SUMMARY AND CONCLUSION

The usage of pharmaceuticals is always a balance of benefits and risks but the same concept is not true for impurities and degradation products (DPs) present in pharmaceuticals, impurities and DPs always convey risks only. Hence it is important to analyze, evaluate and control impurities in drug substances (API, Active pharmaceutical ingredient) and drug products (FPP, finished pharmaceutical products). It is very difficult to maintain the level of impurities and DPs to almost zero, but can be reduced to the lowest level that is why regulatory guidelines provide the specifications for control of impurities and DPs.

In the present work a detailed study and systematic profiling of impurity and degradation behaviour of three drugs Pidotimod, Edaravone and Ciclopirox Olamine have been carried out by various techniques like RP-HPLC, HPTLC, LC-MS/MS, FT-IR and FT-NMR. Stress degradation studies were carried out to facilitate the development of stability indicating assay method (SIAMs) and to gain a better understanding of stability of API and FPP, to identify and quantify DPs and to establish degradation pathways of DPs formed during stress degradation. Among number of DPs formed, some major DPs were isolated based on degradation kinetic study and were characterized.

To study complete impurity profiling and degradation behavior of Pidotimod, the research work carried out in chapter-3 was bifurcated into 5 sections and were summarized as:

Section A: This section comprises of development and validation of SIAMs by utilizing principle of quality by design. For this stress degradation was carried out as per ICH prescribed conditions, the samples generated for different stress degradation conditions (viz acid, base, neutral, oxidative, photolytic, dry heat induced and thermal-humidity induced degradation) were mixed to optimize and develop QbD based SIAMs with LC-PDA detection. Risk assessment by cause- effect relationship and CNX approach was performed to identify high risk factors or variables that affected the responses, which was then subjected to 4^2 full factorial design to optimize the chromatographic conditions.

An RP-HPLC method was developed in isocratic mode. The final optimized condition that give well resolved asymmetric peaks of API and DPs comprised of mixture of ammonium acetate buffer (10 mM, pH adjusted to 4.5 with glacial acetic acid) and MeOH/ACN (90:10) in the ratio 97:03, v/v at a flow-rate of 1.0 ml/min. The analysis was performed at 39^0C using column oven with injection volume of 20 µL. The separation was accomplished on a Phenomenex RP- C18 column (250 ×4.6 mm, 5 µm) at wavelength of 215 nm. The retention time (Rt) of Pidotimod was found to be 8.8. Six degradation products were observed at Rt of 2.8 (DP-1), 3.4 (DP-2), 3.7 (DP-3), 12.2 (DP-4), 2.4 (DP-5) and 7.3 (DP-6). The developed method was validated as per ICH and ISO-17025 guideline based on total error approach. The linearity was found in the concentration range of 50-250 µg/ml. LOD and LOQ were found to be 1.422 and 4.309 µg/ml. The percentage relative bias for trueness study is in between -0.0059 % to 0.2082 % and hence acceptable. The percentage recovery lies within 95% confidence interval. The uncertainty, uncertainty of bias, expanded uncertainty and relative expanded uncertainty values fall with in limit.

Section B: Degradation rate kinetics of Pidotimod was studied by HPLC method in this section. Pidotimod was found to be most sensitive towards oxidative degradation. It undergoes significant degradation after acid and base degradation. Slight degradation was observed under neutral, photolytic and thermal-humidity degradation conditions and was most stable under dry heat induced degradation. Acid, base and oxidative degradation followed first order degradation rate kinetic while neutral and photolytic degradation followed zero order degradation kinetic. Arrhenius plots were constructed and half life ($t_{1/2}$) and activation energy (Ea) were calculated.

