP was named as (*4S, 5S)*-5-hydroperoxy-*N-((E)*-4-((E)-(((R)-5-oxo-1, 3, 2-dioxazolidin-4-yl)imino)methyl)benzylidine)isoxazolidin-4-aminium that matches with chemical formula $C_{13}H_{15}N_4O_6$.

The DPs reported in this study are not reported in literature at the time of study and reported DPs are cited with suitable reference.

The degradation kinetics study was completed by Conventional kinetics study method. Degradation kinetics study was performed for acid, alkaline, neural and oxidative conditions.

For degradation kinetics different stressor concentration and different temperatures were used. The study was completed using following conditions;

Acid: 0.1N, 0.3N and 0.5N HCl/25, 40 and 60^0C/ 40minutes (4 points)

Alkali: 0.1N, 0.3N and 0.5N NaOH/25, 40 and 50^0C/20minutes (4 points)

Neutral: Water/25, 40 and 60^0C/120minutes (4 points)

Oxidative: 1%, 3% and 6% H_2O_2/ 25, 40 and 60^0C/ 40 minutes (4 points)

The degradation kinetics parameters were calculated for each conditions and relationship between kinetic parameters and stressor condition were evaluated.

TRZ in acid degradation followed first order kinetics and the degradation kinetic parameters value decreased (Except rate constant) with increase in stressor conditions. The increase in rate constant with increase in stressor condition indicate that degradation rate of reaction in acid condition is increasing. The positive enthalpy value indicates that reactions were endothermic. The negative and close entropy values indicate that the arrangements of

molecules were not affected by acid in reaction. The TRZ and acid reaction was acid catalyzed reaction that produced new DPS.

The alkali catalyzed reaction was first order for TRZ, degradation kinetic parameters and stressor conditions relationship was same as described in acid kinetic study. The positive enthalpy values indicate that reaction was endothermic but the difference between entropy values indicate that high level of molecular disarrangement was occurred during TRZ and alkali reaction. This was the reason DPs were not identified in this conditions.

The TRZ in aqueous solution followed first order to form DPs. The relation between degradation kinetics parameters and stressor conditions were same as described in acid degradation kinetics study.

In oxidative condition TRZ followed first order kinetics to form DPs. The degradation kinetics parameters decreased (except rate constant) with increased in stressor conditions. The rate constant increased with increase in stressor conditions indicate that reaction of degradation of TRZ was boosted by stressor conditions. The activation parameters indicated that reaction was endothermic and less molecular rearrangements were occurred during reaction.

The TRZ is highly unstable in hydrolytic solution and in presence of oxidizing agent. To store TRZ, it should be stored away from hydrolytic solutions, and oxidizing agent.

The **Bedaquiline (BDQ)** was obtained as gift sample from Dishman pharmaceuticals, Gujarat and identification of sample was completed by IR study, UV study, melting point study and solubility study, the results of data were compared with available reported data and these studies confirmed the sample as BDQ.

The UV spectroscopic method was developed in methanol for 0.01-0.07mg/ml and validated. The stability indicating method was developed using 0.01M sodium dihydrogen ortho phosphate and methanol as mobile phase in gradient scheme. The method was validated as per ICH Q2 (R1) guidelines , the linearity was taken for the range 0.03-0.18mg/ml, the RSD values for precision , linearity and accuracy study was <2.0% indicates that method was precise, linear, reproducible and accurate. The recovery of BDQ was in the range 98.9-99.7% indicates that good recovery was obtained. The robustness study was performed to prove that method is robust and can be used with deliberate changes.

The stress degradation study was performed in hydrolytic solutions, in presence of oxidative agent, photolytic degradation and thermal degradation. BDQ was degraded in acid and oxidative conditions while it was stable or slightly degraded under alkaline, neutral, thermal and photolytic conditions.

