A PROJECT REPORT ON

Verification of Pharmacopeia Assay by HPLC test method for Methylprednisolone Hydrogen Succinate (drug substance)

Submitted to Mangalore university in partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE

In

CHEMISTRY

Submitted by

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M.Sc. IV Semester Chemistry

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Project work was carried out at

Mylan laboratories limited, Bangalore.

Department of studies in chemistry

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APRIL 2018

DECLARATION

I hereby declare that the matter embodied in this project report is the result of work carried out by me at Mylan laboratories limited, Bangalore under the combined guidance of Dr. R. Maddani, Assistant Professor, Department of chemistry, Mangalore University, Mangalagangothri and Mr. Sudhakar Pujari, group leader, quality control Mylan laboratories Limited, Bangalore.

I further declare that the work presented in this report is a genuine and bonafide work and that it has not previously formed the basis for the award of any degree, fellowship or other similar titles.

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WHOMSOEVER IT MAY CONCERN

This is to certify that **Mr. Muhammed Alkali**, student of Mangalore University- Mangalagangothri, has successfully completed his project Training on the topic **"Development & Validation of Assay by HPLC method for Methylprednisolone Hydrogen succinate"** in QC Department at **Mylan Laboratories Limited from 15th June 2017 to 12th July 2017** under the guidance of Mr. Sudhakara Pujari.

During this period, we found him sincere and diligent. We wish him all the very best in his future endeavors.

For Mylan Laboratories, Limited,

Balakrishna Bhate
Manager – HR

CERTIFICATE

This is to certify that this project work on the topic '**Verification of Pharmacopeia Assay by HPLC test method for Methyl Prednisolone Hydrogen Succinate drug substance**' was carried out at Mylan laboratories limited, Bangalore by MUHAMMED ALKALI, a student of M.Sc. IV Semester Chemistry, in partial fulfilment for the award of degree of Master of Science in chemistry, Mangalore university.

The project work was carried out under the combined guidance of Dr. R. Maddani, Assistant Professor, Department of chemistry, Mangalore University, Mangalagangothri and Mr. Sudhakar Pujari, group leader, quality control Mylan laboratories Limited, Bangalore.

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ABSTRACT

This project incorporates the understanding of the analytical techniques especially on high performance liquid chromatographic technique (HPLC) in quality control laboratories of the pharmaceutical industry. The analytical methods which are used to test the drug substances or in drug products are either adopted from pharmacopeia or in-house developed methods. High performance liquid chromatographic technique is used wide range in the pharmaceutical industry to estimate the potency and related compounds of drug substance or drug product. Hence, project initiative taken to understand the complete instrumentation technique of HPLC and the analytical method verification process of a drug molecule (Methylprednisolone hydrogen succinate) was performed.

PROJECT OBJECTIVES

The objectives of this project are to understand the principle & instrumentation techniques of HPLC and column chromatography. Subsequently, to **verify the Assay by HPLC** test method of **"*Methylprednisolone Hydrogen Succinate*"** drug substance. This project was initiated at the Quality Control Department, **Mylan Laboratories Limited**, Bangalore.

COMPANY PROFILE

Mylan is one of the world's leading global pharmaceutical companies, with a significant and growing presence in India. The company having workforce of more than 30,000 people, approximately half of whom are based in India. Mylan's broad portfolio includes generic and brand name products in a variety of dosage forms, which are sold in about 165 countries and territories. Mylan's history in India began in 2007 with the acquisition of Matrix Laboratories Limited. The deal transformed Mylan overnight into one the world's largest manufacturers of active pharmaceutical ingredients (API) and allowed the company to vertically integrate the production of its finished dosage form (FDF) medicines.

Today, Mylan's manufacturing platform includes more than 40 facilities, 25 of which are located in India. Those 25 facilities include all nine of its API facilities and 15 FDF facilities, which manufacture Mylan's high-quality medicines for markets all over the world, including emerging markets. Mylan's manufacturing capabilities in India include a range of dosage forms, such as tablets, capsules and injectables, in a wide variety of therapeutic categories.

Mylan's commercial businesses based in India markets high quality API to third parties around the world and antiretroviral products for people living with HIV/AIDS. Today, nearly 50% of those receiving treatment for the disease in the developing world rely on a Mylan product, all of which are made in India. In fact, Mylan is India's third largest pharmaceutical exporter.

In addition, Mylan has a growing commercial presence domestically. its current franchises include Critical Care, Hepato Care, HIV Care, Onco Care and Women's Care (Mylan.in, 2017).

CHAPTER ONE.