Section C: The goal of this section was to isolate and characterize major DPs of pidotimod. Two major degradants formed (DP-2 at Rt 3.4 and DP-4 at Rt 12.2) during acid degradation were isolated and characterized. Both DP-2 and DP-4 were isolated by liquid-liquid extraction and preparative TLC and purified by recrystallization. These were characterized by LC-MS/MS, FT-IR and FT-NMR spectral studies. The mass and IR spectra of DP-4 matched with reported degradation product, 3-[3,5,6,7,8,8a-hexahydro-5,8-dioxo-1Hthiazo-[3,4a]pyrazine-6-yl]propionic acid (IM-X), hence further characterization was not done. Based on spectral data DP-2 was characterized as 3,8-Dihydroxy-tetrahydro-bisthiazolo[3,4,4a,3’,4’-d]pyrazine-5,10-dione.

Section D: Impurity profiling and degradation study of Pidotimod was carried out by LC-PDA and LC-MS/MS. LC- PDA showed total 6 degradants while LC-MS/MS study showed 11 different masses of degradants. Among 11 DPs, two were isolated and characterized as discussed above. Two process related impurities INH-1 (Pyroglutamic acid) and INH-2 (Thiazolidine carboxylic acid) were also formed during stress degradation and were identified as DP-1 and DP-3 respectively. Remaining 9 DPs were characterized based on molecular mass and fragmentation pattern obtained from LC-MS/MS study. Considering the structures of DPs, the degradation pathways for DPs were proposed.

Section E: Pidotimod and isolated DP-2 were isomeric in nature, hence enantiomeric separation was carried out and stability indicating RP-HPLC method was also developed and validated as per ICH guideline. The separation was carried out on CHIRADEX Lichrocart RP- C18 column (150 ×4.6 mm, 5 µm) using mobile phase, 10 mM ammonium acetate buffer (pH adjusted to 5.5 with glacial acetic acid) and MeOH in the ratio 80:20, v/v at a flow-rate of 0.8 ml/min with isocratic elution. The analysis was performed at ambient temperature with injection volume of 20 µL and wavelength of 215 nm. The Rt of R and S form Pidotimod was found to be 4.8 and 5.8 min respectively. Three isomers of isolated DP-2 were retained at Rt of 6.5, 7.7 and 8.5 min (DP-2(e1), DP-2(e2), DP-2(e3) respectively). The peaks of Pidotimod and DP-2 were confirmed by LC/MS-MS study. Two other degradation products were retained at Rt of 3.1 and 4.0 min. The developed method was found to be linear in the concentration range of 50-250 µg/ml. The LOD and LOQ were found to be 1.2072 and 3.6583. The percentage recoveries were found to be above 99 %.

To study complete impurity profiling and degradation behaviour of Edaravone, the research work carried out in chapter-4 was divided into 6 sections and were summarized as:

Section A: This section comprises of development and validation of quality by design based stability indicating RP-HPLC method of Edaravone. For this stress degradation was carried out as per ICH prescribed conditions, the samples generated for different stress degradation conditions (viz acid, base, neutral, oxidative, photolytic, dry heat induced and thermal-humidity induced degradation) were mixed to optimize and develop QbD based SIAMs with LC-PDA detection. A gradient RP-HPLC method was developed. Plackett Burman Screening was carried out to identify significant factors affecting the selected responses, which was further optimized by Box Behnken design to optimize the chromatographic conditions. The final optimized conditions that give well resolved asymmetric peaks of API and DPs comprised of mixture of mobile phase A, 10 mM ammonium acetate buffer (pH adjusted to 5.8 with glacial acetic acid) and mobile phase B MeOH/ACN in the ratio 90:10, at a flow-rate of 0.8 ml/min. The analysis was performed at 34^0C using column oven with injection volume of 20 µL. The separation was accomplished on a Thermo Scientific BDS Hypersil RP- C18 column (250 ×4.6 mm, 5 µm) at wavelength of 244 nm. The gradient program consists of ratio of A/B; 95/05, 85/15, 75/25, 55/45, 75/25, 85/15, 95/05, 95/05; at times 0.01, 04, 10, 12, 14, 16, 17 and 20 min respectively. The retention time (Rt) of Edaravone was found to be 11.5 min. Ten DPs were observed at Rt of 5.9 (DP-1), 6.5 (DP-2), 7.1 (DP-3), 12.5 (DP-4), 14.3 (DP-5), 15.3 (DP-6), 16.3 (DP-7), 9.6 (DP-8), 13.9 (DP-9) and 13.5 (DP-10) min respectively. The method validation was carried out as per ICH Q2(R1) guideline. The linearity of the method was found to be in the concentration range of 10-300 µg/ml. LOD and LOQ were found to be 0.5341 and 1.6186 µg/mL respectively. The percentage recoveries of Edaravone in marketed formulation were found to be more than 99.0 %.