The acid degradation study was performed in 0.5N HCl at 80^0C for 75 minutes. Six new peaks were observed other than BDQ bulk drug peak (Bulk drug peak area 25.18%). The major DP was observes at Rt 26.48minutes. The ESI/MS spectrum showed 8 DPs among which one was major DP which was isolated using HPLC (Preparative) and DPs were identified using ESI/MS, the major DP was characterized using NMR(^{1}H, ^{13}C and APT). The major DP was named as 6-bromo-3((*1R, 2S*)-4-(dimethylamino)-2-hydroxy-2-(napthelen-1-yl)-1-phentlbutyl)-2-hydroxyquinolin-1-ium which matched with chemical formula $C_{31}H_{30}BrN_2O_2$.

There were no major DPs obtained in alkaline and neutral conditions therefore no DPs were reported or identified for these conditions.

In the solution containing oxidizing agent BDQ was degraded and three new peaks appeared other than BDQ bulk drug peak. The major DP was obtained at Rt 32.1 minutes. The ESI/MS spectrum showed three *m/z* values. The major DP was identified by ESI/MS *m/z* 571.2 and characterized by NMR (^{1}H, ^{13}C and APT). The major DP was named as 2-((*1R.2S)*-1-(6-bromo-2-hydroxyquinolin-3-yl)-4-(-dimethylmino)-2-hydroxy-2-(napthlen-1-yl) butyl) benzene-1, 4-diol and chemical formula was $C_{31}H_{28}BrN_2O_4$.

The DPs reported in this study are not reported in literature at the time of study.

The degradation kinetics study was completed by two methods

1. Conventional kinetics study method
2. Multi-factorial tool and DoE approach

Degradation kinetics study was performed for acid, alkaline and oxidative conditions.

For degradation kinetics different stressor concentration and different temperatures were used. The study was completed using following conditions;

Acid: 0.1N, 1.0N and 2.0N HCl/25, 50 and 80^0C/ 180minutes (7 points)

Alkali: 0.1N, 1.0N and 2.0N HCl/25, 50 and 80^0C/ 12hrs (7 points)

Oxidative: 1%, 6% and 12% H_2O_2/ 25, 50 and 80^0C/ 6 hrs (7 points)

The degradation kinetics parameters were calculated for each conditions and relationship between kinetic parameters and stressor condition were evaluated.

BDQ in acid degradation followed first order kinetics and the degradation kinetic parameters value decreased (Except rate constant) with increase in stressor conditions. The increase in rate constant with increase in stressor condition indicate that degradation rate of reaction in acid condition is increasing. The positive enthalpy value indicates that reactions were endothermic. The negative and close entropy values indicate that the arrangements of molecules were not affected by acid in reaction. The BDQ and acid reaction was acid catalyzed reaction that produced new DPS.

The alkali catalyzed reaction followed first order for BDQ, degradation kinetic parameters and stressor conditions relationship was same as described in acid kinetic study. The positive enthalpy values and negative entropy value indicate that reaction was endothermic and the close entropy values indicate that less disarrangement was occurred during BDQ and alkali reaction. Slight degradation was occurred in these reactions.

In oxidative condition BDQ followed first order kinetics to form DPs. The degradation kinetics parameters decreased (except rate constant) with increased in stressor conditions. The rate constant increased with increase in stressor conditions indicate that reaction of degradation of BDQ was boosted by stressor conditions. The activation parameters indicated that reaction was endothermic and less molecular rearrangements were occurred during reaction.

The BDQ is highly unstable in acidic solution and in presence of oxidizing agent. To store BDQ, it should be stored away from acidic pH, and oxidizing agent.

The multi-factorial tool was used for degradation kinetics study of BDQ and it was successfully applied to predict the value.

Rifabutin was obtained as a gift sample from Lupin Pharmaceuticals, Gujarat. The sample identification was done by IR, UV, melting point and solubility study. The sample was confirmed as rifabutin. The literature study showed that no stability study and DPs are reported in literature for rifabutin.

UV spectrophotometric method was developed in methanol for 0.01-0.06mg/ml and validated. The stability indicating method was developed in 0.01M ammonium acetate buffer (pH 4.0 by glacial acetic acid) and acetonitrile in gradient program. The method was validated for linearity of range 0.050-0.25mg/ml, RSD for precision, linearity and accuracy study was <2.0%indicated that method was precise, linear and accurate. The accuracy study showed good recovery in the range of 99.2-100.5%. The robustness study showed that small but deliberated changes do not affect the method sustainability.

