“Analytical method development and validation of pharmaceutical products using HPLC”

Submitted By:

Md. Zahid Hossain

(ID: 2014-1-79-001)

Research Supervisor:

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A thesis report submitted to the Department of Pharmacy, East West University in partial fulfillment of the requirements for the degree of Master of Pharmacy.

Declaration by Research Candidate

I am Md. Zahid Hossain (ID: 2014-1-79-001), hereby declare that the thesis report entitled "Analytical method development and validation of pharmaceutical products using HPLC" submitted by me to the Department of Pharmacy, East West University in the partial fulfillment of the requirement for the award of degree of Master of Pharmacy is a genuine and authentic record of original research work carried out by me during Spring 2015 to Fall 2015 under the supervision and guidance of Dr. Repon Kumer Saha, Assistant Professor, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/ Diploma/ Fellowship or other similar title to any candidate of any University.

Md. Zahid Hossain

ID: 2014-1-79-001

Department of Pharmacy

East West University

CERTIFICATE

This is to certify that the thesis report "Analytical method development and validation of pharmaceutical products using HPLC" submitted to the Department of Pharmacy, East West University, Aftabnagar, Dhaka, in the partial fulfillment of the requirement for the award of degree of Master of Pharmacy (M. PHARM) was carried out by Md. Zahid Hossain (ID: 2014-1-79-001) under our guidance and supervision and no part of the thesis has been submitted for any other degree. We further certify that all the source of information availed in this connection is duly acknowledged.

Dr. Repon Kumer Saha

Thesis Supervisor & Assistant Professor

Department of Pharmacy

East West University

CERTIFICATE

This is to certify that the thesis report "Analytical method development and validation of pharmaceutical products using HPLC" submitted to the Department of Pharmacy, East West University, Aftabnagar, Dhaka, in the partial fulfillment of the requirement for the award of degree of Master of Pharmacy (M. PHARM) was carried out by Md. Zahid Hossain (ID: 2014-1-79-001) under our guidance and supervision and no part of the thesis has been submitted for any other degree. We further certify that all the source of information availed in this connection is duly acknowledged.

Dr. Shamsun Nahar Khan

Chairperson & Associate Professor

Department of Pharmacy

East West University

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ABSTRACT

This study presents the development, optimization and validation of a simple HPLC method for the determination of different pharmaceutical products using HPLC. Method development was carried out by using different column specially C18 column. In first study HPLC method development and validation was carried out on Metformin. By changing mobile phase composition found Symmetrical peak. Different column was used to get satisfactory result. It is also found that among Octyl and octadecyl columns and Oyster BDS C18 column, oyster gives symmetric peaks with high theoretical plates and low tailing factor. Simple, fast, economical, accurate, precise and reproducible HPLC methods also were developed for the determination of COX 2 inhibitors. The methods were validated in terms of specificity, linearity, precision accuracy, and robustness. The proposed method's results were found to be satisfactory and are suitable for determination of COX 2 inhibitors for routine quality control of drugs in bulk drug and formulation. A simple and reproducible method was developed for anticancer drugs (Capcitabine, Cladribine, Fludarabine, Gemcitabine, Methotrexate, Epirubicin, Carmustine, Dacarbazine, Docetaxel, Paclitaxel, Vinblastin, Imatinib Mesylate) by Reverse phase high performance liquid chromatography (RP-HPLC). The separation was performed by C18 column at different temperature for different methods, as mobile phase different buffer, acetonitrile, methanol and water were used at different flow rate. The detection was performed by PDA (Photodiode array detection) detector, photo diode array UV-Visible detector were used at different wavelength. LOD (Limit of Detection) and LOQ (limit of quantification) ranged from 0.011 µg /mL to 1.16 µg/ml and from 0.047 µg /mL to 1.413 µg/mL respectively. The method which is developed is also validated in complete compliance with the current regulatory guidelines by using well developed analytical method validation techniques and tools which comprises with the analytical method validation parameters like linearity, accuracy, method precision, specificity with forced degradation, system suitability, robustness, ruggedness etc. adopting the current method the linearity obtained is near to 0.999 and thus this shows that the method is capable to give a good detector response, the recovery calculated was within the range of 98% to 102% of the specification limits.

Keywords: Metformin, COX 2 inhibitors, HPLC, method validation, RP-HPLC, Photodiode array detection, system suitability, LOD, LOQ.

CHAPTER ONE

01

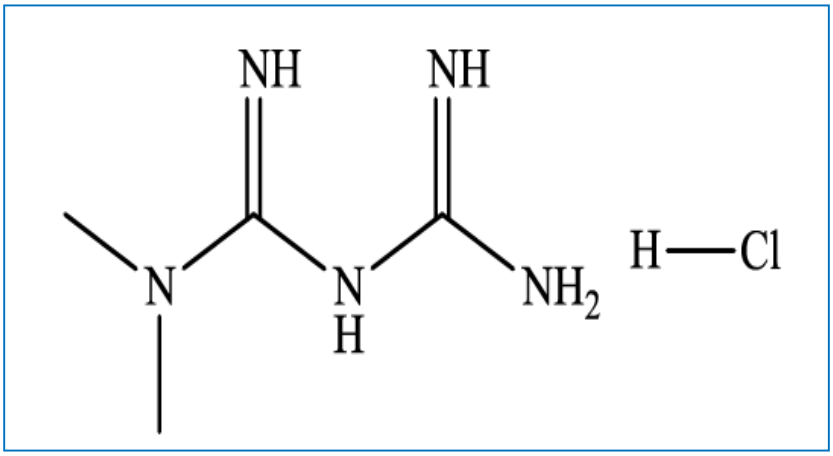
- INTRODUCTION

INTRODUCTION:

The aim of the present study was to develop and validate analytical methods for the estimation of different class of drugs by using HPLC like, antidiabetic, antihypertensive, antimalerial, antipsychotic, antioxidant, antiviral, anthelmintics, antiparasitic, antiparkinsonian, drugs use for breast cancer, bronchodilators, glucocorticoid steroid, HMG CoA reductase inhibitors, hypothyroidism drugs, hair lose treatment drugs, migraine drugs, narcotic drugs, NSAID, opthalmics, quinoline drugs. The target is to obtain an easy, rapid, reproducible as well as a rugged method.

Metformin hydrochloride, chemically known as 1, 1-dimethylbiguanide hydrochloride is a biguanide hypoglycaemic agent commonly used for the treatment of type II diabetes.
Metformin decreases glucose production in the liver, increases insulin sensitivity and enhances peripheral glucose uptake. It does not stimulate secretion of endogenous insulin [1].

Metformin is the first-line drug of choice for the treatment of type 2 diabetes, particular in overweight and obese people and those with normal kidney function. It is used in the treatment of polycystic ovary syndrome and has been investigated for other diseases where insulin resistance may be an important factor. Literature survey reveals that some methods have been reported for determination of metformin by HPLC. This present work describes the development and validation of a new RP HPLC method for simultaneous estimation of metformin in bulk and in tablet dosage formulation [2].



Chemical Structure 01: Metformin hydrochloride

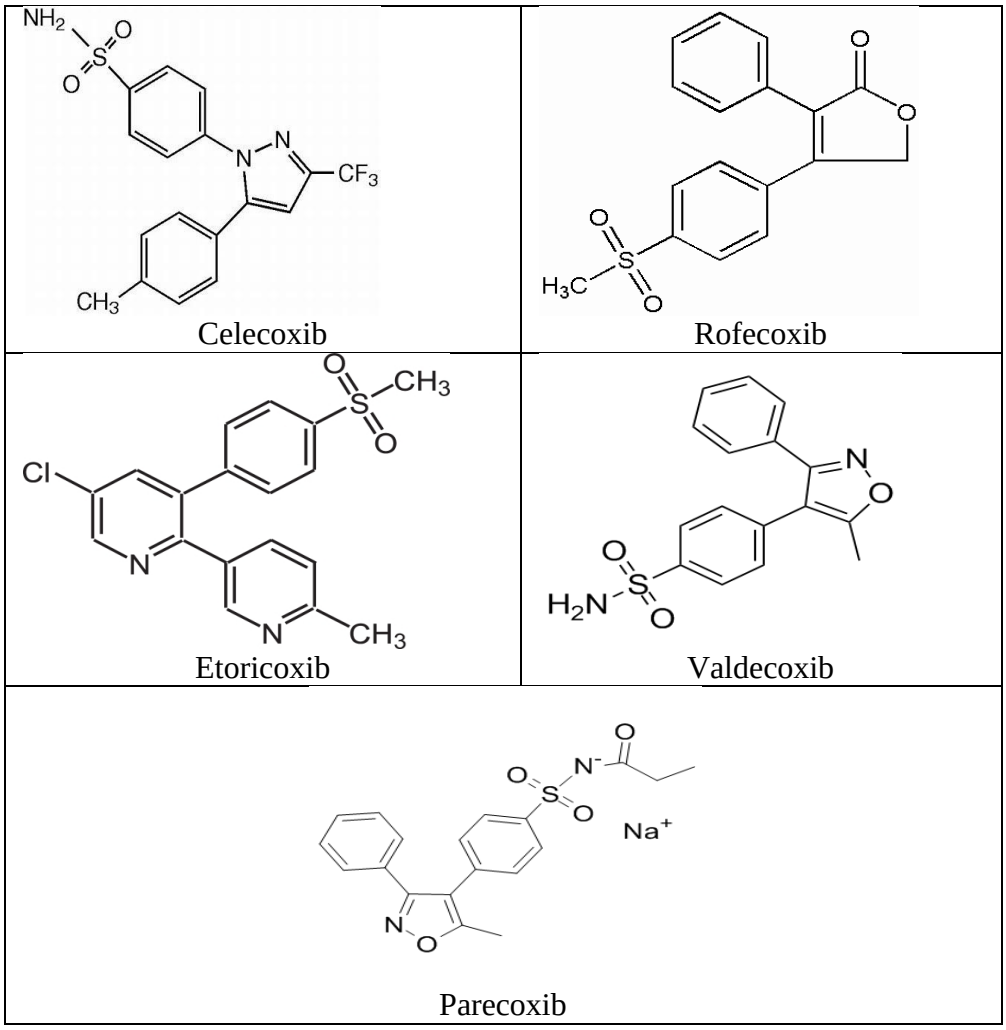
Significant interest has now been shown in the role that inflammation (driven by COX-2) plays in conditions such as Alzheimer's disease and colonic carcinoma. COX-2 is certainly induced around the inflammatory plaques seen widely throughout the central nervous system in Alzheimer's disease, and COX-2 expression is unregulated dramatically in colonic carcinoma. Epidemiological data support the argument that patients taking NSAIDs have a lower incidence and a slower rate of progression of Alzheimer's disease. NSAIDs also reduce the growth rate of colonic polyps in humans and the incidence of colonic tumours in animals [3,4].

Cyclooxygenase-2 (COX-2) inhibitors block cyclooxygenase-2 (COX-2) an enzyme that promotes inflammation. COX-2 enzyme converts arachidonic acid to prostaglandin, causing pain and inflammation. They are mainly present in places of inflammation and are responsible for formation of prostanoids (prostacyclins, prostaglandins and thromboxanes) as part of the inflammatory response. COX-2 inhibitors are used to relieve pain due to inflammation. Discovery of a second cyclooxygenase, COX-2, led to the hypothesis that NSAID side effects could be decreased, as the inhibition of COX-2 is more directly implicated in ameliorating inflammation while the inhibition of COX-1 is related to adverse effects in the GI tract. This stimulated the development of selective COX-2 inhibitors that are better tolerated than nonselective NSAIDs but comparable in analgesic efficacy. Some COX-2 inhibitors are used in a single dose to treat pain after surgery [3,4].

Etoricoxib appears as good as if not better than other pain medications. Celecoxib appears to be about as useful as ibuprofen [3,4].

COX-2 inhibitors appear to work as well as nonselective NSAIDS and may have fewer side effects. They have not been compared to other treatment options such as colchicine or glucocorticoids [3,4].

Total five COX-2 drugs have been developed by HPLC: Celecoxib, Rofecoxib, Etoricoxib, Valdecoxib and Parecoxib (structures are shown below).

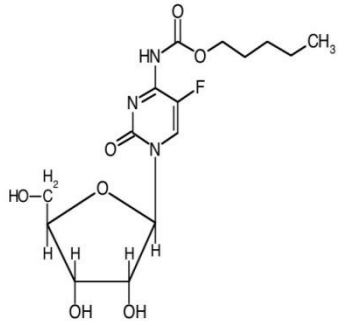
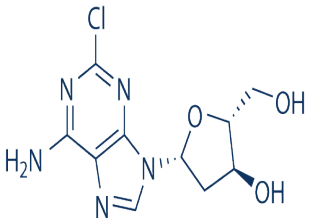
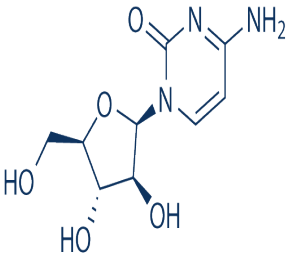


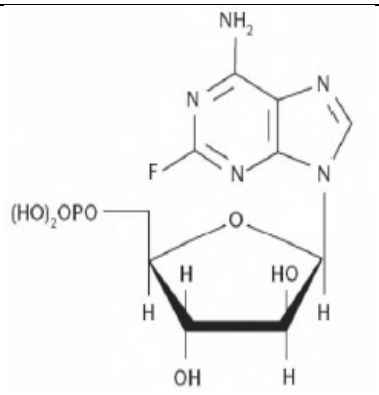
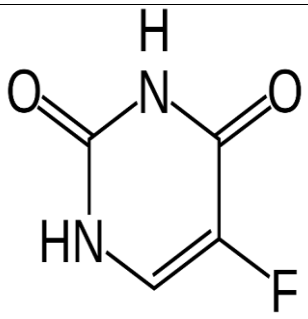
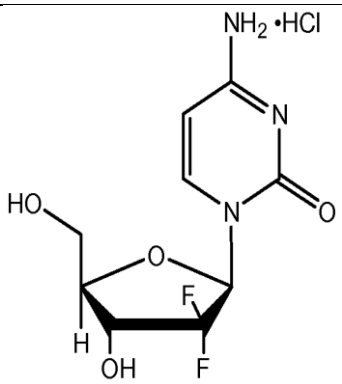
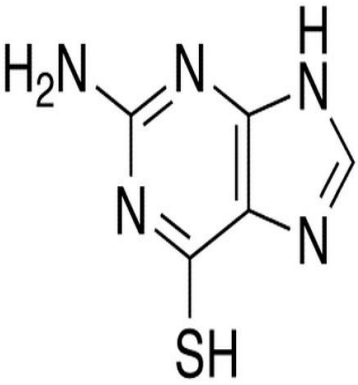
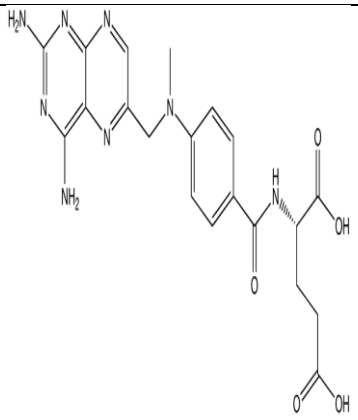
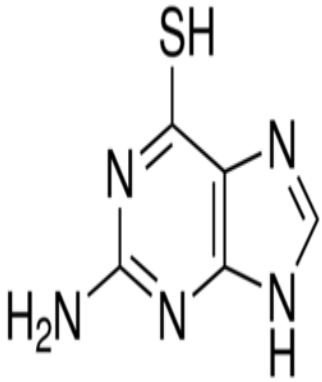
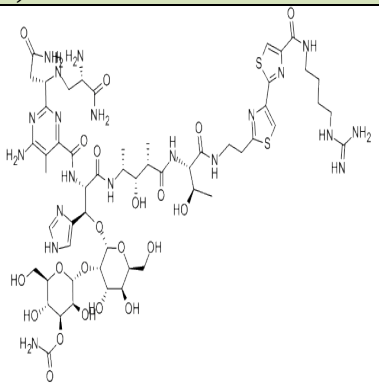
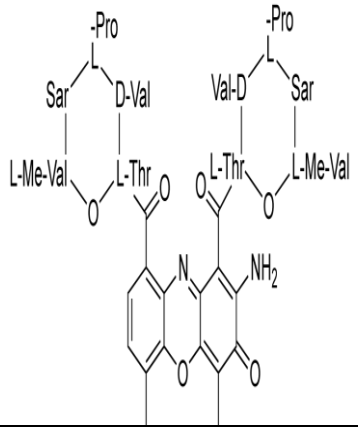
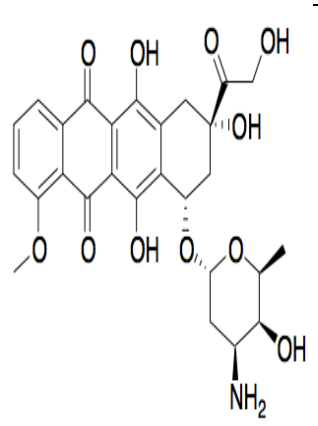
Chemical Structures 02: COX 2 inhibitors