Section B: Degradation rate kinetics of Edaravone was studied by HPLC method in this section. Edaravone was found to be most sensitive towards oxidative degradation. It undergoes significant degradation under acid, base, neutral and dry heat induced degradation. Slight degradation was observed under photolytic and thermal-humidity induced degradation conditions. Nonlinear regression analysis was performed to determine degradation kinetic of acid and base degradation that followed pseudo first order kinetics. To determine degradation kinetic of neutral, oxidative and photolytic degradation, linear regression analysis was performed that followed zero order degradation kinetic. $K_{obs,}$ $t_{1/2}$ and activation energy (Ea) were also determined.

Section C: This section comprises of development and validation of quality by design based stability indicating HPTLC method of Edaravone. For this stress degradation was carried out as per ICH prescribed conditions, the samples generated for different stress degradation conditions were mixed to optimize and develop QbD based SIAMs. CNX risk assessment and Box Behnken design was used to screen and optimize the chromatographic conditions. The final optimized conditions that give well resolved asymmetric peaks of API and DPs consisted of Petroleum ether- Ethyl acetate- Glacial acetic acid (GAA) in the ratio of 3:2:0.01 v/v/v. The TLC plates were developed in a CAMAG 20cm X 20 cm twin trough glass chambers saturated with the mobile phase to a distance of 89 mm. The analysis was performed at wavelength of 244 nm. The retardation factor (Rf) value of Edaravone was found to be 0.52. Six DPs were resolved at Rf value of 0.21 (DP-1), 0.31 (DP-2), 0.41 (DP-3), 0.91 (DP-4), 0.98 (DP-5) and 0.61 (DP-6) respectively. The method validation was carried out as per ICH Q2(R1) guideline. The method was linear in the concentration range of 2-24 µg/spot. LOD and LOQ were found to be 0.327 and 0.989 µg/spot respectively. The percentage recovery from formulation matrix was found to be more than 99.0 %.

Section D: Degradation rate kinetic of Edaravone was studied by HPTLC method in this section. Drug was found to be most sensitive towards oxidative degradation. It undergoes significant degradation under acid, base, neutral and dry heat induced degradation conditions. Slight degradation was observed under photolytic and thermal-humidity induced degradation condition. Nonlinear regression analysis was performed to determine kinetics of acid and base degradation that followed pseudo first order degradation kinetic. To determine kinetics of neutral, oxidative and photolytic degradation, linear regression analysis was performed that followed zero order degradation kinetic. $K_{obs,}$ $t_{1/2}$ and activation energy (Ea) were also calculated.

Section E: The goal of this section was to isolate and characterize major DPs of Edaravone. Two major DPs formed (DP-7 at Rt 16.36 and DP-4 at Rt 12.5) during dry heat induced degradation were isolated and characterized. DP-7 was isolated by column chromatography by using different ratios of petroleum ether and ethyl acetate in gradient mode and purified by recrystallization. DP-4 was also isolated by column chromatography by same mobile phase but the ratio was changed. It was further purified by preparative TLC using mobile phase petroleum ether and ethyl acetate (80:20) and recrystallized. These DPs were characterized by LC-MS/MS, FT-IR and NMR spectral studies. Based on spectral data DP-7 was characterized as 4-(4,5-dihydro-3-methyl-5-oxo-1-phenyl-1H-pyrazol-4-yl-4-(4,5-dihydro-5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one and DP-4 as 3-hydroxy-dihydro-thiazolo(1-(2-methyl-buta-1,3dienyl)-1-phenylhydrazine)5-one.