Stress degradation study showed that rifabutin was stable under neutral, thermal and photolytic conditions. The noticeable degradation was occurred in acid, alkaline and oxidative condition.

For acid degradation condition, the sample was kept in 0.5N HCl at RT for 24hrs; three new peaks were observed other than rifabutin bulk drug. The major DP was observed at Rt 5.5minutes. The major DP was isolated and characterized by ESI/MS and MS/MS, respectively. The ESI/MS detected 9 DPs and the major DP was identified by ESI/MS (*m/z* 451) and characterized by MS/MS; named as (11*E*,13*Z*)-7,9-dihydroxy-3-methoxy-4,6,8,10-pentamethyl-15-oxo-15-(vinylamino)pentadeca-1,11,13-trien-5-yl-acetate match with chemical formula $C_{21}H_{29}N_3O_5$.

For alkaline degradation, rifabutin was kept in 0.1N NaOH at RT for 60minutes; three new peaks were observed other than rifabutin bulk drug peak. The major DP was observed at Rt 6.9 minutes. The ESI/MS Spectrum identified 7 DPs among which one was major DP, which was identified by ESI/MS (*m/z* 805.2) and NMR, named as (2Z, 4E, 6S, 7R, 9R, 11S, 12S, 13S)-11-acetoxy-1-(((2S,9S)-6,9-dihydroxy-2-(-2isobutyl(methyl)amino)ethyl)-7,9-dimethyl-5,10-dioxo-3,5,9,10-tetrahydro-2H-furo[2',3':7,8] napthol[1,2-d]imidazol-4-yl)amino)-9-hydroxy-13-methoxy-2,6,12-trimethyl-1-oxopentadeca-2,4,14-trien-7-olate matched with chemical formula $C_{45}H_{63}N_3O_{10}$.

For oxidative degradation, rifabutin was kept in 30% hydrogen peroxide at 80^0C for 3hrs. The slight degradation was observed and no major DP was obtained. Oxidative DPs were reported.

The DPs are reported for the first time in literature for rifabutin.

The degradation kinetics study was completed by two methods;

1. Conventional kinetics study method
2. Multi-factorial tool and DoE approach

Degradation kinetics study was performed for acid, alkaline and oxidative conditions.

For degradation kinetics different stressor concentration and different temperatures were used. The study was completed using following conditions;

Acid: 0.1N, 0.5N and 1.0N HCl/25, 40 and 60^0C/ 50minutes (6 points)

Alkali: 0.01N, 0.05N and 0.1N HCl/ RT/ 60minutes (7 points)

Oxidative: 7%, 15% and 30% H_2O_2/ 25, 50 and 80^0C/ 30 minutes (7 points)

The degradation kinetics parameters were calculated for each conditions and relationship between kinetic parameters and stressor condition were evaluated.

Rifabutin followed first order kinetics in acidic solution, in alkaline medium and oxidative medium. The degradation kinetic parameters value decreased (Except rate constant) with increase in stressor conditions. The increase in rate constant with increase in stressor condition indicate that degradation rate of reaction in acid condition is increasing. The positive enthalpy value indicates that reactions were endothermic. The negative and close entropy values indicate that the arrangements of molecules were not affected by stressor in reaction.

The rifabutin is highly unstable in acidic solution, alkaline medium and in presence of oxidizing agent. To store rifabutin, it should be stored away from acidic pH, alkaline pH and oxidizing agent.

The multi-factorial tool was used for degradation kinetics study of rifabutin and it was successfully applied to predict the value.

The result of these studies can be applied for formulation development and to study marketed formulations' stability and shelf life. The DPs formed under different conditions can be further studied to evaluate pharmacological/ toxicological studies. The kinetics data are useful to study the reaction mechanism at molecular level and disarrangements in the molecule during reaction.