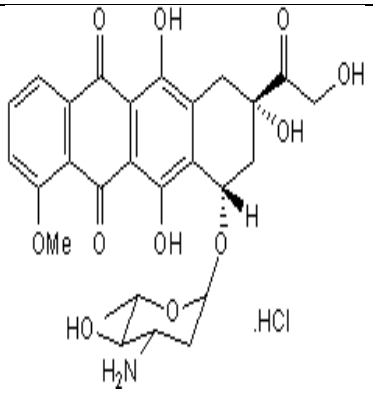
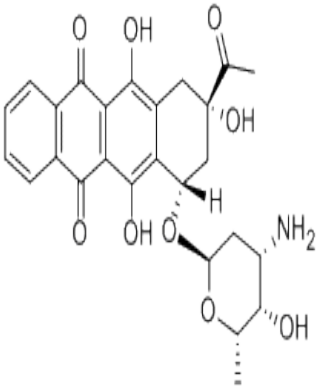
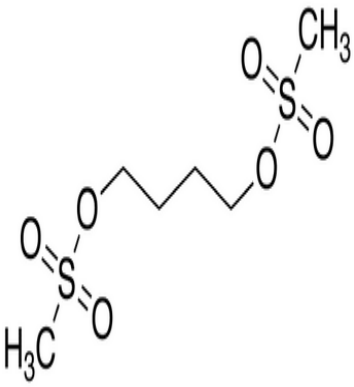
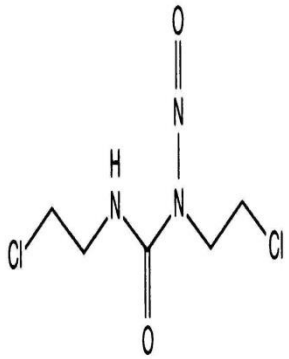
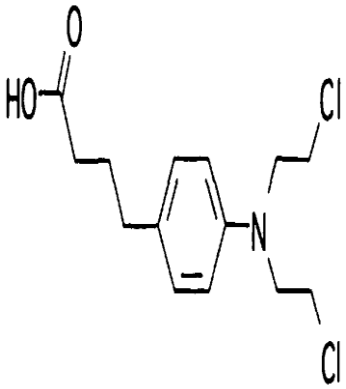
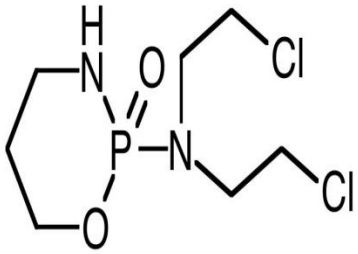
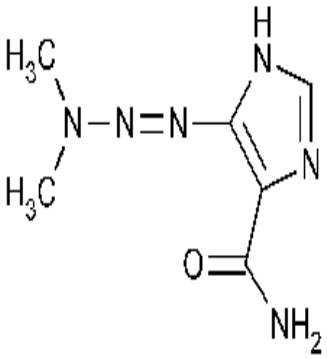
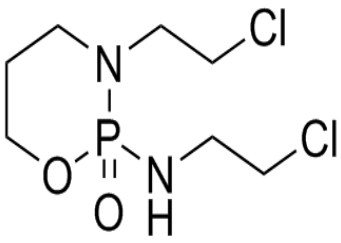
Cancer is the third most lethal disease in the world after cardiovascular, parasitic and infectious diseases, based on reports from American Cancer Society (ACS). In 2011, nearly 13 million people are diagnosed with cancer and hence, cancer continues to be a great threat to people now. Thus, the medical needs for cancer remain one of the most demanding areas in scientific research. Several studies have been carried out to prevent and treat cancers. Chemoprevention is defined as pharmacological intervention with synthetic or naturally occurring compounds that may inhibit or prevent carcinogenesis. Cancer treatment involves surgery, radiation and drugs. Surgery is the first line of therapy, is used for early stage of cancer. Radiotherapy is most often applied in a localized setting and conjunction with surgical procedures. The last one, drugs are

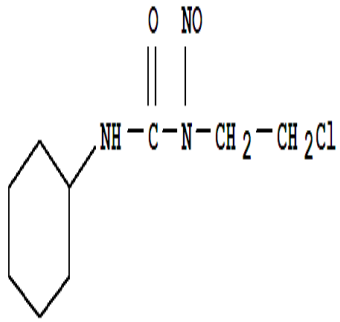
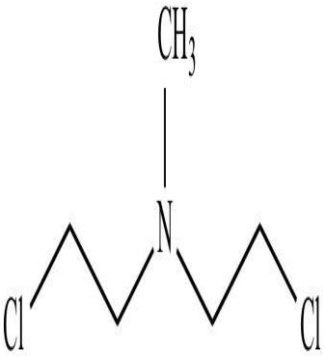
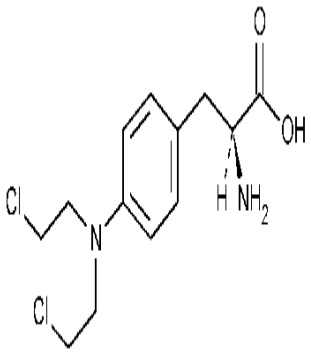
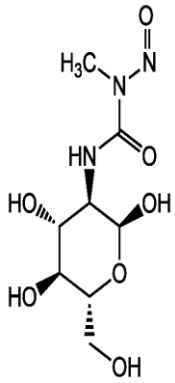
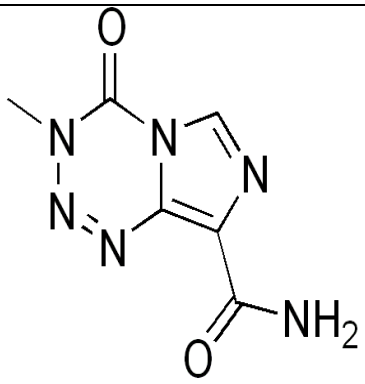
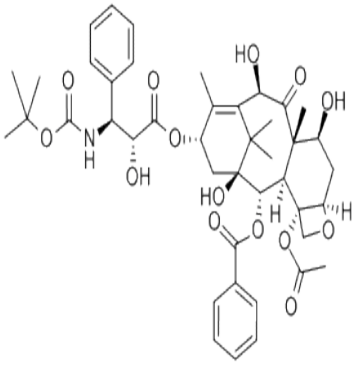
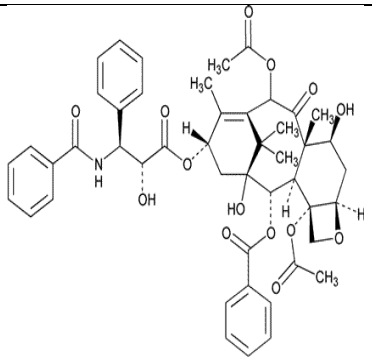
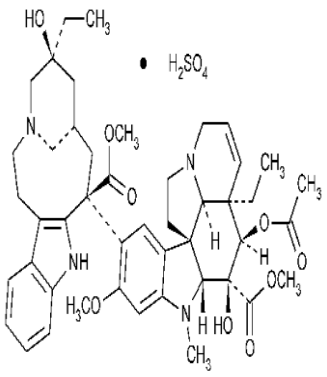
implemented with chemotherapy (CTX), which employs a wide group of drugs that have cytotoxic effects. The anticancer drugs inhibit cell division and proliferation and are less selectivity towards cancer cells. Thus, these drugs not only destroy cancer cells but also destroy normal cells [5].

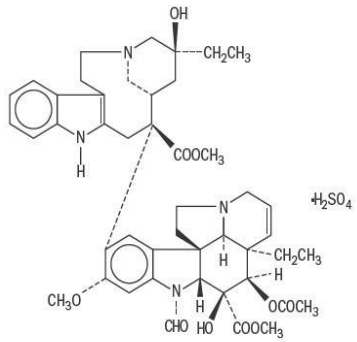
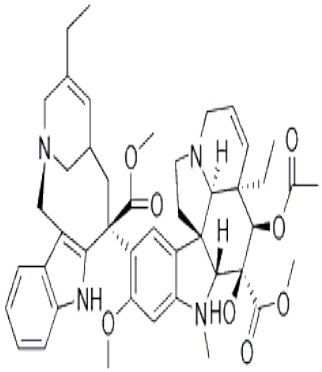
This article describes a new validated high performance liquid chromatography (HPLC) method for the simultaneous determination of anti-cancer drugs. A simultaneous determination method saves cost and time as both drugs can be injected into a single HPLC system without the need to change or re-equilibrate with a new mobile phase. The objective of the study is to develop a simultaneous determination method of two anti-cancer drugs.

Anticancer Drugs		
1) Antimetabolites		
		
Capecitabine	Cladribine	Cytarabine

		
Fludarabine	5-Fluorouracil	Gemcitabine
		
6-mercaptopurine	Methotrexate	6-thioguanine
2) Antibiotics		
		
Bleomycin	Dactinomycin	Doxorubicin

		
Epirubicin	Idarubicin	
3) Alkylating Agents		
		
Busulfan	Carmustine	Chlorambucil
		
Cyclophosphamide	Dacarbazine	Ifosfamide

		
Lomustine	Mechlorethamine	Melphalan
		
Streptozotocin	Temozolomide	
4) Microtubule Inhibitors		
		
Docetaxel	Paclitaxel	Vinblastine

		
Vincristine	Vinorelbine	
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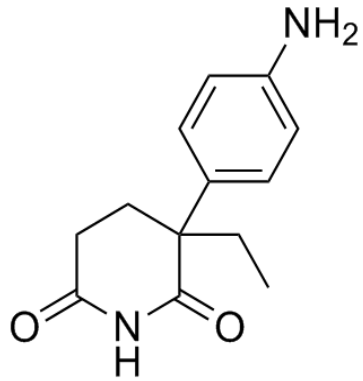
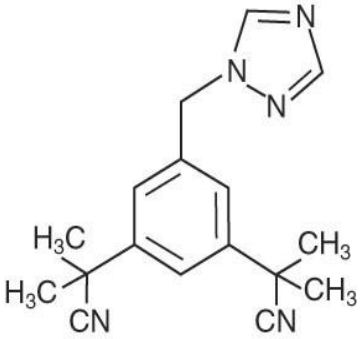
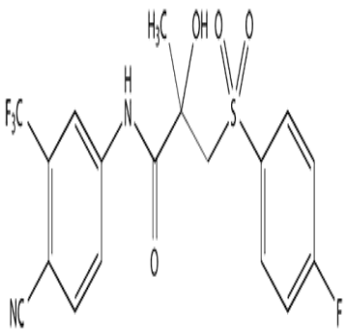
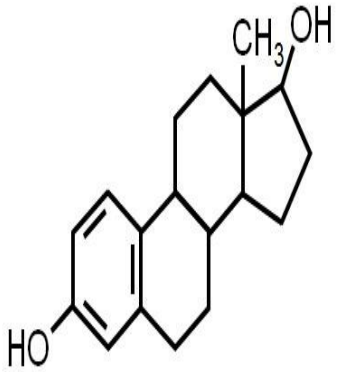
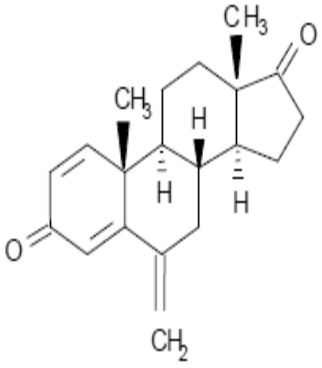
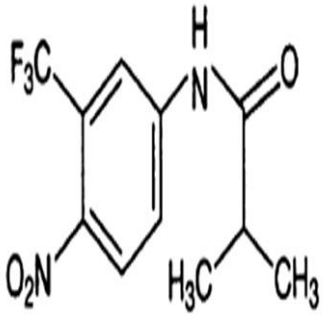
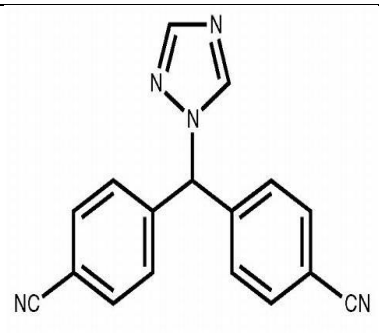
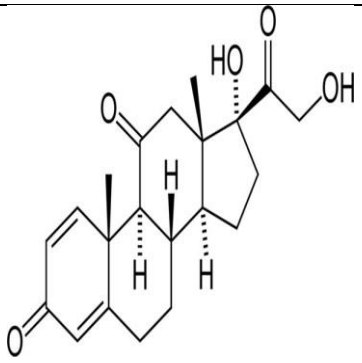
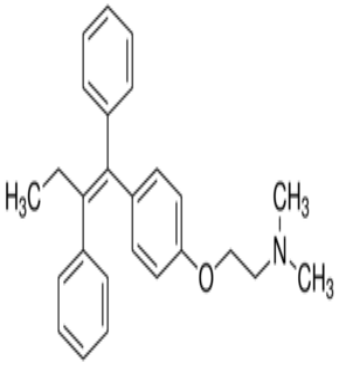
		
Aminoglutethimide	Anastrozole	Bicalutamide
		
Estrogen	Exemestane	Flutamide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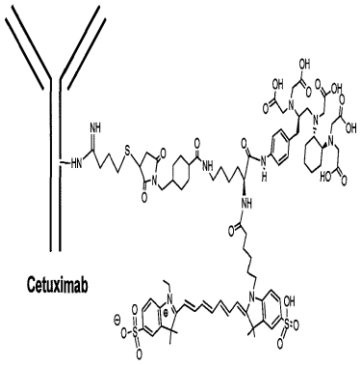
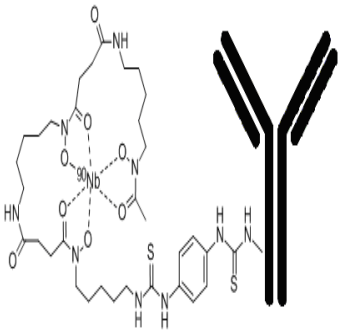
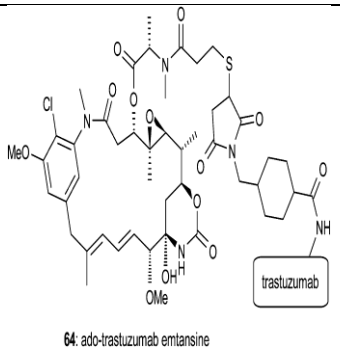
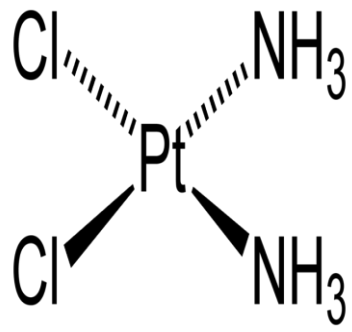
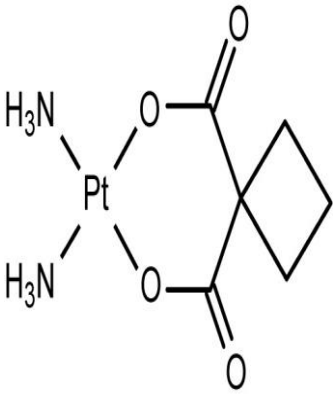
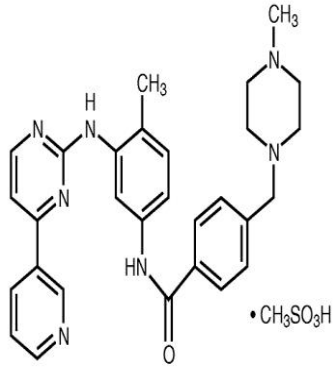
		
Cetuximab	Rituximab	Trastuzumab
7) Others		
		
Cisplatin	Carboplatin	Imatinib

Chemical structures 03: Anticancer drugs

CHAPTER TWO

LITERATURE REVIEW

HPLC is a separation technique that involves the injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 μm) in diameter called the stationary phase, where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. An output from this detector is called a "liquid chromatogram" [6].

In the present paper, the goal of this presentation is to provide pharmacists with auxiliary information concerning the use of high performance liquid chromatography for quality control of compounded preparations. After reading and studying this paper, the reader will be able to know, which type of High Performance Liquid Chromatography technique is most widely used, which is the most commonly used detector in High Performance Liquid Chromatography and why, why C-18 column widely use, why is it necessary to degas the mobile phase, how to calibrate HPLC**,** what is guard column and how to choose, how to perform method development and validation, what is the separation principle in Size Exclusion Chromatography**,** what are the differences between reverse phase HPLC and normal phase HPLC, what are the basic components for HPLC system.

In 1903 the Russian botanist Mikhail Tsvet is considered to have 'invented' the chromatographic technique when he reported separations of different plant pigments into a series of colored bands on a packed column. He called this technique 'chromatography'. The expression "high performance liquid chromatography" was created by Horvath et al. in 1967. As experimenting in his lab at Yale with superficially porous "pellicular" materials, the pressure went up the first time above 1000 psi, Horvath said, "this is not LC anymore, this is high-pressure LC (HPLC)". The consequent and visionary work of another Hungarian, Halasz with small particles 35 years ago laid down the fundaments for columns packed with fine particles and he made separations of 15 compounds possible in 60 s as early as in 1974. The understanding of the fundamentals of

reversed phase chromatography (RPC) performed by Csaba Horvath and his team at Yale, was strongly influencing the future developments in HPLC such as the concepts of the Design Space and DryLab® already in 1976 [7].

In 1979, Agilent Technologies, Inc. introduced a new diode array detector, which provided a rapid optical method for chemical analysis. In 1980s, HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far beyond previous techniques, while computers and automation provided convenience. In 1996, Waters introduced the Alliance ® HPLC system. Targeted mainly at pharmaceutical scientists concerned with the quality of their test results, the Alliance system was positioned as a product that raised the bar of performance by which HPLC would be measured. Alliance was subsequently named as "one of the most successful products in the history of analytical instruments". In 2002, JASCO Corporation introduced the first ultra-high pressure HPLC pump, installed at Imperial College, London. In 2004, Waters unveiled a new category of LC technology known as Ultra Performance LC (UPLC) that would take the science of separation to a new level. This liquid chromatography system was the first of its kind, and designed to provide chromatographic run times up to ten times shorter than those of the fastest existing HPLC systems, with up to two times better peak capacity or resolution, and three times better routine sensitivity. In 2005, ESA Biosciences, Inc. launched the first new HPLC detection technology in 20 years, known as the 'Corona Charged Aerosol Detector.' In 2006, Agilent introduced a liquid chromatography system that removed the seven most abundant proteins in human plasma, unmasking previously undetectable proteins that are potential biological markers of drug toxicity or disease. In 2008 IDEX Health & Science launched a line of Ultra High Performance (UHP) fittings and connectors that increased the ability of a separation system to handle the demands of modern techniques. Used in applications requiring greater efficiency, speed and resolution, these UHP fittings and connectors effectively handled the stresses of higher temperatures and greater column pressures. In 2009, Agilent introduced the 1290 Infinity Liquid Chromatography System, designed to deliver significantly greater power, speed and sensitivity for enhanced performance in the high-end UHPLC market. Later the same year, Agilent also introduced the 1290 Infinity LC System sample injection system, offering superior performance in speed, ultra-low carryover and robustness for customers requiring high throughput. This

injector extended sample capacity to 24 cooled microwell plates or 648 cooled 2-mL vials, for high-throughput usage. In 2010, Phenomenex introduced a unique way of achieving UHPLC performance from existing HPLC system hardware in certain applications with the Kinetex core-shell HPLC columns. Core-shell particles can be used with high mobile phase flow rates to further reduce analysis time without significant losses in separation efficiency, whereas the performance of fully porous particles begins to drop off sharply at high flow rates [8].

