The drug discovery and development process has undergone dramatic changes particularly in the last decade. Progress in drug discovery has been fueled by improvements in methodologies and technologies including automated HPLC, automated method development, LC-MS, LC-NMR and high-throughput purification methods. During the drug discovery and development process, rugged analytical methods are highly desirable to evaluate early drug targets, drug metabolism, pharmacokinetics, process research, preformulation and formulations. In view of the continuous demand for bioanalytical methods in drug discovery and impurity profiling method for maintaining the quality and safety of drugs; the present work has been proposed. The thesis has been divided into six chapters.

Chapter 1 deals with a brief introduction to role of liquid chromatography in pharmaceutical analysis.

Chapter 2 describes development and validation of a high-throughput LC-ESI-MS method for determination of an immunosuppressant drug sirolimus on dried blood spots using monolithic column.

Chapter 3 deals with the synthesis of a molecularly imprinted polymer (MIP) followed by development and validation of a molecularly imprinted polymer based solid phase extraction method for determination of sitagliptin in rat plasma and urine.

Chapter 4 describes the isolation and characterization of one of the potential process related impurities of phenazopyridine.

Chapter 5 describes the enantiomeric separation and determination of adrafinil and its related substances.

Chapter 6 discusses the evaluation of polysaccharide-based chiral stationary phases for resolution of mirtazapine and its process related substances.
Chapter 1: Role of liquid chromatography in pharmaceutical analysis.

This chapter gives a brief introduction to various roles of liquid chromatography in pharmaceutical analysis. In the modern pharmaceutical industry, HPLC is the major and integral analytical tool applied at all stages of drug discovery, development, and production. At each phase of development the analyses of varieties of samples are performed to adequately control and monitor the quality of the prospective drug candidates, excipients, and final products, which require bioanalytical as well as impurity profiling methods.

Effective and fast method development requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization. HPLC basics, types of chromatography, detectors, method development and validation are discussed in brief in this chapter. Hyphenated techniques such as LC-MS and LC-NMR were also discussed. In addition, a description of the specifics of the latest advancements in fast, chiral and other modern LC techniques are included. In lieu of continuous demand of bioanalytical and impurity profiling method, five drugs viz. sirolimus, sitagliptin, phenazopyridine, adrafinil, mitrazapine were chosen and following objectives had been set:

A. Development and validation of a high throughput LC-ESI-MS method for determination of immunosuppressant drug sirolimus on dried blood spots using monolith column.

B. Development and validation of a molecularly imprinted polymer based solid phase extraction of anti-diabetic drug sitagliptin in rat plasma and urine using zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC).

C. Isolation and characterization of one of the potential process related impurities of phenazopyridine.

D. Enantiomeric separation and determination of adrafinil and its related substances

E. Evaluation of polysaccharide-based chiral stationary phases for resolution of mirtazapine and its process related substances
Chapter 2: Development and validation of a LC-ESI-MS method for determination of sirolimus on dried blood spots.

Immunosuppressive agents are used to avoid rejection of transplanted organs by body immune system. There are various classes of immunosuppressive agents. Sirolimus belongs to mTOR (mammalian target of rapamycin) class of immunosuppressive agents which potently inhibit T-cell proliferation. Therapeutic index of sirolimus is narrow, so its blood level concentration needs to be analyzed regularly in ng/mL level in transplant patients. It is difficult for a patient to go regularly to clinic to provide blood sample for analysis.

In this chapter the suitability of a dried blood spot (DBS) technique was evaluated for sample collection. A patient can collect blood samples from finger prick on a blood spot card without much special training to a clinic for analysis. A high-throughput LC–ESI-MS method for screening of sirolimus on DBS was developed and validated. It involves solvent extraction of a punch of DBS followed by reversed-phase LC on a relatively new monolithic column consisting of a silica rod with bimodal pore structure and detection by ESI-MS.

The analysis was less than 3 min with a very low backpressure at a flow rate of 0.5 mL/min [Mobile phase A/B: 20/80, v/v; A: 0.01% (v/v) formic acid in water, B: methanol containing 0.01% (v/v) formic acid]. The method can analyze more than 100 samples in an 8 h working day, including sample preparation. The assay was linear from 1 to 100 ng/mL. The mean recovery was 92.42%. The mean inter-day and intra-day precisions were 1.23 and 1.41%, respectively.

The developed method is simple, rapid and useful for clinical applications. The validated method was demonstrated to be accurate, precise, and robust and complied with the regulatory guidelines. Robustness of the method was tested by means of DOE technique. The stability of sirolimus on DBS samples stored in dark at 4°C for 90 days demonstrated that the samples can be stored for long periods of time before analysis. The effect of mobile phase flow rate on the performance of monolithic and particulate columns was also evaluated.
Chapter 3: Development and validation of a molecularly imprinted solid phase extraction method for determination of sitagliptin in rat plasma and urine.