1.0 INTRODUCTION AND THE HPLC TECHNIQUE.

Chromatography is defined as a set of techniques which are used for the separation of constituents in a mixture. This technique involves 2 phases namely the stationary and mobile phases. The separation of constituents is based on the differences between partition coefficients of the two phases. The chromatography term is derived from the Greek words namely chroma (color) and graphein (to write). (Duvvada et al. 2016)

The chromatography is a very popular technique and it is mostly used analytically. There are different types of chromatographic techniques namely Paper Chromatography, Gas Chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography and lastly High-Performance Liquid Chromatography (HPLC). This review mainly focuses on the HPLC technique its principle, types, instrumentation and applications.

High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures up to 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities. (Tang M, et al. 2014)

High-performance liquid chromatography (HPLC) is a separation technique that can be used for the analysis of organic molecules and ions. HPLC is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase.

Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases. HPLC can be used to assess the purity and/or determine the content of many pharmaceutical substances. It can also be used to determine enantiomeric composition, using suitably modified mobile phases or chiral stationary phases. Individual separation mechanisms of adsorption, partition and ion exchange rarely occur in isolation since several principles act to a certain degree simultaneously. (international pharmacopeia, 2016).

1.1 HISTORY OF HPLC

Preceding HPLC researchers utilized standard liquid chromatographic methods. Liquid chromatographic systems were to an extent inefficient because of the flow rate of solvents being reliant on gravity. Separations took numerous hours, and some of the time days to finish. Gas chromatography (GC) at the time was more effective than liquid chromatography (LC), in any case, it was trusted that gas stage partition and investigation of extremely polar high atomic weight biopolymers was impossible. GC was ineffectual for some organic chemist's due to the thermal instability of the solutes. Accordingly, alternative techniques were hypothesized which would soon bring about the advancement of HPLC.

Taking after on the original work of Martin and Synge in 1941, it was anticipated by Cal Giddings, Josef Huber, and others in the 1960s that LC could be worked in the high-proficiency mode by decreasing the pressing molecule measurement generously beneath the run of the mill LC (and GC) level of 150 μm and utilizing pressure to expand the versatile stage velocity. These expectations experienced broad experimentation and refinement all through the 60s into the 70s. Early developmental exploration started to enhance LC particles, and the innovation of Zipax, an externally permeable molecule, was promising for HPLC technology. The 1970s achieved

numerous advancements in equipment and instrumentation. Specialists started utilizing pumps and injectors to make a simple configuration of a HPLC system. Gas amplifier pumps were perfect since they worked at consistent pressure and did not require release of free seals or check valves for steady flow and great quantitation. (Duvvada et al. 2016)

1.2 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid, where sample ions or molecules are dissolved.

It is carried out either in a column or a plane. The sample with the mobile liquid will pass through the column or the plane, which is packed with a stationary phase composed of irregularly or spherically shaped particles. Due to the differences in ion-exchange, adsorption, partitioning, or size, different solutes will interact with the stationary phase to different degrees, and therefore the separation of the compounds can be achieved and the transit time of the solutes through the column can be determined by utilizing these differences.

Conventional LC is commonly used in preparative scale work to purify and isolate some components of a mixture. Nowadays liquid chromatography generally utilizes very small packing particles and a relatively high pressure for analytical separations of solutions, detection & quantification, referred to as high performance liquid chromatography (HPLC). HPLC can provide a very high resolution (up to parts per trillion) and a fast analysis time.

1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. This difference in rates cause the separation of various components. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography).

HPLC is a separation technique that involves the solid stationary phase & liquid mobile phase, also that depends upon the adsorption, partition & ion exchange process.

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 micron (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". In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior. (Agilent Technologies, Inc. 2017)

1.4 TYPES OF HPLC

These are the following variants of HPLC, depending upon the phase system (stationary) in the process:

Normal Phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

In this mode, the column packing is polar (e.g. silica gel, cyanopropyl-bonded, amino-bonded, etc.) and the mobile phase is non-polar (e.g. hexane, iso-octane, methylene chloride, ethyl acetate). Normal phase separations are performed less than 10% of the time. The technique is useful for water-sensitive compounds, geometric isomers, cis-trans isomers, class separations, and chiral compounds.

Reverse Phase HPLC

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained. The column packing is non-polar (e.g. C18, C8, C3, phenyl, etc.) and the mobile phase is water (buffer) + water-miscible organic solvent (e.g. methanol, acetonitrile). RPC is, by far, the most popular mode, over 90% of chromatographers use this mode. The technique can be used for non-polar, polar, ionizable and ionic molecules making RPC very versatile. For samples containing a wide range of compounds, gradient elution is often used. One begins with a predominantly water-based mobile

phase and then adds organic solvent as a function of time. The organic solvent increases the solvent strength and elutes compounds that are very strongly retained on the RPC packing.