In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior. These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow through device (detector) that measures their amount. An output from this detector is called a “liquid chromatogram” [9].

Operation procedure of HPLC is not quite simple. At first turn on the switches of the pump and detector, computer first and then printer. Double click at "Instrument online HPLC system”. The monitor will show the word "HPLC system" which gives "peak" (when the sample is injected), the flow rate of mobile phase or solvent, wave length and pressure. For the pump, click the button "pump on". For the detector, click the button "lamp on". After that, adjust the magnitude of wavelength. Adjust the condition of the pump by keying in the values to the computer at the icon "the Instrument" and "Set up pump". Adjust the flow rate of mobile phase starting from 0.1 ml/min. Then select the proper kind of solvent to be the mobile phase; for examples, acetonitrile, water, acetic acid, methanol etc. Gradually increase the flow rate of the mobile phase by the step of 0.1 ml/min. As the time passes, the pressure increases. When the word "Ready" appears on the monitor, the pressure is constant. Then increase the flow rate of mobile phase one more step. Repeat the same way until reaching the preferred flow rate. Wait until the base line steady then prepare to inject the sample. Clean the syringe with the sample before injecting the sample into HPLC. Then inject and load the sample into HPLC, the components of the sample will be detected by the detector and the time is counted. Peak of each component will be shown on the monitor at the different retention time. When there are no more peaks detected, stop detecting the sample. Computer will calculate and integrate the area of each peak. Then the results will be presented in the relation between area and the retention time. Peaks and other results can be

viewed by clicking at the following icons; "View", "Data analysis", "Load signal" and "Integration result" [10].

Mobile phase is the most important parameter in HPLC. Type of mobile phase used may have a big effect on the retention. It can promote or suppress an ionization of the analyte molecules, and it also can shield an accessible residual silanol or any other active adsorption centers on the adsorbent surface. Proper selection of the mobile phase is the second most important step in the development of the separation method (the first one is the selection of the adsorbent type). The main requirement for the mobile phase is that it has to dissolve the analytes up to the concentration suitable for the detection. Variation of the eluent composition provides the great flexibility of HPLC separations. Choice of mobile phase depends on the mode of HPLC operation. In reverse phase applications water is usually the base solvent. Other polar solvents such as Methanol, Acetonitrile or Tetrahydrofuran are added in fixed or varying proportions. pH is adjusted by buffers to modify separations of ionizable solutes. Ion-pairing reagents also enhance separation selectivity of charged analytes by increasing retention on hydrophobic bonding phases. Typically non polar solvents such as hexane, heptane, iso-octane are used in combination with slightly more polar solvents such as isopropanol, ethyl-acetate or chloroform. Retention increases as the amount of non polar solvent increases in the mobile phase. Mobile Phases in Ion Exchange Chromatography aqueous salt solutions are generally used as mobile phases. Moderate amounts of water miscible polar organic solvents such as methanol can be added to buffered mobile phases. Solvent strength and selectivity can be adjusted by control of pH, buffer and salt concentrations. Mobile Phases in Size Exclusion Chromatography, mobile phase composition is not varied as the detector is sensitive to such changes. Choice of mobile phase is dependent on its ability to dissolve sample and maintain consistent viscosity at operating temperature. High polarity solvents such as acetone, alcohols, dimethyl sulfoxide and water are not used with polystyrene packings. Ionic strength is maintained by addition of salts. Mobile phase degassing is important prior use it. Mobile phases entrap air from the atmosphere and this trapped air gets released as small bubbles under high pressures encountered during the HPLC analysis. Such bubbles can lead to noise in detector response or hinder flow of mobile phase through columns. In order to overcome such problems degassing of mobile phase becomes essential. The HPLC column stationary phase is where the separation occurs and is the most important part of the system. Different types of analysis are classified based on the type of

stationary phase and mechanism behind the separation in the column. The interactions are basically of three types: 1) Polar Interactions, where differences in polarity between the sample components and the bonding entities on stationary phase result in preferential retention. 2) Ionic Interactions, where separation based on charge properties of sample molecules. Analyte ions have affinity for oppositely charged ionic centers on the stationary phase. 3) Molecular Size where separation takes place due to entrapment of small molecules in the stationary phase pores. Large molecules pass through first followed by elution of smaller trapped molecules. There are many types of stationary phases used in HPLC including: 1) unmodified silica, alumina or porous graphite, used in normal-phase chromatography, where separation is based on differences in adsorption; 2) a variety of chemically modified supports prepared from polymers, silica, or porous graphite, used in reversed-phase HPLC, where separation is based principally on partition of the molecules between the mobile phase and the stationary phase; 3) resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase; 4) porous silica or polymers [11].

There are five major HPLC components found in HPLC. 1) Pump: Pumps role is to force liquid (called the mobile phase) through the liquid chromatography at a specific flow rate, expressed in milliliters per min (mL/min). Normal flow rates in HPLC are in the 1 to 2 mL/min range. Typical pumps can reach pressures in the range of 6000-9000 psi (400 to 600 bar). Pump Module–types: a) Isocratic pump delivers constant mobile phase composition; solvent must be pre-mixed and lowest cost pump. b) Gradient pump -delivers variable mobile phase composition; can be used to mix and deliver an isocratic mobile phase or a gradient mobile phase. Binary gradient pump delivers two solvents and quaternary gradient pump delivers four solvents. 2) Injector: The injector serves to introduce the liquid sample into the flow stream of the mobile phase. Typical sample volumes are 5 to 20 microliters (μL). The injector must also be able to withstand the high pressures of the liquid system. An auto sampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical. How is a sample actually put into an LC system? User manually loads sample into the injector using a syringe and then turns the handle to inject sample into the flowing mobile phase which transports the sample into the beginning (head) of the column, which is at high pressure Auto sampler. User loads vials filled with sample solution into the auto sampler tray (100 samples) and the auto sampler automatically

measures the appropriate sample volume, injects the sample, then flushes the injector to be ready for the next sample, etc., until all sample vials are processed for unattended automatic operation. 3) Column: Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters. The small particles inside the column are what because the high back pressure at normal flow rates. The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph. There are four Types of columns found in HPLC: a) Analytical [internal diameter, 1.0 -4.6-mm; lengths 15 –250 mm], b) Preparative [> 4.6 mm; lengths 50 –250 mm], c) Capillary [0.1 -1.0 mm; various lengths] d) Nano [< 0.1 mm, or sometimes stated as < 100 μm] [12].

Basically column contains solid support backbone for bonded phases. Usually 10μ, 5μ or 3μ silica or polymeric particles. Bonded Phases is functional groups firmly linked (chemically bound) to the solid support which is extremely stable and reproducible. Common bonded phases are, C_8 [octyl: Si-$(CH_2)_7$-CH_3], C_{18} [octadecyl: Si-$(CH_2)_{17}$-CH_3], C_6H_5 [phenyl: Si-$(CH_2)_3$-C_6H_5], CN [cyanopropyl: Si-$(CH_2)_3$-CN], NH_2 [aminopropyl: Si-$(CH_2)_3$-NH_2], Diod-$(CH_2)_3$-OCH(OH)-CH_2-OH [13].

The guard column is a miniature column, ideally packed with the same packing material as the analytical column. This is mounted just upstream from the analytical column, to protect the analytical column. Guard columns come in a variety of dimensions. One popular column supplier and for a C18 guard column to go with a 150 x 4.6 mm i.d. column packed with 5-μm particles, they list three different options: 10 x 3.0, 10 x 2.1, and 10 x 1.0 mm i.d, all packed with 5-μm particles. Its volume should not be more than 10% of the analytical column. The packing should be similar to the one in the analytical column. If this is not available, we may use a guard column which has weaker retention to our analytes than the analytical column. 4) Detector: The detector can see (detect) the individual molecules that come out (elute) from the column. A detector serves to measure the amount of those molecules so that the analyst can quantitatively analyze the sample components. The detector provides an output to a recorder or computer those results in the liquid chromatogram (i.e., the graph of the detector response). There are different types of detector, like, a) UV-VIS Detector**,** The most commonly used detector in HPLC is the UV-VIS detector. The reason for its predominant use is that it gives specific response to a

particular compound or class of compounds. Most of the organic compounds absorb at specific wavelengths covered in the available wavelength range of the detector**.** Its response is specific to a particular compound or class of compounds depending on the presence of light absorbing functional groups of eluting molecules. Some compounds which do not have such light absorbing groups can give suitable response after post column derivatization to introduce light absorbing entities. b) Photo Diode Array Detector**,** incorporation of large number of diodes which serve as detector elements makes possible simultaneous monitoring of more than one absorbing component at different wavelengths. This provides benefit of time saving and cost reduction on expensive solvents. c) Fluorescence Detector, its detection offers greater sensitivity than a UV-VIS detector. However, the number of naturally fluorescent compounds is smaller in comparison to light absorbing compounds. This limitation is overcome by post column derivatization. d**)** Mass Spectroscopic Detector, it offers very high sensitivity and selectivity. Detection is based on fragmentation of molecules by electric fields and separation on basis of mass to charge ratios of fragmented molecules. There are four major separation modes that are used to separate most compounds. 1) Reversed phase chromatography where alkyl hydrocarbons are the preferred stationary phase; octadecyl (C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications [13].

Common stationary phase is silica which has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. Common Reverse Phase Solvents are, methanol (CH_3OH), acetonitrile (CH_3CN), tetrahydrofuran $(CH_2)_4O$, water (H_2O). The matrix usually consists of spherical silica beads (3-5 micron) which have linear octadecane groups (C18) attached to the surface via co-valent bonds. These beads are usually porous in order to increase the surface area of the beads available for binding. The C18 groups are very hydrophobic (non-polar) and can bind quite polar molecules such as charged peptides in a highly polar solvent such as water. The name "reversed phase" is derived from the opposite technique of "normal phase" chromato-graphy which involves the separation of molecules based upon their interaction with a polar matrix (silica beads without octadecane groups attached) in the presence of a non-polar solvent. Now the most strongly retained compound is the more non-polar, as its attraction to the non-polar stationary phase is greatest. The polar, being weakly retained, is won

in competition by the polar, aqueous mobile phase, moves the fastest through the bed, and elutes earliest like attracts like [13,14].

Today, because it is more reproducible and has broad applicability, reversed-phase chromatography is used for approximately 75% of all HPLC methods. Most of these protocols use as the mobile phase an aqueous blend of water with a miscible, polar organic solvent, such as acetonitrile or methanol. This typically ensures the proper interaction of analytes with the non-polar, hydrophobic particle surface. A C18–bonded silica [sometimes called ODS] is the most popular type of reversed-phase HPLC packing. Reverse phase chromatography also has the advantage of being able to use pH selectivity to improve separations. The columns are inexpensive, and are generally considered disposable. 2) Normal phase chromatography, typical stationary phases for normal-phase chromatography is silica or organic moieties with cyano and amino functional groups. In normal phase chromatography, the stationary phase is hydrophilic and therefore has a strong affinity for hydrophilic molecules in the mobile phase. Thus, the hydrophilic molecules in the mobile phase tend to bind (or "adsorb") to the column, while the hydrophobic molecules pass through the column and are eluted first. In normal phase chromatography, hydrophilic molecules can be eluted from the column by increasing the polarity of the solution in the mobile phase. There are several reasons why reverse phase HPLC has become more of a standard means of HPLC separation than normal phase. Reverse phase columns have a hydrophobic stationary phase which works well for retention of most organic analytes. This also means that water can be used as a mobile phase in conjunction with less polar solvents such as CH_3CN and CH_3OH which can be adjusted at highly controlled rates to improve chromatographic performance. Gradient separations in normal phase are much more complicated because of UV cutoff variation as well as differences in compress ability of common hydrophobic solvents, which would have an effect on flow rate. Reverse phase chromatography also has the advantage of being able to use pH selectivity to improve separations. So the short answer is reverse phase offers many more options to a chromatographer, but it depends on analytes, detection method and what type of separation you are trying to achieve. Several popular reverse phase, bonded stationary phases are shown. Octadecylsily (C18) is commonly used, as it is a highly robust hydrophobic phase, which produces good retention with hydrophobic (non-polar) analyte molecules. This phase can also be used for the separation of polar compounds

when used with mobile phase additives. C18 columns are only used in reverse phase HPLC in which mobile phase is polar and stationary phase (column) is non-polar, better to go for silica column for better illusion. If we use, DCM or Hexane there is a chance of dissolution of stationary phase in that so better don't use C18 column for those separations**.** A polar stationary phase with a much less polar [non-polar] mobile phase. This classical mode of chromatography became known as normal phase. In case of normal-phase chromatography, the stationary phase is polar and retains the polar most strongly. The relatively non-polar is won in the retention competition by the mobile phase, a non-polar solvent, and elutes quickly. Since the mobile phase (non-polar), it moves faster. It is typical for normal-phase chromatography on silica that the mobile phase is 100% organic; no water is used **[15].**

There are some differences between normal and reversed phase. If the stationary phase is more polar than the mobile phase, the separation is deemed normal phase. If the stationary phase is less polar than the mobile phase, the separation is reverse phase. In reverse phase HPLC the retention time of a compound increases with decreasing polarity of the particular species. The key to an effective and efficient separation is to determine the appropriate ratio between polar and non-polar components in the mobile phase. The goal is for all the compounds to elute in as short a time as possible, while still allowing for the resolution of individual peaks. Typical columns for normal phase separation are packed with alumina or silica. Alkyl, aliphatic or phenyl bonded phases are typically used for reverse phase separation [16].

3) Ion exchange chromatography, where separations based on Charge. For separations based on polarity, like is attracted to like and opposites may be repelled. In ion-exchange chromatography and other separations based upon electrical charge, the rule is reversed. Likes may repel, while opposites are attracted to each other. Stationary phases for ion-exchange separations are characterized by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that they attract and retain. Cation exchange is used to retain and separate positively charged ions on a negative surface. Conversely, anion exchange is used to retain and separate negatively charged ions on a positive surface. With each type of ion exchange, there are at least two general approaches for separation and elution. 4) In size exclusion chromatography the separation does not involve chemical interactions between eluting molecules and stationary phase. The separation takes place on the basis of molecular size with larger molecules eluting

first and small molecules in the end. Small molecules are retained longer in the pores of the stationary phase therefore they get eluted last. Size exclusion chromatography, where separation of mixtures based on the molecular size (more correctly, their hydrodynamic volume) of the components. Separation is achieved by the differential exclusion or inclusion of solutes as they pass through stationary phase consisting of heteroporous (pores of different sizes) cross linked polymeric gels or beads. The process is based upon different permeation rates of each solute molecule into the interior of gel particles. Size exclusion chromatography involves gentle interaction with the sample, enabling high retention of biomolecular activity. For the separation of biomolecules in aqueous systems, SEC is referred to as gel filtration chromatography (GFC), while the separation of organic polymers in non-aqueous systems is called gel permeation chromatography [17].

HPLC has contributed to analytical solutions in diverse fields such as pharmaceuticals, foods, life sciences, environment, forensics, etc. In the present module we shall discuss some application areas in pharmaceuticals and foods. High Performance Liquid Chromatography provides reliable quantitative precision and accuracy along with a high linear dynamic range to allow determination of API and related substances in a single run. A convenient method for sample preparation for solid dosage forms is dispersion in water or aqueous media modified with acetonitrile or methanol .HPLC offers several possibilities for separation of chiral molecules into their respective enantiomers. These include precolumn derivatization to form diastereomers. Alternately, specialty columns prepared with cyclodextrins or special chiral moieties as stationary phases maybe used .In short HPLC, particularly reverse phase HPLC is the most popular choice for quantitative analysis in the pharmaceutical industry. Pharmaceutical drugs like, Analgesic drugs (Antipyrine, Hydroxyantipyrine, Acetaminophen), Androgen drugs (Testosterone Acetate, Testosterone), Antianginal drugs (Verapamil), Antiarrythmic drugs (Quinidine, Disopyramide, Procainamide, N-Acetylprocainamide), Antiasthmatic drugs (Caffeine, Theophylline, Enprofylline, Theobromine), Antibacterial drugs (Penicillin-like Ampicillin, Amoxicillin, Penicillin G, Penicillin V, Tetracyclines Minocycline, Tetracycline, Doxycycline Miscellaneous Hydroxybenzotriazole, Chloramphenicol, Trimethoprim Sulfamethoxazole, Furazolidone, Nalidixic Acid), Anticoagulant drugs (Warfarin), Antidepressant drugs(Bupropion, Trazodone, Maprotiline), Antiepileptic drugs (Caffeine, Phenytoin, Methylphenylsuccinimide, Phenylethylmalonamide,

Carbamazepinepoxide, Ethosuximide, Phenobarbital, Carbamazepine, Primidone), Antiestrogen drugs (Tamoxifen), Antihistaminic drugs (Tetracaine, Promethazine, Chlorpheniramine, Tripelenamine), Antihypertensive drugs(Enalapril, Captopril), Antiinflammatory drugs (Naproxen), Antiprotozoal drugs (Metronidazole), Antitumor drugs (Paclitaxe), Antitussive drugs (Dextromethorphan), Catecholamines (Norepinephrine, Epinephrine, Dihydroxybenzylamine, Dopamine) and many more. Medical Herb Extracts Active Compounds like, Atropa belladonna (Atropine), Cortex cinchonae (Quinine, Quinidine), Dan Shen (Protocatechuic Acid, Protocatechuic Aldehyde, Tanshinone I, Tanshinone IIA, Cryptotanshinone), Ephedra sinica stapf (Ephedrine, Norephedrine), Ginko bilobae (Quercetin, Kaempferol),Rheum palmatum like Rhein, Emodin. High Performance Liquid Chromatography has brought desirable advantages in the field of food analysis. Food matrices are generally complex and extraction of analytes is not an easy task. To further complicate matters both desirable and undesirable components are often found in trace levels and classical extraction and analysis does not provide the required levels of accuracy and precision. HPLC offers viable solutions due to vast choice of stationary phases and mobile phase options. Common applications in foods like, fat soluble vitamins (A,D,E and K), Water soluble vitamins (B-complex vitamins such as B1, B2, B3, B6, Folic acid, Pantothenic acid, B12, VitaminC), Residual pesticides such as 2, 4-D and Monochrotophos, antioxidants such as TBHQ, BHA and BHT, Sugars (Glucose, Fructose, Maltose and other saccharides), cholesterol, sterols, mycotoxins such as Aflatoxins B1,B2,G1,G2,M1,M2 and ochratoxin, amino acids, steroids and flavanoids, aspartame and other artificial sweeteners etc [18].

