Analytical method development and validation play a major role in the discovery, development and manufacture of pharmaceuticals. The official test method that results from these processes are used by quality control laboratories to ensure the identity, purity, potency and performance of drug product ‘quality’, essential for drug safety and efficacy. In the pharmaceutical and biotechnology industries, a current major issue is the high cost of research in introduction of new drugs. In essence, it takes several hundred million dollars to discover, develop and gain regulatory approval. One of the reasons research and development (R&D) is so costly in pharmaceuticals is that most new drug candidates fail to reach the market. Failure can result from toxicity, carcinogenicity, manufacturing difficulties, inadequate efficacy and analytical problems. Therefore, there is a need for high throughput in order to maximize patent lifetime and consequently, generate the profits to support the research and to increase the speed with which the product can be delivered to the market. All the different stages of pharmaceutical R&D are underpinned by analysis so that high throughput is actually dependent on effective and efficient analysis within which simple effective method development and comprehensive analytical method validation is of fundamental importance. A wide variety of materials are used in the pharmaceutical and diagnostic industries. All of these materials must be analyzed in some way or other and, just as importantly, the method of analysis must be validated. There is continued pressure within the pharmaceutical industry to reduce cycle times and increase the number of products that make it to market. Analytical chemists responsible for developing chromatographic methods have responded to this challenge by implementing strategic approaches to get consistent results.
In the pharmaceutical industry, analytical method validation is very much a major issue as analysis is used primarily to control drug quality. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Method validation means establishing, through documented evidence, a high degree of assurance that an analytical method will consistently yield results that accurately reflect the quality characteristics of the product tested.

Safety and efficacy of pharmaceuticals are two fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological and toxicological profile as well as the adverse effects caused by the impurities in bulk and dosage forms. Therefore, it is quite obvious that the products intended for human consumption must be characterized as completely as possible. The quality and safety of a drug is generally assured by monitoring and controlling the impurities effectively. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis. In the pharmaceutical industry, both for quality control and drug development, assessment of the nature and concentration of impurities and/or metabolites of the active pharmaceutical ingredients (APIs) is of utmost importance. At present, the technique most commonly applied for tracing impurities is reversed-phase liquid chromatography (RPLC) combined with UV detection and/or mass spectrometric detection systems (MS or MS/MS).

Four pharmaceutical ingredients (API) selected for the research work, keeping objective to develop three new analytical methods (Ambrisentan, Azilsartan kamedoxomil and Tafluprost) for the determination of related components and assay which are stability indicating analytical methods and one method is developed.
Summary and Conclusion

(Lacosamide) for control of starting material at threshold of toxicological concern (TTC) level. One analytical method was developed for Lacosamide by using derivatization technique for detecting non-chromophoric molecule and the developed methods was extensively validated keeping the current regulatory requirements in mind.

The performance of the developed new analytical methods for its related compounds estimation has been verified by applying the same to evaluate the quality of bulk samples of API during its stability studies. The newly developed analytical methods performed well for the quality evaluation of stability samples of API. The proficient research work has been divided in to five chapters.

Chapter-1 deals with brief introduction on the need for development of new analytical methods for related components estimation, source of impurities in pharmaceutical substances, requirement for control of impurities, pharmacopeial norms, ICH quality guidelines, FDA recommendations, multi-detection system composed of ultraviolet, LC-MS for identification of unknown impurities, new technology of stationary phases, various hyphenated techniques, approaches for development of stability indicating methods with LC contents of method validation parameters and discussion on how to develop new approaches on the general methodology for LC analysis.

Chapter-2 deals with the development and validation of novel stability indicating RP-HPLC method for estimation of related substances and degradation products in Ambrisentan.

This chapter starts with a brief account on Ambrisentan with details such as chemical name, structure, mode of action, characteristics, commercially available
formulations and literature on physicochemical properties and methods reported for Ambrisentan in literature so far. A gradient RP-HPLC method is developed for the estimation of related compounds and assay of Ambrisentan drug substance. The literature survey reveals that, no stability indicating HPLC methods available for the quantification of Sulphonyl pyrimidine impurity, Hydroxy acid impurity, Hydroxy ester impurity, Benzophenone impurity, Pyrimidine ester impurity as process related impurities and Vinyloxy impurity as degradation product and other unknown impurities. No HPLC methods were reported in major pharmacopeia like USP, EP, JP and BP. Hence, an attempt has been made to develop a stability indicating method for Ambrisentan API.