Size-exclusion HPLC

The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

In SEC, there is no interaction between the sample compounds and the column packing material. Instead, molecules diffuse into pores of a porous medium. Depending on their size relative to the pore size, molecules are separated. Molecules larger than the pore opening do not diffuse into the particles, while molecules smaller than the pore opening enter the particle and are separated. Large molecules elute first. Smaller molecules elute later. The SEC technique is used by 10-15% of chromatographers, mainly for polymer characterization and for proteins.

Ion-Exchange HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

In ion exchange, the column packing contains ionic groups (e.g. sulfonic, tetraalkylammonium) and the mobile phase is an aqueous buffer (e.g. phosphate, formate, etc.). Ion exchange is used by about 20% of the liquid chromatographers. The technique is well suited for the separation of

inorganic and organic anions and cations in aqueous solution. Ionic dyes, amino acids, and proteins can be separated by ion exchange because such compounds are salt in brine water.

1.5 APPLICATIONS OF HPLC

HPLC Applications: In Diagnosis, Research & Pharma Industry: HPLC (High Performance Liquid Chromatography) is one of the types of chromatography. It is a highly sophisticated and expensive analytical tool in the present scientific era. It is given prominent importance due to its attributes like

- High sensitivity i.e. ability to evaluate samples of very minute concentrations like in nano-gram and picogram
- Detect precisely the closely similar molecules and also
- Highest accuracy in the identification of components of complex mixtures.

This is possible in HPLC analysis due to efficient separation of molecules under pressure over a large surface area. Besides there is also availability of highly sensitive detectors like UV-visible and florescence spectrophotometers, electrochemical detectors etc.

But unlike other analytical techniques, HPLC analysis is time taking easy and troubleshooting is very important to run the test smoothly. So, one is advised to learn it from an expert in **HPLC** operation.

This method of chromatography finds vast use in

- Clinical diagnosis of diseases, disorders.
- In scientific research for discovery.

- In pharmaceutical labs for analysis.
- In food industry for quality control.
- For standards control by government.
- For separation of similar molecules.

HPLC analysis in clinical diagnosis and health industry: Many disorders related to body metabolism, those related to endocrine and exocrine gland secretion, alteration in body fluids are diagnosed by HPLC analysis of concerned fluids. For example, estimation of metabolites of purines, pyrimidines or other metabolites from plasma, cerebrospinal fluid and urine samples in patients. Estimation of corticoids from plasma in disorders of adrenal gland which secretes an endocrine hormone. Because of time factor, most of the diagnostic methods are replaced by Elisa, electrophoresis and RIA methods. But still for a new a rare problem, HPLC method is preferred to pin point the cause of disorders (i.e. any change in some biochemistry).

HPLC application in scientific research: HPLC system is a mandatory tool in most of the labs involved in research. The fields of research include medical, biological, chemical, biochemical, phytochemical (plant chemical research). When a research is taken up, the scientists are not sure of the actual which need attention in a body fluid or drug sample etc. Then they have to screen every possible molecule to point out the altered change (component). Then HPLC is much suited as it can analyze each and every molecule in the mixture.

It finds it application to analyze and quantify the molecules. Components with similar chemistry and properties are easily distinguished by this method. Due to the principle of separation in HPLC similar molecules get separated and hence their detection, quantification becomes easier.

HPLC applications in pharmaceutical industry: In pharmaceutical industry, HPLC analysis is prominently used in quality control. Beside it also used R& D wings (research and development).