Calibration of HPLC is one of the major important issues in quality control lab to get accurate result. According to ICH definition, the demonstration that a particular instrument or device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements. HPLC calibration involves four steps. First step is leakage test by pressure drop. At first ensure that, the instrument is ready for calibration and Start-up procedure is followed. Place inlet tubing of the Pump in to the Water HPLC grade through suction filter. Allow mobile phase to flow for about 5 min. Block Pump outlet with the block screw. The pressure rises and on crossing the 300 bar, “ERROR P-MAX” appears on the display window. Note the time. Press “CE” key and observe the pressure drop for

5 min. After 5 min., record the pressure in calibration Log. Make entry of the column usage in the Column Usage Log Register. Make entry of the usage into the Instrument Usage Log Register. Compare the result for its compliance against limit given in the Calibration Log and put the remark regarding the HPLC chromatography Calibration Status. In case of non-compliance, should follow the Maintenance Program. Second step is flow rate calibration, ensure that, the instrument is ready for calibration and Start-up procedure is followed. Ensure that, the Pump is passing the "Leakage Test (By Pressure Drop)". Keep the Drain tube in such a way that the mobile phase (Water) drops falls into 10 ml clean, dry volumetric flask without touching the walls of the flask and start immediately the stopwatch when first drop falls into the flask. Wait till the collected mobile phase reaches 10 ml mark of the volumetric flask and Stop the stopwatch. . Record the time required to collect the 10 ml mobile phase in calibration log. Repeat the procedure for 1.0 ml, 1.5 ml and 2.0 ml/ min. flow rates. Repeat the step 3 to 6 but using methanol HPLC grade as mobile phase instead of water. Compare the results for its compliance against limits given in the Calibration Log and put the remark regarding the Calibration Status. Make entry of the usage of the instrument and column in the Instrument Usage Log Register and Column Usage Log Register respectively. Prepare Calibration Status Label and display on the instrument at the designated place. In case of non-compliance, follow the Maintenance Program. Third step is to test reproducibility and linearity of injection volume. . Ensure that, the instrument is ready for calibration and Start-up procedure is followed. Ensure that, the instrument is set according to the Chromatographic conditions. Follow the Instrument Operating procedure, Inject 10 µl in triplicate and record the chromatograms. Repeat the injection of above solution by injecting 15, 20, 25, 30 µl in triplicate. Take the print out of the chromatograms and attach to the Calibration Log. Record the Area and Retention times of the of the Benzene and Toluene peaks in the Calibration Log. Make entry of the usage of the instrument and column in the Instrument Usage Log Register and Column Usage Log Register respectively. Plot the curve for area corresponding to Benzene to Toluene peaks v/s injection volume, Find out the RSD (reproducibility) and record in the Calibration Log. Find out the Correlation coefficient "r2" for each peak at five levels and record in the Calibration Log. Compare the result for its compliance against limit given in the Calibration Log and put the remark regarding the Calibration Status. Prepare Calibration Status Label and display on the instrument at the designated place. In case of non-compliance, follow the Maintenance Program. Third step is detector calibration (D2 Lamp

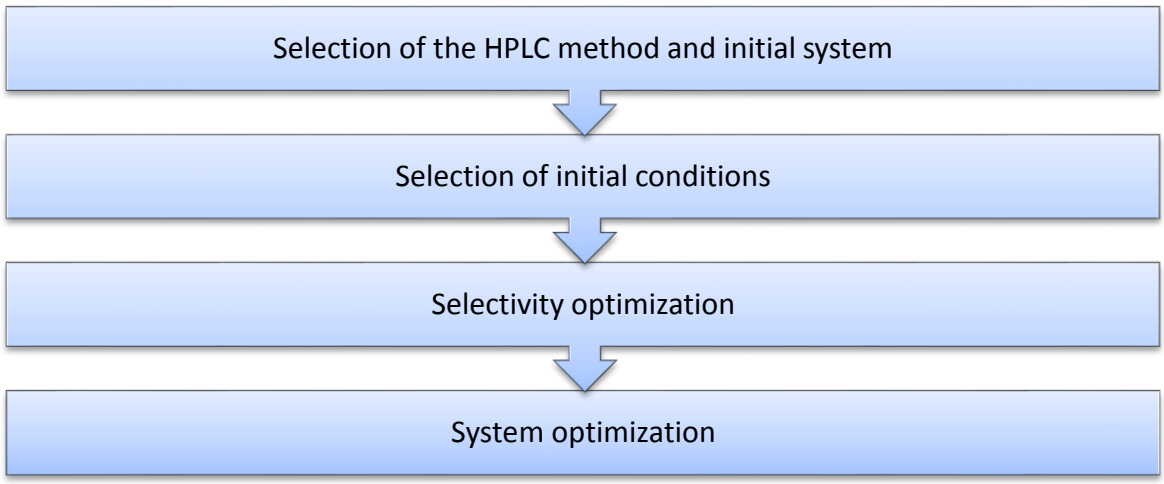
Enregy check). Ensure that, the instrument is ready for calibration and Start-up procedure is followed. On the Detector's display window, some values at the functions "l(nm)", "abs(AU)", "range(AUFS)" and "lamp" appears. On the display, previously set value blinks at "l (nm)" function, enter the wavelength to 254 nm by pressing numeric keys. Press "Func Back" key and select "lamp" functions, enter 1 to select D2 lamp. Further press "Func Back" key till "REF EN" appears. Record the Reference Energy of the D2 Lamp at 254 nm in the Calibration Log. Compare the result for its compliance against limit given in the Calibration Log and put the remark regarding the Calibration Status. In case of non-compliance, follow the Maintenance Program. Last step is to check linearity of detector response. Ensure that, the instrument is ready for calibration and Start-up procedure is followed. Ensure that, the instrument is set according to the Chromatographic conditions. Follow the Instrument Operating procedure, inject each of the Solutions in triplicate and record the chromatograms. Take the print out of the chromatograms and attach to the Calibration Log. Record the Area and Retention times of the of the Benzene and Toluene peaks in the Calibration Log. Make entry of the usage of the instrument and column in the Instrument Usage Log Register and Column Usage Log Register respectively. Calculate the Area Ratio of Benzene to Toluene, Find out the Mean value of the ratios and record in the Calibration Log. Find out the Correlation coefficient "r2" from the Mean area ratio values of the three levels and record in the Calibration Log. Plot the curve for area ratio corresponding to Benzene to Toluene peaks v/s concentration, Find out the RSD (reproducibility) and record in the Calibration Log. Compare the result for its compliance against limit given in the Calibration Log and put the remark regarding the Calibration Status. Prepare Calibration Status Label and display on the instrument at the designated place. In case of non-compliance, follow the Maintenance Program [19].

HPLC method development and validation play important role in the discovery, development and manufacture of pharmaceutical products.

Step 1 (HPLC method development selection of the HPLC method and initial system):

Sample preparation: Does the sample require dissolution, filtration, extraction, preconcentration or clean up? Types of chromatography: If the sample includes polar analytes then reverse phase HPLC.If the sample is nonpolar use normal phase HPLC. Gradient HPLC: Gradient HPLC will

give greater sensitivity, particularly for analytes with longer retention times, because of the more constant peak width (for a given peak area, peak height is inversely proportional to peak width [20].



Flow chart: Steps in method development. [20]

Column dimensions: For most samples (unless they are very complex), short columns (10–15 cm) are recommended to reduce method development time.). Detectors: Fluorescence or electrochemical detectors should be used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without overloading the detector.

UV wavelength: UV wavelengths below 200 nm should be avoided because detector noise increases in this region. The wavelengths were selected by scanning in the range of 200 -400 nm against mobile phase as a blank. Drugs show maximum absorbance at which wavelength, select this wavelength [20].

Step 2 (selection of initial conditions):

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Mobile phase solvent strength *(*in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one). Determination of initial conditions *(*The

recommended method involves performing two gradient runs differing only in the run time. A binary system based on either acetonitrile/water (or aqueous buffer) or methanol/water (or aqueous buffer) should be used) [20].

Step 3 - selectivity optimization:

The aim of this step is to achieve adequate selectivity [20].

Step 4 - system parameter optimization:

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity [20].

As per ICH method validation can be defined as (ICH) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will be consistently produced a desired result or product meeting its predetermined specifications and quality characteristics" (ICH, 2005).

Method validation study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments [21].

1) Accuracy: The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value [21-23].

2) Precision: Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample". A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types: a) Repeatability, b) Intermediate precision and c) Reproducibility [21-23].

Repeatability is the precision of a method under the same operating conditions over a short period of time. Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory.

Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments [21-23].

3) Specificity / Selectivity: The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other [21-23].

4) Limit of detection: Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions [21-23].

5) Limit of quantification: Limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions [21-23].

6) Linearity: The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity [21-23].

7) Range: The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample [21-23].

8) Stability: To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g., one day, one week, and one month, depending on need). Therefore, a few hours of standard and sample solution stability can be required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability [21-23].

Characteristics	Acceptance criteria
Accuracy	Recovery, 98-102 %
Precision	RSD < 2 %
Repeatability	RSD < 2 %
Intermediate Precision	RSD < 2 %
Specificity/ selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	R^2 < 0.9999
Range	80-120 %
Stability	> 24 h or >12 h

Table 01: Acceptance criteria of validation for HPLC (ICH, 2005).

9) System suitability:

9.1) Plate number or number of theoretical plates (N): This is a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP uses the peak width at half the height [21-23].

9.2) Capacity factor or Capacity ratio (K): This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase) [21-23].

9.3) Separation Factor or Relative retention (α): This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase [21-23].

9.4) Tailing factor and Asymmetry factor: If the peak has to be quantified is asymmetric, a calculation of the asymmetry would also be useful in controlling or characterizing the chromatographic system [21-23].

9.5) Resolution (Rs): The resolution of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width [21-23].

9.6) Relative standard deviation: Relative standard deviation of retention time checked after 6 injections of standard and sample [21-23].

Parameter name	Limit
Number of theoretical plates or Efficiency (N)	> 2000
Capacity factor (K)	≥ 2
Separation or Relative retention (α)	≥ 1
Resolution (Rs)	≥ 2
Tailing factor or Asymmetry (T)	≤ 2
Relative Standard Deviation	≤ 2

Table 02: Limits of system suitability parameters (USP, 2003).

HPLC is currently the most widely used method of quantitative and qualitative analysis in the pharmaceutical industry and in pharmaceutical analysis laboratories [23].

CHAPTER THREE

03

- **A REVIEW ON ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF PHARMACEUTICAL PRODUCTS USING HPLC**

Classification of drugs	Mobile Phase	Column	Wave length	LOD	LOQ	Retention Time	Reference
1)Anti-diabetic Drugs							
i)Sensitizer							
a)Thiazolidinedione							
➢ Pioglitazone hydrochloride	Acetonitrile-methanol-water (30:20:50, v/v)	Hiber, 250-4.6 RP-18 column	230 nm	-	-	-	[24]
	Methanol-water-acetonitrile (80:10:10, v/v/v)	C18 column (5 µm, 25 × 0.46 cm)	230 nm	0.93 µg/mL	3.11 µg/mL	-	[25]
	Methanol-water (90 : 10, v/v)	-	235 nm	1.30 µg/mL	4.28 µg/mL	-	[26]
	Methanol-phosphate buffer (75:25, v/v)	Inertsil-ODS-3 (C-18) Column (250 x 4.60 mm, 5 microm)	258 nm	-	-	0.5 min	[27]
	Acetonitrile- ammonium acetate (pH 4.5; 20mM) in proportion of 60:40 (v/v)	-	230 nm	-	-	7.0(+/-)0.1 min	[28]
	Methanol-acetonitrile-mixed phosphate buffer (40:12:48, v/v/v)	Apollo C18 column	269 nm	-	50 ng/ml.	-	[29]
	Buffer(10mM disodium hydrogen phosphate and 5mM sodium dodecyl sulphate in double-distilled water) andacetonitrile (66:34, v/v)	Zorbax XDB C(18), 15-cm analytical column	226 nm				[30]
➢ Rosiglitazone	Acetonitrile and 0.02 M phosphate buffer (60: 40, v/v)	150 mm × 4.6 mm, 5 µm particle size Symmetry® C18 column	235 nm	0.04 µg/mL	0.13 µg/mL	3.7 min	[31]

	Methanol:Water:NH_4Cl 1% w/v (5:4:1 v/v/v)	Symmetry C18 column	312 nm	-	-	-	[32]
b)Biguanide							
➢ Metformin	Methanol-water (30:70 v/v)	C(18) analytical reverse-phase column	233 nm	0.1 µg/mL	0.3 µg/mL	4.4 min	[33]
	Acetonitrile-methanol-water (30:20:50 v/v)	Hiber, 250-4.6 RP-18 column	230 nm	-	-	-	[34]
	Methanol-phosphate buffer, in the ratio of 75:25 v/v	Inertsil-ODS-3 (C-18) Column (250 x 4.60 mm, 5 microm)	258 nm	-	-	2.66 min	[35]
	Methanol and acetonitrile (30 : 70, v/v)	Octylsilyl (C8) 5 microm particle size column	230 nm			-	[36]
	Buffer(10mM disodium hydrogen phosphate and 5mM sodium dodecyl sulphate in double-distilled water) andacetonitrile (66:34, v/v)	Zorbax XDB C(18), 15-cm analytical column	226 nm				[37]
c)Sulfonylurea							
➢ Gliquidone	Methanol-water-acetonitrile (80:10:10 v/v/v)	C18 column (5 µm, 25 × 0.46 cm)	230 nm	0.24 µg/mL	0.80 µg/mL	-	[38]
	methanol-water (90 : 10, v/v)	-	235 nm	0.30 µg/mL	0.98 µg/mL	-	[39]
	Methanol-phosphate buffer in the ratio of 75:25 v/v	Inertsil-ODS-3 (C-18) Column (250 x 4.60 mm, 5 microm)	258 nm	-	-	7.12 min	[40]
➢ Glimepiride	Acetonitrile and ammonium acetate (pH 4.5; 20mM) in proportion of 60:40 (v/v)	-	230 nm	-	-	10.2+/- 0.1 min	[41]
d)Insulin	Phosphoric acid and acetonitrile	Shimadzu Shim-	214 nm	8 ng	20 ng	-	[42]

entrapment	(73:27, v/v)	pack VP-ODS column (150 mm x 4.6 mm)					
2)Antihypertensive							
i)β-blocker							
a) Atenolol	0.01 M, 4.0 pH aqueous phosphate buffer-acetonitrile (50 + 50, v/v)	Reversed-phase column, C18 column	230 nm	0.04 microg /mL	-	1.5 minute	[43]
b)Bisoprolol fumarate	0.1M potassium dihydrogen Phosphate buffer and acetonitrile (70:30, v/v)	Inertsil ODS 3V (25cmx4.6mm) 5microm column	228nm	0.01 microg /mL	0.03 microg/ mL	-	[44]
ii)Thiazide Diretic							
a)Hydrochlorothiazide	0.1M potassium dihydrogen phosphate buffer and acetonitrile (70:30, v/v)	Inertsil ODS 3V (25cmx4.6mm) 5microm column	228nm	0.01 microg /mL	0.05 microg/ mL	-	[44]
iii)Vasodilator							
a)Aminexil	Phosphate buffer and acetonitrile (78:22, v/v)	BDS Hypersil C18 column(250mm×4.6 mm×5µ)	238nm	0.31 µg/mL	0.92µg/ mL	2.3min	[45]
b) Minoxidil	Phosphate buffer and acetonitrile (78:22, v/v)	BDS Hypersil C18 column(250mm×4.6 mm×5µ)	238nm	0.31 µg/mL	0.92µg/ mL	3.9min	[45]
iv) Calcium Channel Blocker							
a)Amlodipine	Acetonitrile, phosphate buffer, methanol (35::30:35 v/v)	Hibar C18 (250 × 4.6 mm) mmx25 cm & 0.5 mm	237 nm	-	-	3.4 minutes	[46]
b)Diltiazem	Acetonitrile-methanol-water (30:20:50 v/v)	Hiber, 250-4.6 RP-18 column	230 nm	-	-	-	[47]
c) Verapamil	Methanol-water-acetonitrile (80:10:10 v/v/v)	C18 column (5 µm, 25 × 0.46 cm)	230 nm	0.40µg /mL	1.36 µg/mL	-	[48]