The chromatographic separation was optimized using Waters symmetry C18 column. A gradient elution was involved with 2.72 g potassium dihydrogen orthophosphate in 1000 mL of Milli-Q water and adjusted its pH to 3.0 with diluted orthophosphoric acid as a mobile phase A and acetonitrile: water in the ratio of 90:10 (v/v) as mobile phase B. The flow rate of the mobile phase and the column temperature was set as 1.0 mL/min and 25°C respectively. The detection wave length was optimized at 210 nm. The column loading was finalized as 8 µg of Ambrisentan in 10 µL injection volume.

The specificity of the developed LC method for Ambrisentan was established in presence of its known impurities. Degradation was not observed under stressed conditions like photolytic, thermal, sunlight, 75% relative humidity and oxidation. The degradation of drug substance was observed only under acid, base and water hydrolysis. Acid and water degradations lead to formation of Vinyloxy impurity and base degradation leads to formation of Hydroxy acid impurity. The purity angle was within the purity threshold limit...
for all the stressed samples, demonstrating the homogeneity of the analyte peak. This method is validated as per ICH guidelines, w.r.t. to precision, limit of detection, limit of quantification, accuracy, linearity, ruggedness and robustness. The calibration curve was drawn by plotting average area of the impurities on the Y-axis and concentration on the X-axis which has shown linear relationship with a correlation coefficient greater than 0.998 for all impurities. The relative standard deviations of intra and inter day experiments were less than 8.0%. The detection limits ranged from 0.0017 to 0.0039% and quantification limits ranged from 0.0073 to 0.0132. The accuracy of the method was calculated at 50%, 75%, 100%, 125% and 150% to the impurities specification limit. % recovery of impurities was in between 100.7 to 105.0.

**Chapter-3** deals with the trace level determination of D-Serine, an aliphatic amino acid, in Lacosamide by pre-column derivatization RP-HPLC method and confirmation of N-Fmoc-D-Serine derivative by LC-MS.

This chapter starts with the a brief introduction giving some details, such as chemical name, structure, mode of action, characteristics and literature reported for Lacosamide and D-Serine. D-Serine is a key starting material in the synthesis of Lacosamide drug substance. Hence, to control the D-Serine TTC level the present work was carried. From the literature no trace level (3.5 ppm) method for D-Serine content in Lacosamide drug substance. D-Serine is less UV active amino acid and basically amino acids are not well retained in reverse phase HPLC conditions. Hence, it is challenge to develop a method for trace level D-Serine estimation in Lacosamide drug substance. Hence, a rapid, accurate, sensitive and validated derivatization method for D-Serine content in Lacosamide was developed.
The method was developed by using YMC Pack Pro C18 (150 x 4.6 mm, S-3 µm, 12 nm) HPLC column. The separation was achieved by using gradient elution. The mobile phase-A contains 1 mL of ortho phosphoric acid in 1000 mL of milli-Q water and the mobile phase-B contains a mixture of water and acetonitrile in the ratio 10: 90 (v/v). The flow rate of mobile phase was 1.0 mL/min. The column temperature was maintained at 25°C. The detection was monitored at a wavelength of 265 nm. The injection volume was 5 µL.

D-Serine was derivatized (N-Fmoc-D-serine) at room temperature using a pre column procedure. The total time required for the derivatization procedure was less than 5 min. Derivative was confirmed by using LC-MS m/z values were identified by using mass detector. m/z values for N-Fmoc-D-Serine and FMOC-Cl were observed 327.2 and 258.7 respectively.