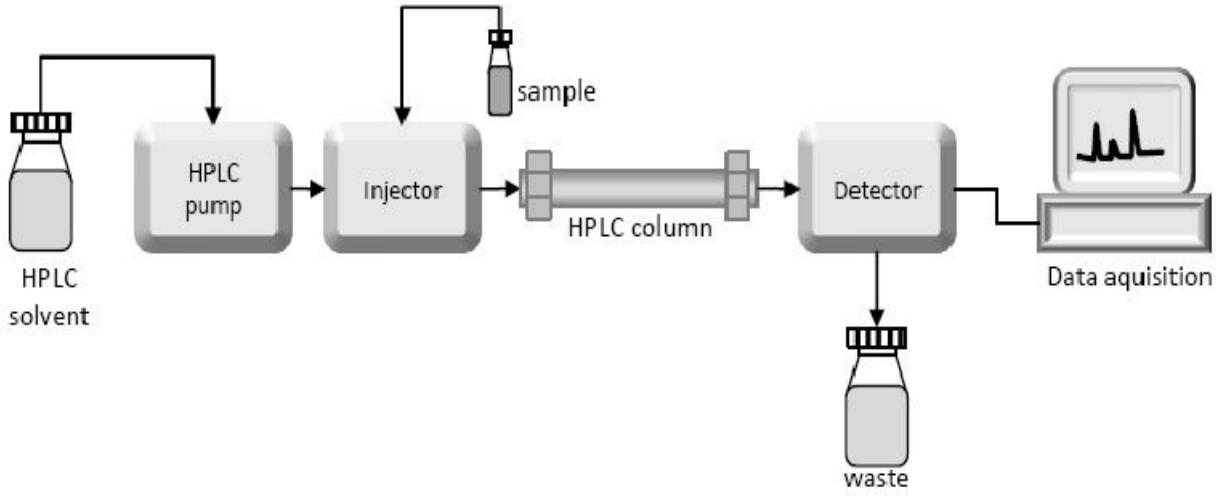
- In quality control, it is used to check if the prepared or manufactured products are in compliance with the specified standards as per the pharmacopoeia and other drug regulating bodies. For quality control, the manufactured product must comply with the quality guidelines referring to pharmacopoeia will give an idea of how the peak of the drug in the formulation should look, at the specified HPLC solvents and mobile phases used. If the peaks do not correspond to those shown in pharmacopeia, the batch cannot be passed for quality check.
- In R & D as discussed in research before, it is used to identify the specific molecule or component in mixture under research. Further it is used for bioavailability studies, drug release from the formulation, dissolution studies etc. After a formulation is designed, the drug release over a period is tested in bioavailability studies. Then the sample released is taken and injected into HPLC system to note the individual molecules released in terms of quantity. Since the molecules might be similar, their separation is easier over the column under pressure. Further, their detection becomes easier as the system is connected UV Visible detector or other specified detectors. For this the formulation like injections, solutions, dissolved form of solid dosage forms are injected into HPLC to record the peaks of the individual constituents.
- Also, any new molecule under development or preclinical trial, are analyzed to see their concentration in the blood after certain intervals of administration. This helps to evaluate the metabolic profile, plasma concentration, bio-availability etc. of the formulation or chemical moieties under development.

- In plant constituents, there are many molecules with similarity in chemistry like isoflavones, glycosides, saponins etc. but different activity or nutritional value. These compounds can't be precisely determined by other methods; hence they are determined by HPLC analysis by means of separation into individual components and there by identification.

For standards control by governments: The pharmacopeia making bodies like United States Pharmacopeia (USP), Indian Pharmacopeia (I.P), British Pharmacopeia (B.P), use HPLC extensively. They fix standards of control for any drug formulation the industry makes. The companies send the formulation to the pharmacopeia bodies for standardization. Most formulations are estimated by HPLC to see the peaks of active ingredients (drug molecules). The peaks are then published in the official volumes of USP, IP or BP for reference by the industries for quality control. This gives an idea of how the peaks for active ingredient in a formulation appear under the specified mobile phase solvents. The effectiveness and use HPLC application in recent days is further enhanced due to coupling with detectors like Mass Spectroscopy, Nuclear Magnetic Resonance etc.

CHAPTER TWO

2.0 Instrumentation of HPLC



As shown in the schematic diagram in Figure above, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

Solvent Reservoir: Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

Pump: A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated

An ideal pump should have the following desirable characteristics

- Solvent compatibility and resistance to corrosion
- Constant flow delivery independent of back pressure
- Convenience of replacement of worn out parts
- Low dead volume for minimum problems on solvent changeover

Three commonly used pump types are Syringe type pumps, Constant pressure pumps and Reciprocating piston pumps.

***Constant pressure pumps*:** Provide consistent continuous flow rate through the column with the use of pressure from a gas cylinder. Valving arrangement allows rapid refill of solvent chamber. A low-pressure gas source is needed to generate high liquid pressures.