3)Antimalerial							
a)Desbutyl lumefantrine	Acetonitrile and 0.05% trifluroacetic acid (70:30, v/v)	HS C18 RP (150mm×4.6mm, 5µm) column	335nm	10.0ng /ml	18.0ng/ ml	-	[49]
4)Antipsychotic							
a) Haloperidol	Phosphate buffer pH 6.5 and acetonitrile	Zorbax Eclipse XDB C18 Rapid Resolution HT 4.6 mm x 50 mm, 1.8 mum particle size, column	230 nm	-	-	5.5 min	[50]
5)Anti-oxidant							
a)Glutathion	Isocratic flow of a 50/50 (v/v) mixture of water	C18 column	215 nm	0.6µg/ mL	1.8 µg/mL	-	[51]
6)Antiviral							
a) Entecavir	Acetonitrile-water (95 + 5, v/v)/potassium phosphate buffer (0.01 M, pH 4; 9 + 91, v/v)	Gemini C18 column (150 x 4.6 mm id)	253 nm	0.39microg/mL	0.5 microg/ mL	4.18 min	[52]
b) Oseltamivir	Acetonitrile and triethylamine	RP-HPLC method utilizes Kromasil C(18), 5 microm, 250 mm x 4.6 mm i.d	215 nm	-	-	-	[53]
7)Anthelmintics							
a) Triclabendazole	Acetonitrile-methanol-water-acetic acid (56 + 36 + 7.5 + 0.5, v/v/v/v)	C18 RP column	245 nm	0.03 microg /mL	0.08 microg/ mL	-	[54]
8)Antiparasitic							
a)Ivermectin	Acetonitrile-methanol-water-acetic acid (56 + 36 + 7.5 + 0.5, v/v/v/v)	C18 RP column	245 nm	0.03 microg /mL	0.08 microg/ mL	-	[54]

9)Antiparkinsonian agent							
a)Biperiden	Methanol-buffer(sodium dihydrogen phosphate (50 mM) and 1-heptanesulfonic acid sodium salt (5 mM) (50:50, v/v, pH 2.50)	C8 column	205 nm	0.03 microg /ml	0.1 microg/ ml	-	[55]
10)Breast cancer treatment							
a)Exemestane	Acetonitrile-water (60:40, %v/v)	C18 reverse phase column (Phenomenex, size: 250 × 4.60 mm, particle size 5 µm)	242 nm	-	-	-	[56]
11)Bronchodilator							
a)Albuterol sulfate b)Ipratropium bromide	Solvent A (solution containing 2.5g of potassium dihydrogen phosphate and 2.87g of heptane-1-sulfonic acid sodium salt per liter of water, adjusted to pH 4 with orthophosphoric acid) and solvent B (acetonitrile)	Inertsil C8-3, 250mmx4.6mm, 5mum column	210nm using photodiode array (PDA)detector	-	-	-	[57]
12)Glucocorticoid steroid							
a) Betamethasone dipropionate	Water (mobile phase A) and acetonitrile (mobile phase B)	RP18 analytical column (150 × 4.6 mm)	240 nm	0.02 µg/mL	0.05 µg/mL	-	[58]
13)HMG CoA reductase inhibitors							

a) Atorvastatin	Methanol-water (90 :10, v/v)	-	235 nm	0.57 µg/mL	1.90microg/mL	-	[59]
14)Hypothyrodism treatment							
a)Levothyroxine	Trifluoroacetic acid (0.1%, v/v, pH 3)-acetonitrile	C18 column	223 nm	-	-	-	[60]
15)Hair loss treatment							
a) Aminexil	Phosphate buffer and acetonitrile (78:22)	BDS Hypersil C18 column (250mm×4.6mm×5µ) as stationary phase	238nm	0.31µg/mL	0.92µg/mL	2.3min	[61]
b) Minoxidil	Phosphate buffer and acetonitrile (78:22)	BDS Hypersil C18 column (250mm×4.6mm×5µ) as stationary phase	238nm	0.31µg/mL	0.92µg/mL	3.9min	[61]
16)Insecticide							
a) Permethrin	Methanol and 0.025 mM Phosphoric acid (85:15 v/v)	C-18 Nucleosil (Macherey-Nagel, Germany) column (250 × 4.6 mm)	272 nm	1.782 µg/mL	48.0 µg/mL	-	[62]
17)Migraine treatment							
a) Pizotifen malate	Acetonitrile and acetate buffer pH 7.0 (60:40)	C(18) ODS column	231 nm	-	-	2.838 min	[63]
b) Almotriptan	Sodium phosphate buffer (pH adjusted to 7.6): acetonitrile (80:20, v/v)	C-18 column	227 nm	-	1.5 ug/mL	-	[64]
18)Narcotic							
a)Buprenorphine	Orthophosphoric acid and	C-18 column,	210 nm	-	-	2.4	[65]

hydrochloride	acetonitrile (17:83	Perfectsil Target ODS3 (150 mm x 4.6mm i.d., 5 microm)					
b**)** Naloxone hydrochloride	Orthophosphoric acid and acetonitrile (17:83	C-18 column, Perfectsil Target ODS3 (150 mm x 4.6mm i.d., 5 microm)	210 nm	-	-	3.8	[65]
c)Noroxymorphone	Orthophosphoric acid and acetonitrile (17:83	C-18 column, Perfectsil Target ODS3 (150 mm x 4.6mm i.d., 5 microm)	210 nm	-	-	8.1 min	[65]
19)NSAID							
a) Meloxicam	Acetonitrile-20mM potassium hydrogen phosphate (40:60, v/v, pH 3.5)	Reverse phase Sunfire C18 column (150 mm x 4.6mm, 5 microm)	355 nm	-	10 ng/ml	11.6 min	[66]
20)Opthalmic							
a) Nepafenac	Acetonitrile: Water (40: 60v/v)	C18 column	254 nm	0.0195 β g/ml	0.039 β g/ml	7.49 minutes	[67]
21)Quinoline							
a)Camptothecin	Triethyamine buffer pH 5.5 and acetonitrile	C18 column	360nm	-	-	9.8 min	[68]

CHAPTER FOUR

04

- METHOD DEVELOPMENT OF METFORMIN BY USING HPLC

Method Development of Metformin and its combination by using HPLC

The aim of the present study was to develop a new rapid RP-HPLC method for simultaneous quantification of combination drug metformin in pharmaceutical preparations and to validate that method according to ICH, USP and FDA guidelines.

Experiment 1

Using different columns for method development

Initially, a mobile phase composed of ammonium dihydrogen orthophosphate (ADP) solution (10 mM) and acetonitrile (1:1) flowing at a rate of 0.3 mL/min over Inertsil ODS-3 (50- mm × 2.1-mm, 2-µm particles) and Zorbax XDB (C-18, 50-mm × 2.1-mm, 1.8-µm particles) columns were employed for metformin HCL. The drug was eluted with highly asymmetric peak shape (tailing was more than 2.0). The variation in the organic modifier proportions in the mobile phase also did not produce a pure, symmetrical peak from the Inertsil and Zorbax and few other columns. But by using Acquity BEH C-18 (100× 2.1 mm), 1.7 µm column, found symmetrical peak which was satisfactory [69].

Sl.No.	Trails taken	Observations	Remarks
1	Inertsil ODS-3 (50 × 2.1 mm), 2-µm	Asymmetrical peak with fronting and tailing	Not satisfactory
2	Eclipse Plus C-18, RRHD (50 × 2.1 mm), 1.8 µm	Asymmetrical peak with tailing	Not satisfactory
3	XDB C-18, (50 × 2.1) mm, 1.8 µm	Split peak	Not satisfactory
4	Acquity BEH C-18 (100 × 2.1 mm), 1.7 µm	Symmetrical peak	Satisfactory

Table 03: Observations and remarks of method development using different columns [69].

Experiment 2

Comparison between Compositions of mobile phase for method development

A change in a strength of the salt (buffer) in the aqueous phase exerted imperceptible effects on the retention time, resolution and peak shape of the drug, while the use of acetonitrile in different ratios (30–80%) resulted in peak-tailing proportional to the acetonitrile (organic modifier) concentration. Replacing acetonitrile with methanol resulted in peak-fronting. Hence, a mixture of acetonitrile, methanol and buffer were employed to obtain the optimum peak shape [70].

Sl.No.	Trails taken	Observations	Remarks
1	Acetonitrile :Water (20:80 v/v)	Asymmetrical peak with tailing and early elution	Not satisfactory
2	Methanol:Water (20:80 v/v)	Very broad peak	Not satisfactory
3	Methanol:Buffer (pH 3.2, 20:80 v/v)	Broad peak	Not satisfactory
4	Acetonitrile:methanol:Buffer (pH 3.2) (10:10:80 v/v/v)	Symmetrical peak	Satisfactory

Table 04: Observation of method development for different organic solvent [70].

Experiment 3

Comparison between pH for method development

The criticality was observed *via* the buffer pH and was extensively studied for the buffer pH optimization. At pH 4, 5, 6 acceptable peaks were not observed. A buffer pH of 3.2 was found to be satisfactory while increasing the pH from 2.5 to 6.0 while keeping the other chromatographic parameters unchanged [71].

Sl.No.	Trails taken	Observations	Remarks
1	Acetonitrile:methanol :Buffer (pH 4)	Peak eluted early with less theoretical plates	Not satisfactory
2	Acetonitrile:methanol :Buffer (pH 5)	Completely split peak	Not satisfactory
3	Acetonitrile:methanol :Buffer (pH 6)	Completely split peak	Not satisfactory
4	Acetonitrile:methanol :Buffer (pH 3.2)	Symmetrical peak	Satisfactory

Table 05: Observation and remarks of method development for different pH conditions [71].

Experiment 4:

Degradation studies of Metformin:

Degradation studies were mainly performed to get the suitable condition for metformin analysis. Different heating, lighting condition were used and finally get the result for analysis [71].

No Degradation:

Degradation was not observed when MFH was subjected to light and heat conditions. No significant degradation was observed when the drug was subjected to hydrolysis (water at 80 °C for 3 h); acid (0.1N HCl at 80 °C for 3 h) [71].

Degradation

But degradation was observed when the drug was experimented in basic condition (0.1M NaOH , 80 °C, 3 hours) [71].

Degradation condition	% degradation
Acid hydrolysis (0.1M HCl , 80 °C, 3 hours)	No degradation
Base hydrolysis (0.1M NaOH , 80 °C, 3 hours)	99.9 %
Oxidation (5% H_2O_2 , 80 °C, 3 hours)	No degradation
Thermal (80 °C, 24 hours)	No degradation
Photolytic (1.2 million lux hours)	No degradation

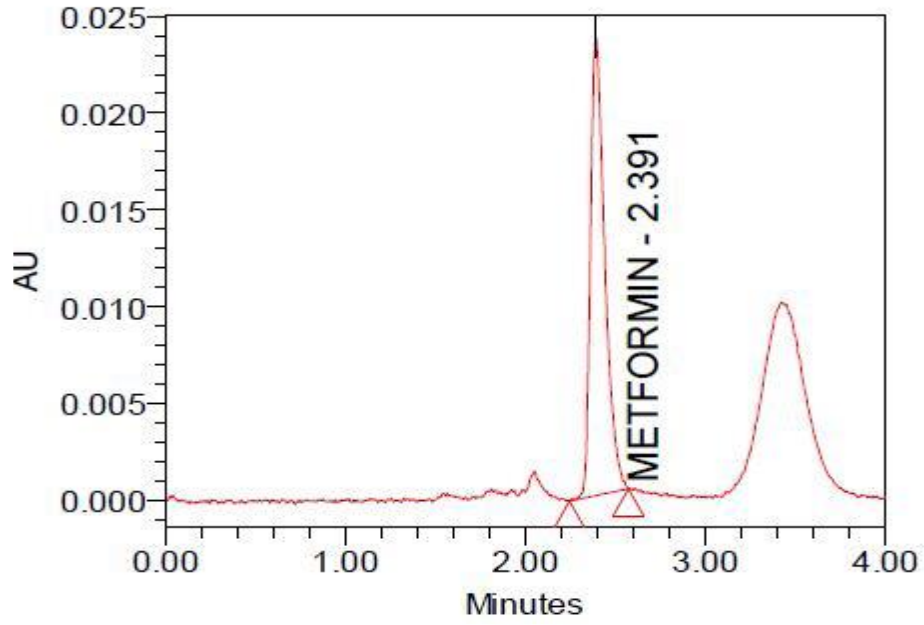
Table 06: The results from the forced degradation studies are presented in table [71].

Experiment 5:

Different solvent use for method development of metformin:

Trial: 1

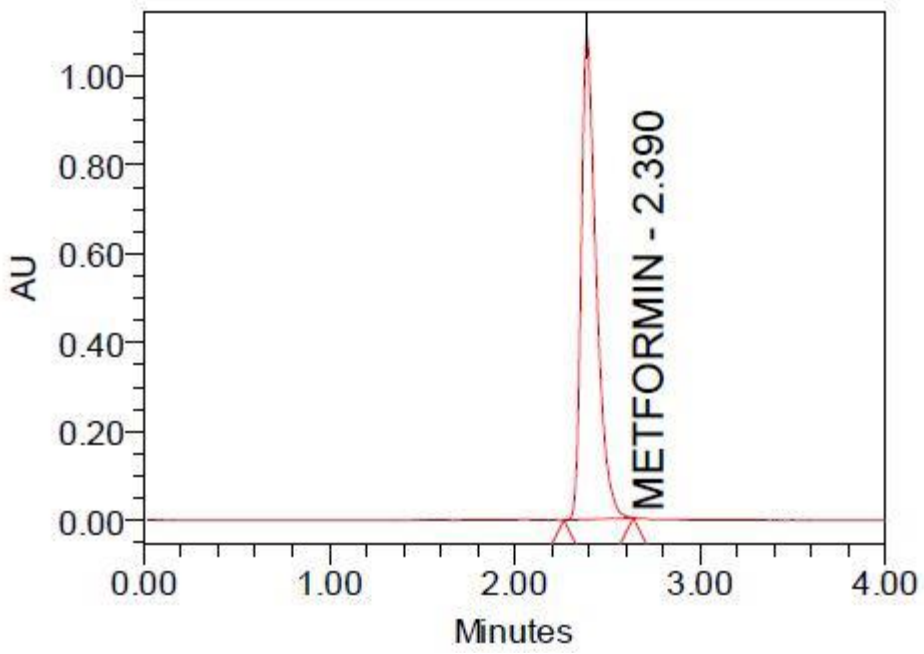
Mobile Phase: Water and Methanol were mixed in the ratio of 50:50 V/V and sonicated to degas. 520 mg metformin hydrochloride drug was weighed and transferred 50ml of volumetric flask and 25 ml of water and sonicated for 5 minutes and make up with water. Trasnsfer above solution of 5ml into 50ml volumetric flask dilute to volume with water [72].



Observation: Theoretical plates are less, peak shape is not good and asymmetry is more than limit [72].

Trail: 2.

Mobile Phase: Methanol: ortho phosphoric acid (50:50)



Chromatogram of trial 2

Observation: The retention time is good and no asymmetry has occurred, peak tailing was not observed [72].

Experiment 6:

Metformin HCL, gliclazide and pioglitazone combination

Difference between initial and optimized condition of mobile phase

Initial trial:

Initially methanol and water in different ratios were tried. But Metformin HCL gave broad peak shape [73].

Optimized condition:

Water was replaced by potassium dihydrogen buffer (20 mM), and mixture of methanol and potassium dihydrogen phosphate buffer in different ratios were tried. It was found that methanol: potassium dihydrogen phosphate buffer (20 mM) in ratio of 85: 15, v/v gave acceptable retention time (tR 2.15 min for Metformin HCL, tR 3.78 min for gliclazide and tR 4.575 min for pioglitazone), plates, and good resolution for all at the flow rate of 1.2 ml/min [73].

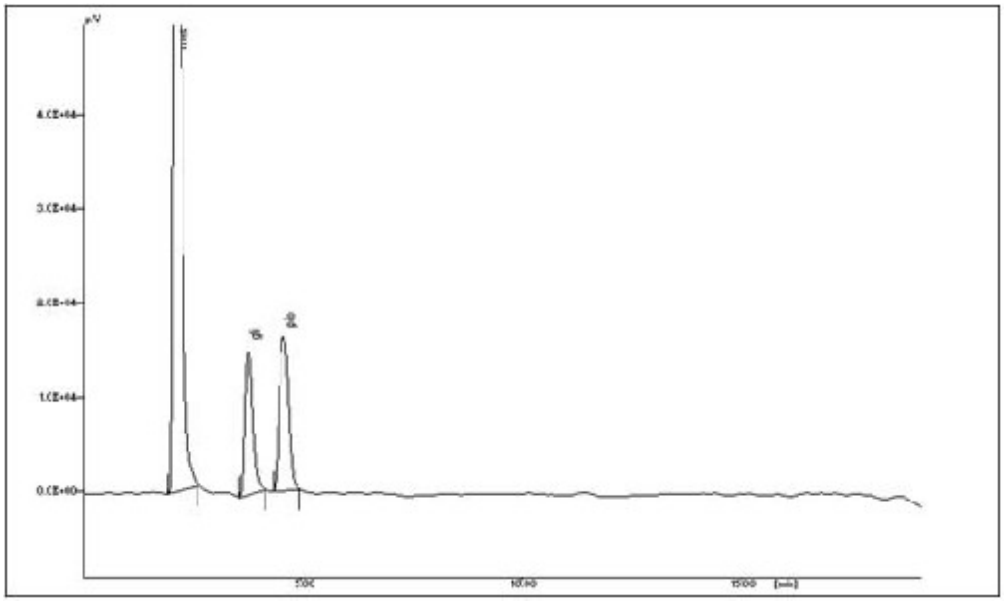


Figure: Chromatogram of Metformin HCl, Gliclazide, Pioglitazone HCl [73].

Experiment 7:

Metformin and Glibenclamide Combination

The objective of this study was to develop a rapid and sensitive HPLC method for estimation of Metformin and Glibenclamide in tablet formulations using the most commonly employed RP C-18 column with UV detection. The mobile phase was optimized with Methanol: Acetonitrile: Water in 30:60:10 (v/v). From the overlain spectrum of Metformin and Glibenclamide, wavelength was selected, at 228nm, isoabsorptive point for both the drugs. Good resolution was carried out at 228 nm and both drugs showed good absorbance at this wavelength with minimum interference of the other drug. All parameters of these proposed method was validated as per the ICH guidelines [74].