The proposed method is specific, linear, accurate and precise. The calibration curve was drawn by plotting average area of the N-Fmoc-D-Serine on the Y-axis and concentration on the X-axis which has shown linear relationship with a regression coefficient greater than 0.999 for N-Fmoc-D-Serine. The relative standard deviation of precision study was less than 0.9%. The limit of detection was 0.23 ppm and limit of quantification was 0.7 ppm. The accuracy of the method was calculated at 50%, 100%, 150% and 200% to the N-Fmoc-D-Serine specification limit (3.5 ppm). The average % of recovery was 97.4.
Chapter-4 deals with the rapid novel HPLC method for estimation of eight related compounds in Azilsartan kamedoxomil and identification of degradation compounds by using LC-MS.

This chapter starts with a brief account on Azilsartan kamedoxomil (AZL) with details such as chemical name, structure, mode of action, characteristics, degradation pathway, formation of AZL from two different routes of synthesis, commercially available formulations and literature on physicochemical properties and assay methods reported for AZL in literature so far. In order to have cost-effective process, AZL was synthesized from two different routes. Hence, the present work was taken up to have a suitable HPLC method that can quantify the process related and degradation impurities from two different route of synthesis. AZL was not listed in major pharmacopeia like USP, EP, JP and BP. The literature survey reveals that, no stability indicating HPLC methods were available for the quantification of related compounds in AZL drug substance.

A new stability indicating HPLC method was developed and validated for quantitative determination of AZL along with its eight related substances in drug substance. The chromatographic separation was performed on YMC Pack pro C18, column (150 x 4.6 mm, 3 µm). Mobile phase consists of two components. Mobile phase A which contains 2.72 g of potassium dihydrogen orthophosphate and 4 g of 1-octane sulfonic acid sodium salt anhydrous dissolved in 1000 mL of Mill-Q water, the pH adjusted to 2.5 with diluted orthophosphoric acid (Milli-Q water in the ratio of 1:1). Mobile phase B contains a mixture of water and acetonitrile in the ratio 10: 90 (v/v) in a gradient mode. The flow rate of mobile phase was 1.0 mL/min. The detection was measured at wavelength 220 nm. The injection volume was 5 µL.
Summary and Conclusion

Slight degradation was observed when the drug was subjected to photo degradation [UV direct for 200 (Wh/m²) and LUX direct for 1.2 million (lxh)]. Degradation was observed when the drug was subjected to 75% relative humidity (5 days), sunlight (30 h) and water (at 60°C for 1 h) and significant degradation was observed with acid (0.1 N HCl at 25°C for 16 h), base (0.01 N NaOH at 25°C for 10 min) and peroxide (1% H₂O₂ at 25°C for 1 h). Acid degradation leads to the formation of impurity-4. Base, peroxide, water and 75% relative humidity degradations lead to the formation of impurity-3. AZL is well resolved from all its related substances and degradants, proving the stability-indicating power of the method.

An LC–MS study was carried to determine the m/z value of the major degradation product formed under acid, base, peroxide and water degradation conditions. The ESI mass spectrum of the acid degradation major peak showed [M+H]⁺ at m/z 541.2, which corresponds to impurity-4. The APCI mass spectrum of base, peroxide and water degradation studies showed major peaks [M-H]⁻ at m/z 455.3, 455.2 and 455.3, respectively, which corresponding to impurity-3.

This method is validated as per ICH guidelines, w.r.t. to precision, limit of detection, limit of quantification, accuracy, linearity, ruggedness and robustness. The calibration curve was drawn by plotting average area of the impurities on the Y-axis and concentration on the X-axis which has shown linear relationship with a correlation coefficient greater than 0.998 for all impurities. The relative standard deviations of intra and inter day experiments were less than 1.77%. The detection limits ranged from 0.00210 to 0.00420% and quantification limits ranged from 0.0074 to 0.0152. The
accuracy of the method calculated at 50%, 75%, 100%, 125% and 150% to the impurities specification limit. % recovery of impurities was in between 99.6 to 102.

**Chapter-5** deals with separation and estimation of a critical pair of Tafluprost and its geometric isomer and other related impurities by using RP-HPLC.