***Syringe Type Pumps*:** Are suitable for small-bore columns. Constant flow rate is delivered to column by a motorized screw arrangement. Solvent delivery rate is set by changing voltage on the motor. These pumps deliver pulseless flow independent of column backpressure and changes in viscosity but major disadvantages are limited solvent capacity and limitation on gradient operation

***Reciprocating Piston pumps*:** Deliver solvent(s) through reciprocating motion of a piston in a hydraulic chamber. On the backstroke, the solvent is sucked in and gets delivered to the column in the forward stroke. Flow rates can be set by adjusting piston displacement in each stroke. Dual and triple head pistons consist of identical piston chamber units which operate at 1800 or 1200 phase difference. The solvent delivery of reciprocating pump systems is smooth because while one pump is in filling cycle the other is in the delivery cycle. High pressure output is possible

at constant flow rate and gradient operation is possible. However, pulse dampening is required for further elimination of pressure pulses.

We now have a fair understanding of different pumps and their operation principles and our next post will deal with injectors for injection of samples into the flowing mobile phase stream.

Sample Injector: The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

Columns; Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 μm. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally, the temperature of the mobile phase and the column should be kept constant during an analysis. The column is one of the most important components of the HPLC chromatograph because the separation of the sample components is achieved when those components pass through the column. Normally, columns are filled with silica gel because its particle shape, surface properties, and pore structure help to get a good separation. Silica is wetted by nearly every potential mobile phase, is inert to most compounds and has a high surface activity, which can be modified easily with water and other agents. Silica can be used to separate a wide variety of chemical compounds, and its chromatographic behavior is generally predictable and reproducible.

Usually, analytical columns are protected by a guard column, which is an essence a disposable (or sacrificial) top of the main analytical column. The guard column is the final filter both mechanical

and chemical. In addition to removing debris, it also can adsorb undesirable sample components that otherwise might irreversibly bind and possibly change the stationary phase of the analytical and preparative columns. Although economy alone is not a persuasive argument for the use of guard columns, the need for a long stable life of the analytical column to obtain reliable and reproducible results is perhaps even more important.

There are several **column types**, according to their function, they can be classified as:

***Normal phase*:** In this column type, the retention is governed by the interaction of the polar parts of the stationary phase and solute. For retention to occur in normal phase, the packing must be more polar than the mobile phase with respect to the sample. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these.

***Reverse phase*:** In this column, the packing material is relatively nonpolar and the solvent is polar with respect to the sample. Retention is the result of the interaction of the nonpolar components of the solutes and the nonpolar stationary phase. Typical stationary phases are nonpolar hydrocarbons, waxy liquids, or bonded hydrocarbons (such as C18, C8, etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.

***Size exclusion*:** In this column type, molecules are separated according to size. Small molecules penetrate into the pores within the packing while larger molecules only partially penetrate the pores. The large molecules elute before the smaller molecules.

***Ion exchange*:** In this column type the sample components are separated based upon attractive ionic forces between molecules carrying charged groups of opposite charge to those charges on

the stationary phase. Separations are made between a polar mobile liquid, usually water containing salts or small amounts of alcohols, and a stationary phase containing either acidic or basic fixed sites. This HP 1090 Chromatograph is also equipped with an oven in the column compartment. The function of the oven is to provide a homogenous air-bath temperature when it is required for some methods, such as the carbohydrate.

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:

- **Polar Interactions**

  Differences in polarity between the sample components and the bonding entities on stationary phase result in preferential retention

- **Ionic Interactions**

  Separation based on charge properties of sample molecules. Analyte ions have affinity for oppositely charged ionic centers on the stationary phase

- **Molecular Size**

  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.

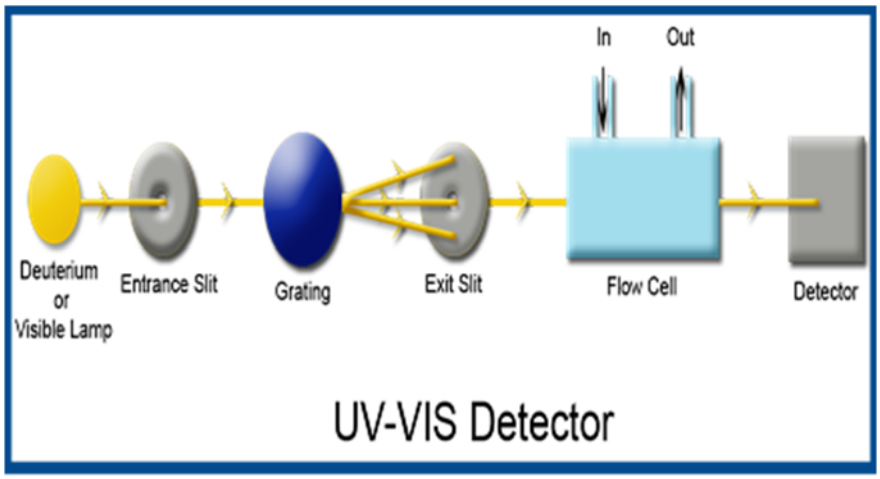
Detector: The HPLC detector, located at the end of the column detect the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

The desirable **features of a detector** are:

- Sensitivity towards solute over mobile phase
- Low cell volumes to minimize memory effects
- Low detector noise
- Low detection limits
- Large linear dynamic range.