Experiment 8:

Optimized chromatographic condition of metformin and sitagliptin:

Selection of mobile phase for method Optimization and experimental condition

Several trials have been taken for the proper optimization of RP HPLC method by changing different mobile phase with different ratio. And finally the mobile phase for optimized condition was selected and given follows. The optimized parameters were for MET and SIT were given in the table [75].

Chromatographic conditions	Mode of separation
Column	Hypersil BDS C18 (100 × 4.6 mm, 5µm particle size)
Flow rate	1.0 ml/min
Solvent	Water
Column temperature	30ºC
Wavelength	215 nm
Injection volume	10 µl
Run time	6 minutes
Mobile phase	Methanol: buffer (50:50, v/v)

Table 07: Optimized chromatographic condition of Metformin & Sitagliptin [75].

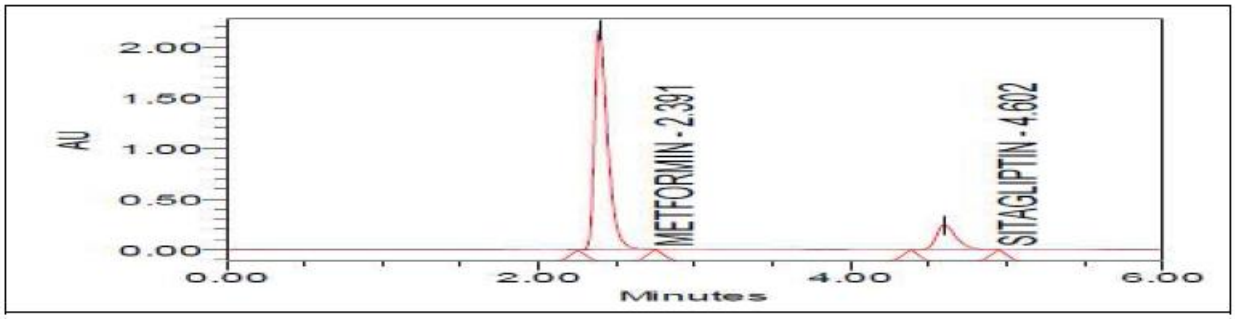


Figure: Optimized Chromatogram [75].

Experiment 9:

Metformin Hydrochloride and Rosuvastatin Calcium combination:

Several trials were performed using acetonitrile and 50 mm phosphoric acid (sodium) buffer solution (pH=2.8) in different proportions, under different pH conditions, by varying wavelength and flow rate conditions and a range was obtained for each factor and optimized chromatographic conditions were selected from fractional factorial design. The optimization parameters were listed in the table [76].

Factor	Range
Organic Phase proportion	60: 65 % v/v
Mobile phase	3.8-3.9
Wave length	251-253 nm
Flow rate	1- 1.2 ml/min

Table 08: Optimization parameters of Metformin HCL and Rosuvastatin Calcium [76].

Experiment 10:

Metformin hydrochloride and Glibenclamide combination:

Different column use for method development:

Trial 1:

Initial development trials have performed with octyl and octadecyl columns with different types, configurations and from different manufacturers. But asymmetric peaks and tailing factors were observed [77].

Trial 2:

Good separation of both the drugs Metformin hydrochloride and Glibenclamide was achieved in Oyster BDS C18 column (250x 4.6 mm, 5µm). In this column both the drugs were resolved and symmetric peaks with high theoretical plates and low tailing factor were observed [77].

Trial No	Column used	Obsevation
Trial 1	Octyl and octadecyl columns	asymmetric peaks and tailing factors
Trial 2	Oyster BDS C18 column (250x 4.6 mm, 5µm)	symmetric peaks with high theoretical plates and low tailing factor

Table 09: Optimized chromatographic conditions for Metformin and Glibenclamide [77].

CHAPTER FIVE

05

- METHOD DEVELOPMENT & VALIDATION OF COX-2 INHIBITORS BY USING HPLC

HPLC method Development and Validation of COX 2 inhibitors

The aim of the present study was to develop a new rapid RP-HPLC method for simultaneous quantification of COX-2 inhibitors in pharmaceutical preparations and to validate that method according to ICH, USP and FDA guidelines.

1) Materials and Methods

1.1. Materials and Reagents:

Celecoxib was obtained as a gift sample. The commercially available tablet, "Zycel" 200 mg (Zydus Healthcare) containing 200 mg of Celecoxib was procured from the local market and used for analysis. The pure reference standards rofecoxib, was obtained from TDM Labs. Working standard of Etoricoxib was gift samples from Eskayef Bangladesh Ltd. The valdecoxib reference standard was generously supplied by Pfizer Laboratories (Kalamazoo, USA). The HPLC-grade acetonitrile was purchased from Tedia (Fairfield, USA). All chemicals used were of pharmaceutical or special analytical grade. For all analyses, double-distilled water filtered through a 0.45 μm membrane filter was used. All the glassware employed in the study was cleaned with hot water, followed by acetone and dried in hot air oven whenever required [78,79].

1.2. Chromatographic conditions:

For Celecoxib the isocratic method was employed with the mobile phase consisting of 70 volumes of methanol and 30 volumes of acetonitrile. The chromatographic column used was a HiQ Sil C-18 Column with dimensions of 250×4.6 mm with 5μm particle size [78, 79].

For Rofecoxib chromatographic separation was performed on a C18 150·4.1 mm I.D., particle size 5 lmsize Hypersil ODS analytical column (Agilent Technologies, Palo Alto, CA,

USA). A C18 precolumn 5·4.1 I.D., particle size 5 lm (Agilent), was used to protect the chromatographic column [80].

For etoricoxib RP-HPLC analysis was performed by isocratic elution with a flow rate of 1 ml/min at 30º C temperature. The mobile phase was prepared with Buffer and acetonitrile (50: 50 v/v). The mobile phase was filtered through 0.22 μm filter tips [81].

For valdecoxib a Vankel VK7010 (VanKel Technology Group, Cary, USA), a paddle-stirrer type of apparatus, was used integrated with a VK 8000 dissolution sampling station, VK type bidirectional peristaltic pump. A Shimadzu HPLC system (Shimadzu, Kyoto, Japan) was used equipped with an SCL-10AVP system controller, LC-10 ADVP pump, DGU-14A degasser, SIL- 10ADVP auto sampler and an SPD-M10AVP photodiode array (PDA) detector [82].

1.3. Preparation of standard stock solutions

A 10 mg amount of celecoxib reference substance was accurately weighed, dissolved in mobile phase and diluted to volume in a 100 ml volumetric flask to obtain 80 ppm concentrated solution [83].

Accurately weighed 251.2 mg of rofecoxib (99.54 %) was taken in a 25.0 mL volumetric flask. This was dissolved in minimum quantity of acetonitrile and diluted up to the mark with acetonitrile to get 10,000 μg/mL of rofecoxib [84].

Accurately weighted (about 50 mg) Etoricoxib working standard was transferred to a 100 ml volumetric flask. Dissolved and diluted up to the mark with mobile phase to obtain a standard stock solutions having concentration Etoricoxib (500 μg/ml) [85].

The stock solution of valdecoxib was prepared by weighing 10 mg of the reference standard which was transferred into a 10 mL volumetric flask and diluting to volume with methanol [86].

2) Result and Discussion:

2.1. Method development and optimization:

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. For method development and optimization mobile phase, different pH were tried to get better resolution and peak symmetry, such as phosphate, ammonium formate, ammonium acetate, trifluro acetic acid with different pH composition. A variety of mobile phases were investigated in the development of an HPLC method suitable for analysis of CXB in the bulk drug and in microemulsions. These included methanol–water, 75:25 (% v/v), acetoni-trile–water, 75:25, methanol–water, 50:50,methanol–water, 95:5, methanol–phosphate buffer (pH 3.5–6.5), 80:20, and acetonitrile–phosphate buffer (pH 3.5–6.5), 80:20. The suitability of the mobile phase was decided on the basis of the sensitivity of the assay, suitability for stability studies, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents [87].

Best suitable chromatographic separation for rofecoxib was performed using an isocratic elution. The mobile phase components were Water: Acetonitrile (77:23), delivered at a flow rate of 0.8 mL/min [88].

The mobile phase of etoricoxib was chosen after several trials with Ammonium Acetate buffer and acetonitrile in various proportions like 15:85, 25:75, 50:50 and 35:65 at different pH values. A Mobile phase consisting Ammonium acetate buffer and Acetonitrile (50:50) was selected to achieve maximum separation and sensitivity. Different flow rates in between 0.50 to 1.20 ml /min were studied. A flow rate of 1.0 ml /min gave an optimal signal to noise ratio with a reasonable separation time. Etoricoxib scanned separately in the UV region. Then, the spectrum of product was taken. While performing HPLC scan active and standard was measured at 228, 235, 245, 278, 314, and 332nm. Finally, all the analysis was done at 235 nm as at this wavelength, drug absorb light better and peak could be distinguished properly [89].

Drug	Detection wavelength	Mobile phase	Retention time
Celecoxib	254 nm	Methanol : acetonitrile (70 : 30v/v)	3.25 min
Rofecoxib	272 nm	Water: Acetonitrile (77:23)	15 min
Etoricoxib	235 nm	Ammonium acetate buffer and Acetonitrile (50:50)	5.337 min
Valdecoxib	210 nm	Water and acetonitrile (52:48, v/v)	5.51 min
Parecoxib	265 nm	Acetonitrile: ammonium acetate (10 mM; pH 5.0) 55:45	0.82 min

Table 10: Optimized parameters for COX-2 inhibitor drugs [83-89].

2.2. Validation of the method:

Method validation was performed by evaluating LOD, LOQ, specificity, linearity, accuracy, robustness and system suitability parameters in accordance with the ICH guideline.

2.2.1 System suitability:

System suitability tests are an integral part HPLC method. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done [83-89].

Plate number or number of theoretical plates (n):

This is a measurement of the sharpness of the peaks and therefore the efficiency of the column.

$n = 16 (t / wb) 2$

Where, t=retention time of peak,

wb= peak width at base.

Capacity factor (K)

This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).

$K = tR - tM / tM$

Where, tM= unrestrained peak retention time,

tR =retention time of peak of interest.

Separation Factor (α)

This describes the relative position of two adjacent peaks. The peaks' separation depends on the components' interaction with the stationary phase.

$\alpha = KB / KA$

Where, KB= capacity factor of peak B,

KA=capacity factor peak A

Resolution Factor (RS)

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima to the mean value of the peak width at base (Wb).

$RS = 2(tR2 - tR1) / wb1 + wb2$

Where, tR1 and tR2 = retention time of two adjacent peak,

wb1, wb2 = peak width of respective peak.

Peak Asymmetric Factor (Af) and Peak Tailing Factor (T)

The deviation from symmetry is measured by the Asymmetry Factor, Af or Tailing Factor T. Asymmetric factor, Af is described by the following equation:

$Af = A10\% h / B10\%h$

Where, A and B= sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height

Tailing factor, T is described by following equation:

$T = (A5\%h + B5\%h) / 2A5\%h$

Drug	Celecoxib	Rofecoxib	Etoricoxib	Valdecoxib	Parecoxib
Theoretical plates (N)	13017	9429	2340	9828	9828
Resolution (Rs)	2.53	2.80	2.421	3.67	5.529
Tailing factor (Tf)	1.04	0.62	1.60	1.20	0.376
Retention time (Rt)	3.25 min	15 min	5.337 min	5.51 min	0.82 min

Table 11: System suitability parameter for COX-2 inhibitors [83-89].

2.2.2 Linearity:

The linearity of the proposed method was evaluated by using calibration curve to calculate coefficient of correlation, slope, and intercept values.

Drug	Regression equation	Correlation coefficient	Linearity range
Celecoxib	y = 48415x + 54359	0.996	0.27–80 µg/ml
Rofecoxib	y = 21.99x + 11.49	0.9970	2.5–100.0 µg/ml
Etoricoxib	Y=257408.17857x-5760.10714	0.99773	1.598-90µg/ml
Valdecoxib	-	-	0.05-150 µg/mL
Parecoxib	-	0.9989	0.9-18.4 µg/mL

Table 12: Linearity data of COX-2 inhibitors [83-89].

2.2.3 Limit of detection and Limit of quantification:

LOD

Limit of detection is the lowest concentration of the substance that can be detected, not necessarily quantified by the method.

$$LOD = \sigma / S \times 3.3$$

Where, S= Slope of the calibration curve

σ = Residual standard deviation

LOQ

It is defined as lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. LOQ is expressed as a concentration at a specified signal to noise ratio.

$$LOQ = \sigma / S \times 10$$

Where, S= Slope of the calibration curve

σ = Residual standard deviation

Drug	Celecoxib	Rofecoxib	Etoricoxib	Valdecoxib	Parecoxib
LOD	0.086 μg mL^{-1}	0.8 mg L^{-1}	0.907 (μg/ml)	10 ng/mL	0.29 ng/mL
LOQ	0.2625 μg mL^{-1}	2.5 mg L^{-1}	2.75 (μg/ml)	50 ng/mL	0.85 ng/ml

Table 13: LOD & LOQ of the drug studied [83-89].

2.2.4. Precision:

Precision refers to the closeness of two or more measurements to each other.

Both repeatability (within a day precision) and reproducibility (between days precision) were determined as follows.

Drug	Intraday (n = 3)				Interday (n = 3)		
	Fortified amount	Mean Area	Amount found (ppm)	% RSD	Mean Area	Amount found (ppm)	% RSD
Celecoxib	20 (ppm)	959508	19.57 (ppm)	0.33	970549	19.80 ppm	0.31
	60 (ppm)	2888041	59.79 (ppm)	0.35	2898901	60.02ppm	0.18
	100 (ppm)	4820326	100.09 (ppm)	0.19	4812943	99.94 ppm	0.14
Etoricoxib	100.09 mg	-	102.0 %	1.491	-	97.35	1.490
	100.05 mg	-	98.8 %		-	98.44	
	100.11 mg	-	101.9 %		-	99.75	
	100.04 mg	-	99.3 %		-	101.10	
	100.07 mg	-	99.5 %		-	97.61	
	100.09	-	98.8 %		-	97.17	

	mg						
Parecoxib	5 µg/ml	-	4.91 µg/ml	0.285	-	4.89	0.210
	10 µg/ml	-	9.82 µg/ml	0.212	-	9.79	0.186
	15 µg/ml	-	14.81 µg/ml	0.224	-	14.75	0.206

Table 14: Intraday and inerday precision of COX 2 inhibitors [83-89].

2.2.5. Accuracy, as Recovery

Accuracy refers to the closeness of a measured value to a standard or known value.

The recovery of the method, determined by spiking a previously analyzed test solution with additional drug standard solution, was 98.16– 103.09 %. The values of recovery (%), RSD (%), listed in table indicate the method is accurate [83-89].

Drug	Amount (%) of drug added to analyte	Theoretical content	Concn found (µg mL^{-1}) ± SD	Recovery (%)	RSD (%)
Celecoxib	0	8 µg mL^{-1}	8.02 ± 0.128 µg mL^{-1}	100.29	1.59
	50	12 µg mL^{-1}	12.08 ± 0.125 µg mL^{-1}	100.69	1.03
	100	16 µg mL^{-1}	16.16 ± 0.080 µg mL^{-1}	101.01	0.49
	150	20 µg mL^{-1}	19.97 ± 0.194 µg mL^{-1}	99.84	0.97
Rofecoxib	0	(25.0) + 0.00 µg mL^{-1}	25.04 µg mL^{-1}	100.14	0.1824
	10	(25.0) + 2.50 µg mL^{-1}	27.51 µg mL^{-1}	100.03	0.0654
	20	(25.0) + 5.00 µg mL^{-1}	30.13 µg mL^{-1}	100.43	0.3932
	30	(25.0) + 7.50 µg mL^{-1}	32.49 µg mL^{-1}	99.97	0.0752
Etoricoxib	80	40.96 mg	40.2081 mg	98.16	1.68
		41.27 mg	41.3545 mg	100.20	
		39.51 mg	39.3374 mg	99.50	
	100	50.14 mg	51.0740 mg	101.86	
		50.87 mg	50.8119 mg	99.89	
		49.64 mg	51.1736 mg	103.09	
	120	60.89 mg	59.9766 mg	98.50	
		60.86 mg	60.3254 mg	99.12	
		60.93 mg	59.8589 mg	98.24	
	80	80 µg/mL	79.92 µg/mL		

Valdecoxib	100	100 µg/mL	100.02 µg/mL	100.03	0.12
	120	120 µg/mL	120.18 µg/mL		
Parecoxib	80	80 µg/mL	79.92 µg/mL	100.03	0.12
	100	100 µg/mL	100.02 µg/mL		
	120	120 µg/mL	120.18 µg/mL		

Table 15: Accuracy data expressed as the percentage recovery of the amount added [83-89].

CHAPTER SIX

06

- METHOD DEVELOPMENT & VALIDATION OF ANTICANCER DRUGS BY USING HPLC

METHOD DEVELOPMENT AND VALIDATION OF ANTICANCER DRUGS BY RP-HPLC

The aim of the present study was to develop a new rapid RP-HPLC method for simultaneous quantification of anticancerdrugs in pharmaceutical preparations and to validate that method according to ICH, USP and FDA guidelines.

1) MATERIALS AND METHODS

1.1. Materials and Reagents:

Capecitabine was obtained as a gift sample of Capecitabine was kindly supplied by the Dr. Reddy's laboratories, Hyderabad. Acetonitrile and buffer used were of HPLC and milli- Q grade respectively [90].

Working standard of Cladribine was obtained from well reputed research laboratories. HPLC grade Water, Methanol, Acetonitrile was purchased from E. Merck (Mumbai, India) [91].

Working standard of Fludarabine was obtained from well reputed research laboratories. HPLC grade Acetonitrile, Water, Methanol and Phosphate buffer was purchased from Merck (Mumbai, India). All the chemicals used in the study were purchased from Rankem [92].