This chapter starts with a brief account on Tafluprost (TFL) with details such as chemical name, structure, mode of action, characteristics, origin of impurities and degradation pathway of acid and base degradations, commercially available formulations and literature on physicochemical properties and methods reported for TFL in literature so far. The present work was taken up to separate and estimate the critical pair of Tafluprost and its geometric isomer and other related impurities. TFL was not listed in major pharmacopeia like USP, EP, JP and BP. The literature survey reveals that, no stability indicating HPLC methods were available for the quantification of related compounds in TFL drug substance.

A new gradient method was developed for separating process impurities of TFL from its degradation peaks, thus proving the method to be stability indicating. To separate the TFL and trans isomer different columns are studied finally separation was achieved on Thermo accucore XL C18 (250 x 4.6 mm, 4µm) (Thermo scientific, USA) columns. Mobile phase contains two components. Mobile phase A contains 0.1% orthophosphoric acid in Mill-Q water and methanol in the ratio of 90:10 (v/v) and the mobile phase B contains a mixture of water and acetonitrile in the ratio 10:90 (v/v) in gradient mode. The column temperature was maintained at 50°C. The detection was measured at wavelength 210 nm. The injection volumes 15 µL for related compounds estimation method and 5 µL for assay method were used. acetonitrile: water (50:50 v/v) was used as diluent.
Degradation not observed when the drug was subjected to water (at 60°C for 48 h) and 75% relative humidity (10 days). Slight degradation was observed when the drug was subjected to thermal (at 60°C for 10 days), photo degradation (UV direct for 200 (Wh/m^2) and LUX direct for 1.2 million (lxh.). Degradation was observed when the drug was subjected to under sunlight (20 h), acid (0.05 N HCl at 25°C for 48 h), base (0.05N NaOH at 25°C for 1 h) and peroxide (6% H_2O_2 at 25°C for 44 h). Sunlight degradation leads to the formation of 0.61 RRT impurity and acid degradation lead to the formation of acid impurity. Base degradation leads to formation of acid and 0.68 RRT impurities. Peroxide degradation leads to formation of 0.46 RRT impurity.

An LC–MS study was carried to determine the m/z value of the major degradation products formed under acid and base degradation conditions. In acid degradation, the major degradant in the study was found 4.05% of acid impurity, with total impurities of approximately 4.35%. The APCI mass spectrum of the 4.05% peak showed [M-H]^- ion at m/z 409.2, which corresponds to acid impurity.

In base degradation, the major degradants in the study was found 23.33% of acid impurity and 14.58% of 0.68 RRT impurity, with total impurities of approximately 38.11%. The APCI mass spectrum of the 23.33% peak showed [M-H]^- ion at m/z 409.2, which corresponds to acid impurity and APCI mass spectrum of the 14.58% peak showed [M+H]^+ at m/z 425.3 which corresponds to 0.68 RRT impurity. During base degradation, NaOH was the base and methanol was used for dissolution of the sample. In this condition Tafluprost undergoing trans esterification formed a methyl ester impurity. Based on the structure of the TFL and m/z 425.3 the 0.68 RRT impurity is most probably methyl ester of Tafluprost.
Summary and Conclusion

This method is validated as per ICH guidelines, w.r.t. to precision, limit of detection, limit of quantification, accuracy, linearity, ruggedness and robustness. The calibration curve was drawn by plotting average area of the impurities on the Y-axis and concentration on the X-axis which has shown linear relationship with a correlation coefficient greater than 0.998 for all impurities. The relative standard deviations of intra and inter day experiments were less than 1.84%. The detection limits ranged from 0.00085 to 0.0066% and quantification limits ranged from 0.0026 to 0.0215. The accuracy of the method was calculated at 50%, 75%, 100%, 125% and 150% to the impurities specification limit. % recovery of impurities was in between 94.6 to 96.9.

The gradient RP-HPLC methods developed for quantitative estimation of related impurities and degradation products is accurate, precise, linear, rugged, robust and specific. Acceptable results were obtained from validation of the methods. These methods revealed an excellent performance in terms of sensitivity and speed. The methods were proven as stability-indicating and can be used for routine analysis of production samples and to ensure the stability of drug substances.