Detector:

UV-VIS Detector:



UV-VIS Detector is the most commonly used 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 such light absorbing groups do not have can give suitable response after post column derivatization to introduce light absorbing entities.

***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.

***Fluorescence Detector*:** Fluorescence 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.

Mass Spectroscopic Detector: Mass spectroscopy 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. LC –MS technique has opened up new application areas due to advantages of resolution and sensitivity.

Bulk Property Detectors:

***Refractive Index Detector*:** The response is dependent on changes in refractive index of eluting compounds in the mobile phase. The mobile phase itself should have refractive index different from the sample. Gradient programming is not possible due to resulting changes in refractive index of mobile phase. The detector is less sensitive than UV-VIS detector. Temperature control is necessary as it has high temperature sensitivity. Typical applications are in Size Exclusion Chromatography.

Electrochemical Detector:

Based on electrochemical oxidation or reduction of sample on electrode surface. It is, however, sensitive to changes in composition or flow rate of mobile phase.

***Light Scattering Detectors*:** Light scattering detectors are useful for detection of high molecular weight molecules. After removal of mobile phase by passing through a heated zone the solute molecules are detected by light scattering depending on molecular sizes.

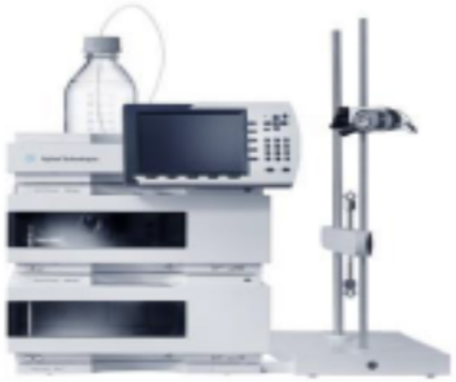
Data Collection Devices or Integrator: Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret. Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The data storage capacity of these devices is usually limited. Modern data stations are computer-based and have a large storage capacity to collect, process and store data for possible subsequent reprocessing. Integration of peak areas and the setting of threshold levels are not normally problematic in an assay since the peak of the substance to be analysed should be free of interference. However, in a test for impurities, the selection of the peak area integrator parameters becomes very important, particularly when baseline separations are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed for peaks of similar size, whereas for peaks of very different size tangent skimming is recommended.

HPLC allows limits to be set for individual impurities and for the sum of impurities, but there must be a threshold below which peaks should not be integrated. This "disregard level" or “reporting threshold” is set in relation to the area of the peak in the chromatogram of the

prescribed reference solution and is usually equivalent to 0.1% or 0.05% of the substance being examined. (international pharmacopeia, 2016).

The schematic representation of a HPLC instrument ordinarily incorporates a sampler, pumps, and a locator. The sampler brings the sample into the mobile phase stream which conveys it into the column. The pumps convey the mobile phase through the column. The detector generates a sign relative to the measure of sample component rising up out of the segment, consequently taking into consideration quantitative investigation of the example parts. A computerized microchip and software control the HPLC instrument and give information data. A few models of mechanical pumps in a HPLC instrument can combine numerous solvents in proportions changing in time, producing a sythesis slope in the portable stage. Most HPLC instruments likewise have a column broiler that considers altering the temperature at which the partition is performed (Reinhardt TA, et al. 1983 and Shintani H. 2013)

2.6 Examples of Different Instruments and Configurations



Modular HPLC System –basic configuration with isocratic pump, manual injector, variable wavelength detector, and hand-held controller

Modular HPLC System –high-end configuration with quaternary pump, autosampler, column thermostat, diode array detector, and computer with control and data analysis SW

Integrated HPLC System "all parts in one box" –different configurations possible, here with gradient pump, autosampler, column oven, VWD, and computer with control and data analysis SW (not shown on picture)