Antidiabetic drugs treat diabetes mellitus by lowering glucose levels in the blood. There are different classes of antidiabetic drugs. Sitagliptin (belongs to DPP-IV inhibitor class) is a relatively new drug approved for treatment of diabetes. The current methods for analyzing sitagliptin in blood and urine are not only time consuming and require expensive instrumentation but also affected by matrix effects and have low recoveries. Plasma phospholipids are one of the major contributing sources of matrix effects in protein precipitation based sample preparation techniques.

A novel water-compatible molecularly imprinted polymer was synthesized and used for selective solid phase extraction of sitagliptin in rat plasma and urine. The effects of progenic solvents, pH, cross-linker and amount of monomer were studied to optimize the efficiency and selectivity. The adsorption kinetics and isotherms were measured. The molecularly imprinted polymer (MIP) showed good specific adsorption capacity with an optimum of 180 mg/g at pH 7.5 and selective extraction of sitagliptin from rat plasma and urine. The recovery of sitagliptin from rat urine and plasma was >98%.

Figure: Synthesis process for sitagliptin imprinted polymer.
Sitagliptin is relatively a polar compound having high solubility in water and not retain properly on C\textsubscript{18} column. Zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) is a best choice to analyze such types of polar compound which were earlier cannot be analyzed without prior derivatization. The HPLC method was developed using Merck ZIC-HILIC Column (100mm×4.6mm×5μm). Mobile phase, ACN and 15 mM ammonium acetate (pH 4.5) 90:10, v/v. Analysis was carried out under isocratic conditions using a flow rate of 0.4mL/min at 25°C. The chromatograms were recorded at 268 nm using a PDA detector. The limits of detection (LOD) and quantification (LOQ) were 0.03 and 0.10 μg/mL respectively.

Chapter 4: Isolation and characterization of a potential process related impurity of phenazopyridine

Drugs can be dangerous if there is no adequate control over their manufacture, storage and distribution. The impurity profile of a drug substance is critical to its safety assessment and its manufacturing process. Because the impurities are usually process related, they are most probably structurally similar to the synthesized target drugs.

During the process development of phenazopyridine HCl bulk drug, a potential impurity was detected in the routine impurity profiles by HPLC. This impurity was collected using semi-preparative HPLC. The semi-preparative HPLC was performed with Inertsil ODS 3V (10mm×250mm; particle size 5μm) column using water/ACN (30:70, v/v) as a mobile phase at a flow rate of 5.0 mL/min and the detector was maintained at 254 nm. Using MS/MS and multidimensional NMR techniques, the trace level impurity was unambiguously identified to be 3-phenyl-5-phenylazo-pyridine-2,6-diamine after its isolation from phenazopyridine HCl by semi-preparative HPLC. The formation of the impurity was discussed.
Chapter 5: Enantiomeric separation and determination of adrafinil and its related substances

Metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and different responses may often be observed comparing the activities of a pair of enantiomers. Chiral molecules are constituents of a large proportion of therapeutic agents, since the role of stereochemical factors in toxicity of chemotherapeutic drugs is vital. Analytical methods for controlling the enantiomeric purity of drug substances and their intermediates play a key role in the process of drug development.

A rapid and reliable high-performance liquid chromatographic method for resolution of enantiomers of adrafinil, a novel vigilance promoting agent, and its synthetic intermediates was developed. The separation was carried out on a Chiralcel OJ-H column using n-hexane–ethanol (62:38 v/v) as a mobile phase. The detection was carried out at 225 nm using a photodiode array (PDA) detector. The optical rotation and order of elution of enantiomers were assigned using a polarimetric detector. Chromatographic parameters: retention factor (k’), resolution (R_s) of R-(+)-adrafinil, S-(−)-adrafinil, I, II, III and IV on Chiralcel OJ-H were also determined. The method is suitable not only for process development of adrafinil but also for quality assurance of bulk drugs and pharmaceuticals.

Figure: The mechanism of formation of the impurity of phenazopyridine.
Chapter 6: Evaluation of polysaccharide-based chiral stationary phases for resolution of mirtazapine and its process related substances.

Mirtazapine is a tetracyclic antidepressant that finds widespread use as a racemate in the treatment of patients with severe depression. Investigations have revealed that the (S)-enantiomer of mirtazapine has better potential in the treatment of depression because of its high binding affinity with α2-adrenergic, 5-HT2 and 5-HT3 receptors. Thus, the development of single enantiomers as a new active pharmaceutical ingredient is of great importance because biological systems interact with and metabolize them differently.

High-performance liquid chromatographic methods were developed for separation of the enantiomers of mirtazapine and its four process-related substances. The direct separations were achieved on chiral stationary phases containing amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak® AD-H), cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel® OD-H) and cellulose tris(4-methylbenzoate) (Chiralcel® OJ-H). The experimental data were utilized to discuss the effects of the mobile phase composition, the nature of the alcoholic modifier and the specific structural features of the analytes on retention and separation. The elution sequence was determined under the optimized separation conditions.