The reference sample of gemcitabine was supplied by M/s Shilpa Medicare Limited, Raichur, India. Theophylline (2) was received as gift sample from Hetero Pharmaceutical Ltd., Hyderabad, India [93].

Methotrexate working standard, Folitrax-5 Tablets, Placebo or Excipient mixture (about 100g), Potassium Dihydrogen Ortho Phosphate, Acetonitrile etc [94].

The working standard of Epirubicin hydrochloride was provided as gift sample from Spectrum Labs, Hyderabad, India. HPLC grade acetonitrile and water were purchased from

E.Merck (India) Ltd, Mumbai, India. Potassium dihydrogen phosphate, orthophosphoric acid and triethylamine of AR grade were obtained from S.D. Fine Chemicals Ltd, Mumbai, India [95].

Carmustine bulk drug was made available from Sun Pharmaceutical Ltd. India (purity 99.3). Potassium dihydrogen phosphate, Orthophospharic acid, Triethylamine were obtained from Qualigens fine chemicals, India Limited. Ethanol, methanol, hydrochloric acid, sodium hydroxide were obtained from Rankem laboratories, India. All chemicals and reagent were used as HPLC grades [96].

Dacarbazine standard was obtained from Taj Pharmaceuticals Ltd (Mumbai, India). Orthophosphoric acid, Triethylamine and Acetonitrile (HPLC grade) were obtained from Rankem Chemicals (Mumbai, India) [97].

Docetaxel reference standard (purity: 100.7%) was from Therdose Pharma (Hyderabad, India). HPLC grade acetonitrile and methanol were purchased from Merck (Mumbai, India). Triethylamine (HPLC grade) and chloroform were purchased from Merck. Deionized water was purified using Milli-Q system [98].

Standard drug paclitaxel was obtained as working standard from Fresenius Kabi Oncology Ltd, Gurgoan, India. HPLC grade acetonitrile, glacial acetic acid and hydrochloric acid were procured from Merck Ltd., India. Analytical grade sodium hydroxide, hydrogen peroxide and other chemicals used in the study were procured from CDH chemicals Ltd, Mumbai, India [99].

Analysis of Vinblastin was carried out using PEAK 7000 isocratic HPLC with rheodyne manual sample injector with switch (77251) and the column used was analytical column kromosil 100-5 C18 (250x4.6mm). Electronic balance used was ELB 300, DIGISUN pH meter was used for all pH measurements [100].

Vinblastine reference standard was a kind gift of V.V.MED Laboratories, Hyderabad and the tablet formulation (VIAL-10mg) used for testing the method was purchased from local market. The solvents used were methanol and Acetonitrile of HPLC grade and phosphoric acid (GR) of Merck manufactures [101].

Imatinib Mesylate pure form was obtained as gifted sample from pharma industry and its pharmaceutical dosage form GLEEVAC Tablets labelled claim 100 mg were purchased from

local pharmacy. Acetonitrile of HPLC grade (Merck India), Water of HPLC grade(Merck India) and o-Phosphoric acid of Analytical grade (SD Fine Chemicals) were used [102].

1.2. Chromatographic conditions:

Capecitabine analysis was performed by using HPLC (Shimadzu), Column used is of Hypersil BDS C8, 250, 5 µm column, with a flow rate of 1.2 ml/min [103].
Cladribine assay was performed on a Series 200 HPLC system PEAK LC7000 isocratic HPLC with PEAK 7000 delivery system. Rheodyne manual sample injector with switch (77251), Analytical column Zodiac 100-5 C18. 250×4.6mm, manual injector Rheodyne valve) with 20µL fixed loop, PEAK LC software was used [104].

For Fludarabine, quantitative HPLC was performed on a high pressure gradient high performance liquid chromatography (PEAK LC-7000 liquid chromatography) with two LC-7000 pumps, manual injector with loop volume of 20µL (Rheodyne), programmable variable wavelength PEAK LC-7000 prominence UV-Vis detector and kromasil C-18 Column (4.6 x 250 mm, 5µm) [105].

For Gemcitabine, Shimadzu Prominence high pressure liquid chromatographic instrument provided with a Luna C-18 column (250mm × 4.6 mm; 5 μ), an LC 20AT-VP solvent delivery system, a universal loop injector (Rheodyne 7725 i) of injection capacity of 20 μL, and an SPD 20A UV-visible detector (λmax 275 nm) was employed in the study [106].

For Methotrexate Agilent 1100 Series with Chromeleon software (Instrument I.D: AL-013) HPLC Analytical column C18, 100mm x 6mm x 5µ was used [107].

For Epirubicin, a mixture of phosphate buffer pH 3.1 and acetonitrile in the ratio of 50:50 v/v was found to be the most suitable mobile phase for ideal chromatographic separation of Epirubucin. The solvent mixture was filtered through 0.45 µ membrane filter and sonicated before use. It was pumped through the column at a flow rate of 1.0 mL/min. Injection volume was 10 µL and the column was maintained at a temperature of 250C. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solution. The detection of the drug was monitored at 233 nm. The run time was set at 7 min [108].

For carmustine, chromatographic system Waters consisting of quaternary solvent delivery pump, a degasser, an auto- injector, column oven and UV detector. The chromatographic column of 250 mm length and internal diameter of 4.6 mm filled with Octadecyl silane YMC ODS-A C18 stationary phase with particle size 5 micron and pore size 100A was used [109].

The chromatographic system used was a Waters 2695 model, which comprised a degasser, quaternary pump, auto injector, column compartment, and photodiode array detector, and the system was controlled through Empower 2 software. Agilent Eclipse XDB C18 (150 x 4.6 mm, 5µ) column maintained at 30°C column oven temperature and a mobile phase flow rate of 1.0 mL/min [109].

Docetaxel was analysed using HPLC system consisting of Waters 2695–Alliance separations module with Millennium32 software, auto injector and Waters 2996 photodiode array detector. Separation was carried out using a Waters Symmetry C18 column (250 × 4.5 mm, 5 µm) [110].

Quantitative HPLC was performed on a binary Shimadzu prominence HPLC in Gradient mode with a 20µl sample injection loop (manual), and SPD 20A Photo diode array UV-Visible detector [111].

Chromatographic analysis of the vinblastine was done using a Kromasil C18, (250x4.6mm, 5µm) column. Development of an Analytical Methodology for Simultaneous Determination of Vincristine and Doxorubicin in Pharmaceutical Preparations for Oncology by HPLC–UV [112].

Imatinib Mesylate optimizations of chromatographic conditions were done by performing different trails by taking different mobile phases and varying their compositions, flow rates. Finally an optimised chromatogram was obtained. The system suitability parameters were, Retention time: 3.25 min Tailing factor: 1.06, Theoretical factor: 3509.474 [113].

1.3. Preparation of standard stock solutions:

100mg of Capecitabine is weighed accurately into a clean 100ml volumetric flask, dissolved and made up to the mark with mobile phase. 3ml is diluted to 25ml to attain a concentration of 12 µg /ml [114].

A 10 mg amount of Cladribine reference substance was accurately weighed dissolved in10ml mobile phase in a 10 ml volumetric flask to obtain 1000ppm concentrated solution. From

standard solution by the serial dilution we prepared required concentrations of 210 to 60 ppm [115].

A 10 mg of Fludarabine reference substance was accurately weighed and dissolved in 10 ml mobile phase in a 10 ml volumetric flask to obtain 1000 ppm concentrated solution. Required concentrations were prepared by serial dilution of this solution [116].

An accurately weighed amount (100mg) of gemcitabine was transferred into 100mL calibrated flask and dissolved in appropriate volume of methanol. Then, the void volume was completed with methanol to produce a stock solution of 1000 μg/mL. The stock solution was further diluted with mobile phase to obtain working solutions (25, 100, and 200 μg/mL) [117].

For Methotrexate Weigh accurately about 25 mg of Methotrexate working standard and transfer to a 25 ml volumetric flask. Add 10 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix [118].

10 mg of Epirubicin was accurately weighed, transferred to 10 mL volumetric flask and is dissolved in 7 mL of the diluent. Sonicated the solution for few minutes to dissolve the drug completely. Then it is filtered through 0.45 µ filter and the volume is made up to 10 mL with diluent to get a concentration of 1 mg/mL stock solution. Further pipetted 0.2 mL of the above stock solution into a 10 mL volumetric flask and diluted up to the mark with diluent to obtain required concentrations [119].

A 50-mg sample of carmustine standard was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with the diluent (1000 μ g mL-1). Further 1.0 ml above solution transferred in a 100-mL volumetric flask, and dissolved with the diluent (10 μ g mL-1) [120].

Stock solution of the drug (pure) was prepared by dissolving 100 mg of Paclitaxel in 50 ml of Acetonitrile in 100 ml volumetric flask and the final volume was made up to 100 ml using Acetonitrile [121].

Vinblastin standard stock solution was sonicated for 10 minutes to degas it. The standard solution was then filtered with 0.45µm membrane filter paper. A series of different dilutions

(3-15ppm) were prepared using above stock solution with mobile phase (Methanol,acetonitrile and orthophosphoric acid in the ratio 90:5:5 (v/v/v) [122].

A stock solution was prepared by taking accurately weighed 10 mg of Imatinib Mesylate in 100 ml volumetric flask and initially dissolved in 50 ml mobile phase. Then the solution was made upto mark with mobile phase to obtain a concentration of 100 µg/ml and the resulting solution was sonicated for 15 min [123].

2) RESULT:

2.1. Method development and optimization:

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice [123].

For method development and optimization mobile phase, different pH were tried to get better resolution and peak symmetry. A variety of mobile phases were investigated in the development of an HPLC method suitable for analysis of Capcitabine, but finally ACN: MeOH: Water (18: 80: 2) composition was chosen. At flow rate 1.1 ml/min [123].

Having optimized the efficiency of a chromatographic separation, the quality of the chromatograph was monitored by applying the following system suitability tests: capacity factor, tailing factor and theoretical plates. The system suitability method acceptance criteria set in each validation run were: capacity factor >2.0, tailing factor ≤ 2.0 and theoretical plates >2000 [123].

Drug	Detection wavelength	Mobile phase	Retention time
Capcitabine	230 nm	ACN : MeOH: Water (18: 80: 2, v/v/v)	6.92 min
Carmustine	230 nm	Acetonitrile : 10mM dipotassium hydrogen phosphate (50:50, v/v)	36.22 min
Cladribine	231 nm	Methanol: Acetonitrile: Water (64:22:14, v/v/v)	5.530 min
Dacarbazine	323nm	Buffer: acetonitrile (96:4, v/v)	4.333 min
Docetaxel	230 nm	Acetonitrile : 0.2% triethylamine pH adjusted to 6.4 (48:52, v/v)	11.5 min
Epirubicin	233 nm	Phosphate Buffer: Acetonitrile (50:50, v/v)	3.437 min
Fludarabine	265nm	Methanol: Acetonitrile: Phosphate Buffer 50:20:30(v/v)	5.97min

Gemcitabine	275 nm	Water: acetonitrile (90: 10, v/v)	3.95 min
Imatinib Mesylate	266 nm	Acetonitrile : o-Phosphoric acid (0.1% v/v) ,(70 : 30 v/v)	3.25 min
Methotrexate	303 nm	Buffer (Potassium dihydrogen orthophosphate): acetonitrile (92:8 v/v)	4.6 min
Paclitaxel	282nm	Buffer: acetonitrile (40:60, V/V), pH 7.4	3.610 min
Vinblastin	268nm	Methanol: Acetonitrile: orthophosphoric acid (pH= 3.5) (90:5:5, v/v/v)	2.82 min

Table 16: Optimized parameters for anticancer drugs [114-123].

2.2. Validation of the method:

Method validation was performed by evaluating LOD, LOQ, specificity, linearity, accuracy, robustness and system suitability parameters in accordance with the ICH guideline.

2.2.1 System suitability:

System suitability tests are an integral part HPLC method. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done [123].

Plate number or number of theoretical plates (n):

This is a measurement of the sharpness of the peaks and therefore the efficiency of the column.

$n = 16 (t / wb) 2$

Where, t=retention time of peak,

wb= peak width at base.

Capacity factor (K)

This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).

$K = tR - tM / tM$

Where, tM= unrestrained peak retention time,

tR =retention time of peak of interest.

Separation Factor (α)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the Choudhary *et. al.,* Am. J. PharmTech Res. 2012; 2(6) ISSN: 2249-3387 317 capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

$\alpha = KB / KA$

Where, KB= capacity factor of peak B,

KA=capacity factor peak A

Resolution Factor (RS)

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima to the mean value of the peak width at base (Wb).

$RS = 2(tR2 - tR1) / wb1 + wb2$

Where, tR1 and tR2 = retention time of two adjacent peak,

wb1, wb2 = peak width of respective peak.

Peak Asymmetric Factor (Af) and Peak Tailing Factor (T)

The deviation from symmetry is measured by the Asymmetry Factor, Af or Tailing Factor T. Asymmetric factor, Af is described by the following equation:

$Af = A10\% h / B10\%h$

Where, A and B= sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height

Tailing factor, T is described by following equation:

$T = (A5\%h + B5\%h) / 2A5\%h$

Drugs	Theoretical plates (N)	Resolution (Rs)	Tailing factor (Tf)
Capecitabine	11644.32	-	1.37
Carmustine	6233	20.1	1.04
Capecitabine	11644.32	-	1.37
Dacarbazine	4500	-	1.23
Docetaxel	8307.82	3.99	1.01
Epirubicin	10968	-	1.30
Fludarabine	6110	-	1.08
Gemcitabine	7716	11.0	1.10
Imatinib Mesylate	3509	-	1.06
Methotrexate	4052	23.6	1.17
Paclitaxel	21108.916	2.20	1.614
Vinblastin	3058.86	-	1.29

Table 17: System suitability parameter for anticancer drugs [114-123].

2.2.2 Linearity:

The linearity of the proposed method was evaluated by using calibration curve to calculate coefficient of correlation, slope, and intercept values.

Drug	Regression equation	Correlation coefficient	Linearity range
Capecitabine	y = 152882x - 414517	0.9985	40-60µg/ml
Carmustine	-	0.9999	50-150 µg/mL
Cladribine	y = 6180.709+ 4200.982x	0.9999	60-210 ppm
Dacarbazine	y = 34222x + 8396.9	0.999	25-150µg/ml
Docetaxel	Y=123130x + 60141	0.9998	0.5–20 µg/mL
Epirubicin	Y= 15149x+3497	0.999	10-60 µg/mL
Fludarabine	y = 5495.9 + 140551x	0.9993	2–800ng/mL
Gemcitabine	Y = 0.0353x + 0.0063	0.9998	0.5–50 μg/mL
Imatinib Mesylate	Y= 1758760x-175341	0.9991	5-30 µg/ml
Methotrexate	y = 10.14x - 1.718R2	0.999	5-70 µg/ml
Paclitaxel	y = 575561x - 4E+06	0.9963	20-100 µg/ml
Vinblastin	Y= 37953.37x+ 11044.06	0.999	3-15 ppm

Table 18: Linearity data of Anticancer drugs [114-123].

2.2.3 Limit of detection and Limit of quantification:

LOD

Limit of detection is the lowest concentration of the substance that can be detected, not necessarily quantified by the method.

$$LOD = \sigma / S \times 3.3$$

Where, S= Slope of the calibration curve

σ = Residual standard deviation

LOQ

It is defined as lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. LOQ is expressed as a concentration at a specified signal to noise ratio.

$$LOQ = \sigma / S \times 10$$

Where, S= Slope of the calibration curve

σ = Residual standard deviation

Drug	LOD	LOQ
Capecitabine	0.088 µg/ml	0.26 µg/ml
Carmustine	0.011 µg /mL	0.066 µg /mL
Cladribine	0.4ppm	1.3 ppm
Dacarbazine	0.451 µg /mL	1.367 µg /mL
Docetaxel	0.1 µg/mL	0.5 µg/mL
Epirubicin	0.06 µg /mL	0.19 µg /mL
Fludarabine	0.05µg/ml	0.16 µg/ml
Gemcitabine	0.1498 μg/mL	0.4541 μg/mL
Imatinib Mesylate	1.16 µg/ml	3.15 µg/ml.
Methotrexate	0.014 µg /mL	0.047 µg /mL
Paclitaxel	0.43 µg/mL	1.413 µg/mL
vinblatine	0.05ppm	0.165ppm

Table 19: LOD & LOQ of the drug studied [114-123].

2.2.4. Precision:

Precision refers to the closeness of two or more measurements to each other.
Both repeatability (within a day precision) and reproducibility (between days precision) were determined as follows.

The intra-day and inter-day variations of the method were determined by using six replicate injections of one concentration and analyzed on the same day and three different days over a period of two weeks. Acceptance criteria of not more than 2.0%. Values for both system precision and method precision in terms of % RSD were found to be <2.0%.

Sample	Intraday			Interday	
	Concentration	Peak Area	% RSD	Mean Area	% RSD
Capcitabine	60 µg/ml	543590	0.66	197408	1.027
		543097		195435	
		543189		197207	
		543765		195871	
		543980		191836	
		543690		195847	
Cladribine	120ppm	513263.7	0.48	524965	0.56
		513921.4		518622	
		512832.9		517249	
		512936.5		520593	
		519253.6		522965	
		513292.5		522954	
Epirubicin	80 µg/ml	630908	1.3	639250	1.23
		626045		639224	
		638246		639344	
		630696		623091	
		614294		624574	
		631633		637855	
Gemcitabine	120 µg/ml	286132.9	1.30979	285700.5	0.988
		282005.2		280403.4	
		289364.1		284920.8	
		281692.3		280248.6	
		281137.2		281020.9	
		288816.3		286157.7	
Imatinib Mesylate	20 µg/ml	30800560 (Mean area)	0.51	33438430	0.77
				32942404	0.62
				33724742	0.57
Fludarabine	80 µg/ml	467060	0.79	470334	1.05
		467677		465681	
		466978		464910	
		476611		466032	
		471297		476007	
		469043		462203	
		227491.7		225898.6	

Vinblastin	6 ppm	224066.6	0.926	221815.0	0.608
		222747.4		223321.3	
		221220.0		224639.5	
		224222.0		223734.7	
		223878.2		224084.6	

Table 20: Intraday and inerday precision of Anticancer drugs [114-123].