CHAPTER THREE

3.0 Literature review

K. Sivarami Reddy, B. Ramachandra, and N. V. S. Naidu from the Department of Chemistry, S. V. University, Tirupati-517502, A.P., India in 2014 published a paper titled Development and Validation of HPLC Assay Method for Determination of Mesalamine in Bulk Drug and Tablet Formulation. The objective of the study was to develop a simple, precise and accurate RP-HPLC assay method and validate for rapid assay of Mesalamine in bulk and tablet dosage form. Isocratic elution at a flow rate of 1.0 mL/min was employed on Waters (alliance) HPLC C18, 100X4.6, 5µ. column using mobile phase of mixed buffer and Acetonitrile (65: 35 v/v) at UV detector. The UV detection wavelength was 258 nm and 20 µL of sample was injected, retention time for Mesalamine was 3.214 min. The method was linear in the drug concentration range of 10-60 µg/ml with a correlation coefficient 0.998. The method was validated for specificity, linearity, precision, accuracy, robustness and solution stability and also found to be robust as indicated by the % RSD values which are less than 2%. The method was validated as per the ICH guidelines, successfully applied for routine analysis of Mesalamine in bulk samples and its formulations.

S. Kumar*, S. Mathkar, C. Romero, and A.M. Rustum in june 2011 carried out the Development and Validation of a Single RP-HPLC Assay Method for Analysis of Bulk Raw Material Batches of Four Parabens that are Widely Used as Preservatives in Pharmaceutical and Cosmetic Products. A stability-indicating, robust, fast, and user friendly reversed-phase high-performance liquid chromatographic (RP-HPLC) assay method was developed and validated for the analysis of commercial raw material batches of methylparaben, ethylparaben, propylparaben, and butylparaben. These four parabens are widely used as preservatives in pharmaceutical and cosmetic products. Accurate assay value of each of the parabens in their respective commercial

lots is critical to determine the correct weight of the paraben that is needed to obtain the target concentration of the paraben in a specific lot of pharmaceutical or cosmetic products. There was no single HPLC assay methods (validated as per ICH requirements) available in the literature that could be used to analyze the commercial lots of each of the four parabens. The analytical method reported analyses all four parabens in less than 10 min. The method presented in the report was successfully validated as per ICH guidelines. Therefore, the method could be implemented in QC laboratories to analyze and assay the commercial bulk lots of the four parabens.

Richa Sah* and Saahil Arora developed and validated HPLC analytical assay method for amlodipine besylate tablets: A Potent Ca^{+2} channel blocker. Amlodipine besylate is a potent calcium channel blocker used for the treatment of hypertension, congestive heart failure and angina pectoris. Amlodipine besylate avoids the adverse effect of amlodipine in racemic mixtures. A highly precise and cost-effective RP-HPLC method with retention time of 2.60 minutes was developed for the estimation of amlodipine besylate in tablet dosage form, by fixing the parameters as WATERS C18 column 250 mm × 4.6 mm (5µm), with mobile phase as acetonitrile: 70mM potassium dihydrogen orthophosphate buffer: methanol (15:30:55) and pH adjusted to 3.0. Mobile phase flow rate was maintained at 1.0 ml/min and detected at 240nm.

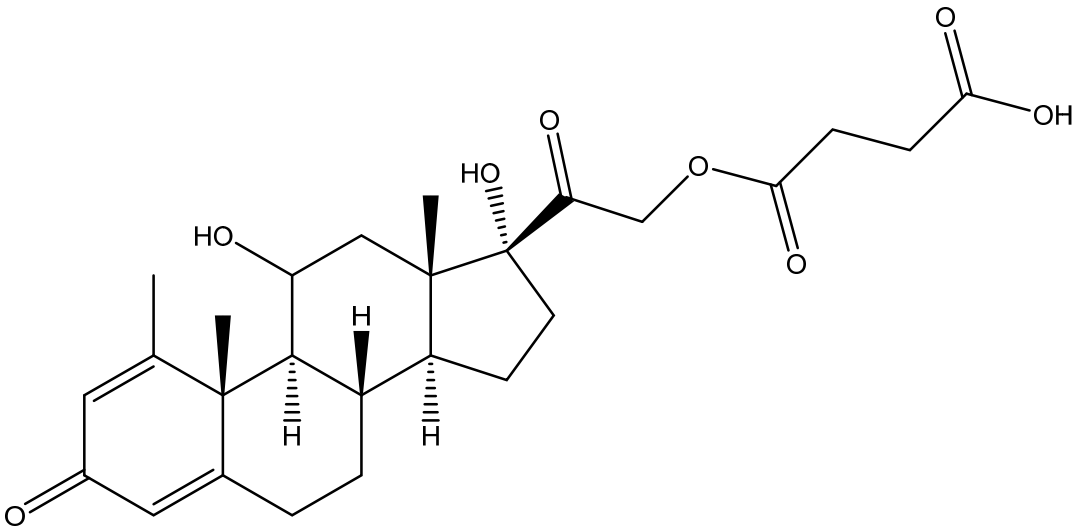
Recently in 2017, MERAL YUCE1 and YILMAZ CAPAN2* from the Department of Pharmaceutical Technology, Faculty of Pharmacy, Trakya University, Balkan Campus, 22030, Edirne, Turkey and from the Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, Sihhiye 06100, Ankara, Turkey respectively published a paper titled DEVELOPMENT AND VALIDATION OF HPLC ANALYTICAL METHODS USED FOR DETERMINATION OF ASSAY, CONTENT UNIFORMITY AND DISSOLUTION OF IMMEDIATE RELEASE CANDESARTAN CILEXETIL 32 MG TABLETS. Method