Section F: Impurity profiling and degradation study of Edaravone was carried out by LC-PDA and LC-MS/MS. LC- PDA showed total 10 DPs while LC-MS/MS study showed 13 different masses of DPs. One DP formed during stress degradation was reported as EDA IM- F and were identified as DP-6. Among 13 DPs, two were isolated and characterized as described above. Remaining 10 DPs were characterized based on molecular mass and fragmentation pattern obtained from LC-MS/MS study. Considering the structures of DPs, the degradation pathways for DPs were proposed.

To study complete impurity profiling and degradation behavior of Ciclopirox Olamine, the research work carried out in chapter-5 was described into 3 sections and were summarized as:

Section A: This section comprises of development and validation of stability indicating RP-HPLC method by using principle of quality by design. For this stress degradation was carried out as per ICH prescribed conditions, the samples generated for different stress degradation conditions were mixed to optimize and develop QbD based SIAMs with LC-PDA detection. Also pre-column derivatization of Ciclopirox Olamine was carried out by methylating N-hydroxyl group of Ciclopirox Olamine by dimethyl sulfate in presence of sodium hydroxide since it has strong chelating property that makes quantification of Ciclopirox Olamine difficult. An isocractic RP-HPLC method was developed. CNX risk assessment was carried out to identify high risk variables, which were subjected to Plackett Burman screening design to identify significant factors effecting the selected response and then optimized by Central Composite design. The final optimized conditions that give well resolved asymmetric peaks of API and DPs comprised of mixture of ammonium acetate buffer (10 mM, pH adjusted to 4.5 with glacial acetic acid) and MeOH/ACN (90:10) in the ratio 97:03, v/v at a flow-rate of 1.0 ml min^{-1}. The analysis was performed at 39^0C using column oven with injection volume of 20 µL. Separation was accomplished on a Phenomenex RP- C18 column (250 ×4.6 mm, 5 µm) at wavelength of 300 nm. The Rt of Ciclopirox Olamine was found to be 12.77 min. Four DPs were observed at Rt of 7.1 (DP-1), 7.5 (DP-2), 8.9 (DP-3) and 10.2 (DP-4) min respectively. The developed method was validated as per ICH Q2(R1) and ISO-17025 guidelines based on total error approach. The linearity was found in the concentration range of 20-120 µg/ml. LOD and LOQ were found to be 0.6247 and 1.8732 µg/mL respectively. The percentage relative bias for trueness study is in between -0.4339 % to 0.4 % and hence acceptable. The percentage recovery lies within 95% confidence interval. The uncertainty, uncertainty of bias, expanded uncertainty and relative expanded uncertainty values fall with in limit.

Section B: Degradation rate kinetics of Ciclopirox Olamine was studied by HPLC method in this section. The drug was most sensitive towards photolytic degradation. Significant degradation was observed in oxidative degradation. It undergoes slight degradation under acid and base degradation and almost stable under neutral, dry heat induced and thermal-humidity induced degradation conditions. Hence degradation kinetic was studied under oxidative and photolytic conditions that followed first order and zero order degradation kinetic respectively. Arrhenius plot was constructed and $t_{1/2}$ and Ea were calculated.

Section C: Impurity profiling and degradation study of Ciclopirox Olamine was carried out by LC-PDA and LC-MS/MS study. LC- PDA showed total four DPs while in LC-MS/MS study five different peaks were observed (observed as bifurcated peak in LC-PDA). The major DP formed i. e. DP-3 was identified as an already reported process related impurity CIO IM-C, hence isolation and characterization of this major DP was not carried out. According to the m/z values and fragmentation pattern, the structures and degradation pathway for DPs were proposed.