2.2.5. Accuracy, as Recovery

Accuracy refers to the closeness of a measured value to a standard or known value.

The recovery of the method, determined by spiking a previously analyzed test solution with additional drug standard solution, was 98.00 % – 101.42 %. The values of recovery (%), RSD (%), listed in table indicate the method is accurate.

Drug	% Type of Level	Amount of drug added	Amount recovered	Recovery (%)	RSD (%)
Capecitabine	80	42.01 (µg/ml)	41.84	99.6	0.35
	100	52.47 (µg/ml)	52.57	100.3	0.26
	120	62.54 (µg/ml)	62.98	100.7	0.25
Carmustine	50	50 ppm	49.94	99.88	-
	100	100 ppm	100.12	100.12	-
	150	150 ppm	150.03	100.02	-
Cladribine	50	90 (ppm)	88.80	98.67	-
	100	120 (ppm)	118.90	99.09	-
	150	150 (ppm)	151.46	100.97	-
Dacarbazine	100	50 µg/mL	50.060	100.121	0.3970
	100	100 µg/mL	100.572	100.572	0.7061
	100	150 µg/mL	149.754	99.83	0.7435
Docetaxel	20	4 mg	3.928	98.2	0.57
	20	8 mg	7.848	98.1	
	20	12 mg	11.76	98.0	
Epirubicin	50	20 µg/mL	19.93	99.71	-
	100	40 µg/mL	40.49	100.24	-
	150	60 µg/mL	60.47	100.79	-
Fludarabine	60	60 µg/mL	59.47	99.11	-
		60 µg/mL	59.50	99.16	-
		60 µg/mL	59.32	98.86	-
	100	80 µg/mL	79.14	98.93	-
		80 µg/mL	78.43	98.04	-
		80 µg/mL	78.77	98.46	-
		100 µg/mL	98.92	98.92	-

	150	100 µg/mL	99.47	99.47	-
		100 µg/mL	99.73	99.73	-
Gemcitabine	50	100 µg/mL	100.11	100.11	0.235
		100 µg/mL	100.21	100.21	
		100 µg/mL	99.83	99.83	
	100	200 µg/mL	200.25	100.12	1.158
		200 µg/mL	199.76	99.88	
		200 µg/mL	201.321	100.66	
	150	300 µg/mL	298.85	99.62	0.720
		300 µg/mL	299.87	99.96	
		300 µg/mL	300.63	100.21	
Imatinib mesylate	80	16 µg/mL	16.22	101.42	1
		16 µg/mL	16.11	100.72	
		16 µg/mL	15.85	99.08	
	100	20 µg/mL	19.36	98.82	1.42
		20 µg/mL	19.85	99.25	
		20 µg/mL	19.23	99.19	
	120	24 µg/mL	24.06	100.29	0.0432
		24 µg/mL	24.04	100.22	
		24 µg/mL	24.05	100.19	
Paclitaxel	-	20 µg/mL	19.963	99.817	4.023
	-	40 µg/mL	40.060	100.150	0.628
	-	60 µg/mL	59.934	99.889	0.365

Table 21: Accuracy data expressed as the percentage recovery of the amount added [114-123].

CHAPTER SEVEN

DISCUSSION

The methods were validated for accuracy, precision, reproducibility, specificity, robustness, and detection and quantification limits, in accordance with ICH guidelines.

This paper has reviewed HPLC method development, optimization and validation of COX-2 inhibitors, metformin & its combination drugs and anticancer drugs. Total five COX-2 inhibitors were selected for HPLC method development and validation; they are Celecoxib, Rofecoxib, Etoricoxib, Valdecoxib, Parecoxib. But it was found that wavelength vary product to product, Celecoxib 254 nm, Rofecoxib 272 nm, Etoricoxib 235 nm, Valdecoxib 210 nm, Parecoxib 265 nm. So maximum wavelength was 272 nm and minimum was 210 nm. Now question arise why wavelength vary drug to drug? The major reason is, Rofecoxib shows maximum absorbance at 272 nm and Valdecoxib shows maximum absorbance at 210 nm. The wavelengths were selected by scanning in the range of 200 -400 nm against mobile phase as a blank.

In case of Celecoxib, Rofecoxib, Valdecoxib no need to use buffer, but Ammonium acetate was used for Etoricoxib, Parecoxib, but why? We know buffer is mainly important for pH control because pH can impact selectivity, resolution, peak shape, and retention. Although change in pH does not affect Celecoxib, Rofecoxib, Valdecoxib estimation.

Retention time of Celecoxib is 3.25 minutes, Rofecoxib 15 minutes, Etoricoxib 5.337 minutes, Valdecoxib 5.51 minutes, Parecoxib 0.82 minutes. So again question arise, why retention time vary drug to drug? The main reason is drugs polarity. In Reversed-phase chromatographic column use nonpolar stationary phase. For reversed phase, alkyl hydrocarbons are the preferred stationary phase; octadecyl (C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications. Rofecoxib's attraction to the non-polar stationary phase is greatest, so it is most strongly retained compound and Parecoxib weakly retained as it is more polar than Rofecoxib.

Among COX-2 inhibitors, maximum theoretical plate found in celecoxib and lowest theoretical plate found in Etoricoxib. Resolution or better peak separation was found in Parecoxib and low resolution was found in Etoricoxib. Rofecoxib shows less tailing factor 0.62 and Etoricoxib shows more, 1.60.

For method development of Metformin different types of column was used, like Inertsil ODS-3 (50 × 2.1 mm), 2-µm, Eclipse Plus C-18, RRHD (50 × 2.1 mm), 1.8 µm, XDB C-18,

(50 × 2.1) mm, 1.8 µm and Acquity BEH C-18 (100× 2.1 mm), 1.7 µm column. At first Inertsil ODS-3 (50 × 2.1 mm), 2-µm and Eclipse Plus C-18, RRHD (50 × 2.1 mm), 1.8 µm columns were used but they show Asymmetrical peak with fronting and tailing. Then try with XDB C-18, (50 × 2.1) mm, 1.8 µm column, but still problem show that was Split peak observed. Finally tried with Acquity BEH C-18 (100× 2.1 mm), 1.7 µm column, then found Symmetrical peak which was satisfactory.

Comparison between Compositions of mobile phase was observed in this paper. Change in mobile phase composition may show satisfactory result. For metformin different mobile phase compositions were tried, like Acetonitrile: Water (20:80 v/v), which shows asymmetrical peak with tailing and early elution. Then tried with Methanol: Water (20:80 v/v), shows Very broad peak. Then use Methanol: Buffer (pH 3.2, 20:80 v/v), shows Broad peak. So all results were not satisfactory at all. Finally tried with Acetonitrile: methanol: Buffer (pH 3.2) (10:10:80 v/v/v) and found Symmetrical peak which was acceptable.

For Metformin method development trying pH was found in this paper. At first pH 4 was selected but it shows peak elution early with less theoretical plates. Then try with pH 5 and 6 but peak completely split. Finally tried with pH 3.2 and symmetric peak was observed.

In another method, at first water and methanol were mixed in the ratio of 50:50 V/V. But the result was not satisfactory, theoretical plates are less, peak shape is not good and asymmetry is more than limit. Then try with methanol: ortho phosphoric acid (50:50, v/v), then found good retention time and no asymmetry has occurred, peak tailing was not observed. Another method was developed, water was replaced by potassium dihydrogen buffer (20 mM), and mixture of methanol and potassium dihydrogen phosphate buffer in different ratios were tried. It was found that methanol: potassium dihydrogen phosphate buffer (20 mM) in ratio of 85: 15, v/v gave acceptable retention time (tR 2.15 min for Metformin HCL, tR 3.78 min for gliclazide and tR 4.575 min for pioglitazone), plates, and good resolution for all at the flow rate of 1.2 ml/min.

In Metformin hydrochloride and Glibenclamide combination different column was tried. Trials have performed with octyl and octadecyl columns with different types, configurations and from different manufacturers. But asymmetric peaks and tailing factors were observed. Good separation of both the drugs Metformin hydrochloride and Glibenclamide was achieved in Oyster BDS C18 column (250 x 4.6 mm, 5µm). In this column both the drugs were

resolved and symmetric peaks with high theoretical plates and low tailing factor were observed.

Study on different anticancer drugs found variation in wavelength, mobile phase composition, retention time. Capcitabine, Carmustine, Docetaxel wave found same, 230 nm. Higher wavelength was found in Methotrexate (303 nm), Paclitaxel (282nm), Gemcitabine (275 nm). One of the major advantage for Capcitabine, Cladribine, Gemcitabine drugs, not need to use buffer in mobile phase composition which may damage column. For Imatinib Mesylate, Carmustine, Dacarbazine, Docetaxel, Epirubicin, Fludarabine, Methotrexate, Paclitaxel, Vinblastin buffer must be used to control pH, the reason that pH control is important in reversed phase HPLC relates to the ionization of the analytes. The retention mechanism for an ionized, and therefore relatively polar, molecule is different to that of the same molecule in an unionized, and relatively less polar, form. If the pH of the mobile phase is not controlled for an ionizable analyte then the chromatography may exhibit broad or misshapen peaks, and if reasonable peak shape is achieved, then it is very likely that the chromatography will be prone to reproducibility problems, including retention time changes, and therefore compromise the overall robustness of the method. It is common practice to control the pH of the mobile phase for ionizable analytes. However, ionizable interferences in the sample matrix may also give rise to chromatographic problems. If the peak resulting from a component in the sample matrix is misshapen, broad or has a changing retention time, then this may interfere with analyte peaks of interest, again compromising the robustness of the method. This explains why the presence of a buffer can make a method more robust, but choosing to control the pH leads to other choices.

Retention time varied drug to drug. Short retention time found in Vinblastin, only 2.82 minutes, where Carmustine's retention time was maximum 36.22 minutes. We know in reverse phase chromatography use non polar stationary phase, if the drug is polar then less interaction occurs with non polar stationary phase, so drug elute first and short retention time observed. But when the drug less polar interaction found more with stationary phase and take more time to elute, that's why increase retention time. From this paper, it is also found that Reverse phase chromatography use more than normal phase. There are several reasons why reverse phase HPLC has become more of a standard means of HPLC separation than normal phase. Reverse phase columns have a hydrophobic stationary phase which works well for retention of most organic analytes. This also means that water can be used as a mobile phase in conjunction with less polar solvents such as acetonitrile and methanol which can be

adjusted at highly controlled rates to improve chromatographic performance. Gradient separations in normal phase are much more complicated because of UV cutoff variation as well as differences in compressability of common hydrophobic solvents, which would have an effect on flow rate. Reverse phase chromatography also has the advantage of being able to use pH selectivity to improve separations.

In system suitability test Theoretical plates, Resolution, Tailing factor and retention time were presented in this paper. All drugs theoretical plate were within range (>2000), where Paclitaxel's Theoretical plate was maximum, 21108.916 and lowest was Dacarbazine, 4500. Higher resolution was observed in Methotrexate, 23.6 and lowest was Paclitaxel, 2.20. Tailing factor of all drugs were found within limit < 2, where Paclitaxel higher value was 1.614 and lowest was Docetaxel, 1.01. Now question is why system suitability test should be performed for method validation, because during the routine analysis of drug and analytes System Suitability Test is one of the most important and integral parts of HPLC method development and calibration. System Suitability Test is generally performed to evaluate the suitability and effectiveness of the entire chromatographic system not only prior to use but also during the time of analysis. Due to several reasons the performance and the capacity of the entire chromatographic system may abruptly or mildly change during their regular uses. This modification of the system can in turn affect the reliability of the entire HPLC analytical results. For this reasons different parameters which is related to the operation of the whole chromatographic system can be monitored and investigated to find out the integrity and reliability of the whole HPLC systems. These parameters are used to determine characteristic chromatographic parameters. After the checking of the parameters, the system is then only can be declared suitable if the responses are within permitted limits.

For method development of anticancer drugs different column, different mobile phases and pH were tried and finally obtained better result. For carmustine method development use C18 column but tailing factor found slightly higher than the range. Then use NH_2 and CN columns still some problems were found. Finally used high carbon loading, double end capped C18 column, which shows better result. For method development of Dacarbazine different mobile phases containing buffers like phosphate, ammonium acetate, and triethylamine with different pH (2–5), and organic modifier (acetonitrile) were used. Finally, the chromatographic separation was achieved using Agilent Eclipse, XDB C18, (150 x 4.6 mm,) column. Changing the composition of mobile phase optimized the chromatographic method. For Paclitaxel, the stressed samples were initially analysed by HPLC using a RP C18 column and

a mobile phase composed of water and acetonitrile (50:50). As the separation and peak shape were not good, therefore, organic modifier concentration was changed from 80 to 95%, but no improvement was observed. Subsequent attempts were made by lowering of pH of the mobile phase (using acetic acid) and replacement of acetonitrile by methanol. In both cases, marked improvement was observed. Further trials were carried out by varying the composition of mobile phase using ACN: Phosphate buffer. Eventually, a mobile phase composition of phosphate buffer: acetonitrile (40:60, pH 7.4) gave the best results. One of the major reason to control pH is, reversed-phase silica-based columns work well from pH 2 to pH 8 or more, providing a wide range when searching for the optimum mobile phase pH for a separation.

For Imatinib, initially various mobile phase compositions were tried, to separate title ingredients. Mobile phase composition and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor) and run time. The system with acetonitrile: o-Phosphoric acid (0.1% v/v), (70: 30 v/v) 0.8ml/min flow rate was found to be quite robust. The optimum wavelength for detection was 266 nm at which better detector response for Imatinib was obtained. The main reason of choosing this mobile phase is, peak shows better resolution, no tailing factor and sharp peak. Flow rate 0.8ml/min was chosen because for better peak separation. Different columns containing octyl and octadecylsilane stationary phases were tried. It was found that X-terra column offered more advantages for better separation and resolution.

In case of docetaxel, when methanol was used in the composition of the mobile phase, resulting peaks were not sharp and shapes of the peaks were also not good. Triethylamine buffer with acetonitrile has shown the elution of both drugs with good peak shapes and resolution. Buffer shows good peak shapes and resolution because buffer resists the pH change, if it is not controlled properly, pH can be a source of many problems. Different columns such as RP-C8, RP-C18, RP–NH2, and Rpphenyl columns were used for the selection of appropriate column. Amino and phenyl columns were not eluted the both drugs and RP-C8 column has shown less sensitivity compared with RP-C18.

In case of Paclitaxel, the stressed samples were initially analysed by HPLC using a RP C18 column and a mobile phase composed of water and acetonitrile (50:50). As the separation and peak shape were not good, therefore, organic modifier concentration was changed from 80 to 95%, but no improvement was observed. Subsequent attempts were made by lowering of pH of the mobile phase (using acetic acid) and replacement of acetonitrile by methanol. In both

cases, marked improvement was observed. Further trials were carried out by varying the composition of mobile phase using ACN: Phosphate buffer. Eventually, a mobile phase composition of phosphate buffer: acetonitrile (40:60, pH 7.4) gave the best results. One of the major reason to control pH is, reversed-phase silica-based columns work well from pH 2 to pH 8 or more, providing a wide range when searching for the optimum mobile phase pH for a separation.

In case of carmustine, C18 stationary phase using ammonium acetate and phosphate buffer at different pH the resolution of carmustine was achieved but broad peak shape of carmustine was obtained having tailing factor about 2.6. To minimize tailing effect, further NH2 and CN columns were tried but it has been observed that (tailing factor 2.5) but resolution of carmustine decreased and in case of CN stationary phase peak shape of carmustine was improved. Finally used high carbon loading, double end capped C18 (YMC ODS-A C18, 25-cm) column, which show better resolution and low tailing factor.

In case of Dacarbazine, the chromatographic method was optimized by using different stationary phases like C18, C8, CN and different mobile phases containing buffers like phosphate, ammonium acetate, and triethylamine with different pH (2–5), and organic modifier (acetonitrile) were used. Finally, the chromatographic separation was achieved using Agilent Eclipse, XDB C18, (150 x 4.6 mm,) column. Changing the composition of mobile phase optimized the chromatographic method. To develop a stability-indicating method assessing the effect of change of proportion, the pH of mobile phase was maintained at 2.4 and the drug was well-resolved from degradation products at mobile phase composition of buffer and acetonitrile (96:4, v/v). From the development studies, it was determined that ortho phosphoric acid in milli-Q water and then pH adjusted to 2.4 with triethylamine and acetonitrile in the ratio of 96:4 (v/v), the flow rate of mobile phase 1.0 mL/min, and column temperature 30°C was optimal.

The wavelengths were selected by scannig in the range of 200 -400 nm against mobile phase as a blank. Drugs show maximum absorbance at which wavelength, select this wavelength.

The C18 column was selected to conduct the method development study based on the polarity of the drugs.

Conclusion:

The proposed methods were found to be simple and rapid for determination of COX 2 inhibitors from pure and pharmaceutical formulations. The sample recoveries in all formulations were in good agreement with their respective label claims suggested non-interference in the estimation. Hence, the methods can be easily and conveniently adopted for routine analysis. The simplicity ensures that the RP-HPLC method can be applied for estimation of COX 2 inhibitors in different dosage forms, the method was found to be accurate, precise, linear, robust and rugged. High performance liquid chromatography at present one of the most sophisticated tool of the analysis. Validated HPLC methods have been developed for determination of anticancer drugs. The proposed stability indicating methods are simple, economical, accurate, precise, specific, and robust. All the mobile phases are simple to prepare and economical. The method is also cost effective with respect to solvent consumption. Accuracy found 98 to 101.42 %, which was within limit. The system suitability parameters were within limit, hence it was concluded that all the systems were suitable to perform the assay.

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