development studies were performed on cyano column. Mobile phase of assay and content uniformity test consisted of mixture of 0.05 M phosphate buffer, pH 4.5 and methanol (40: 60, v/v) adjusted to pH 4.0 with trifluoroacetic acid, whereas mobile phase of dissolution test consisted of mixture of 1M phosphate buffer and acetonitrile (50 : 50, v/v) adjusted to pH 2.0 with trifluoroacetic acid. Mobile phases were pumped at flow rate of 1.0 mL/min, ultraviolet-visible (UV) detector was operated at 254 nm, injection volume was set at 20 µL, column temperature was held at 25∞C. Dissolution medium was 0.05 M phosphate buffer, pH 6.5 including 0.70% (w/v) polysorbate 20. Validation studies met acceptance criteria of system suitability, specificity, linearity and range, accuracy, precision, detection limit (LOD), quantitation limit (LOQ) and robustness parameters.

Also, Mallesh Kurakula, Tariq R Sobahi, AM El-Helw and Magdy Y Abdelaal from Polymer Research Lab, Department of Chemistry, Faculty of Science, Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia, and from the Department of Chemistry, Faculty of Science, Mansoura University, 35516-Mansoura, Egypt respectively developed and validated a RP-HPLC Method for Assay of Atorvastatin and its Application in Dissolution Studies on Thermosensitive Hydrogel-Based Nanocrystals. Chromatographic identification was achieved on C18 (5 µm) column using acetonitrile and 0.025 M potassium dihydrogen ortho-phosphate buffer pH 5 (45:55 v/v) as mobile phase, at a flow rate of 1.5 mL/min and using photo diode array detector (PDA) at 246 nm. The developed HPLC method was validated according to International Conference on Harmonisation (ICH) Q2(R1) guidelines and applied to dissolution studies on atorvastatin thermosensitive hydrogel-based nanocrystal formulation, using Lipitor® as standard. Determination was successfully achieved with good peak resolution from atorvastatin nanocrystals and a commercial formulation brand (Lipitor® tablets)

without interference of polymer or excipients. The retention time of atorvastatin was 4.5 min and drug response were linear in the range of 0.1 - 0.5 µg/mL with a correlation coefficient of 0.9995. Precision was determined to be between 0.16 - 0.61 percent relative standard deviation (% RSD) for the analyzed samples. The limit of detection and of quantification was 35.6 and 71.2 ng/mL, respectively, which was 10 times higher than a previously reported method. The assay of atorvastatin nanocrystal and Lipitor® gave 99.37 and 99.12 % recovery, respectively. Dissolution studies showed atorvastatin release of 40 and 65 % at 40 min from thermosensitive hydrogel nanocrystal formulation and Lipitor®, respectively indicating sustained release.

3.1 METHYLPREDNISOLONE HYDROGEN SUCCINATE



METHYLPREDNISOLONE HYDROGEN SUCCINATE

$C_{26}H_{34}O_8$ Mw 474.6

CHARACTERS

A white or almost white, hygroscopic powder, practically insoluble in water, slightly soluble in acetone and in ethanol. It dissolves in dilute solutions of alkali hydroxides. (European Pharmacopeia, 2005)

Methylprednisolone is a steroid that prevents the release of substances in the body that causes inflammation. Methylprednisolone is used to treat many different inflammatory conditions such as arthritis, lupus, psoriasis, ulcerative colitis, allergic disorders, gland (endocrine) disorders, and conditions that affect the skin, eyes, lungs, stomach, nervous system, or blood cells.

Sample of methylprednisolone hydrogen succinate (an active pharmacopeia ingredient) used for the method verification procedure was prepared in Mylan laboratories limited.

CHAPTER FOUR

4.0 Results and Data interpretation

4.1 SYSTEM SUITABILITY:

To verify that the analytical system is working properly and can give accurate and precise results**.**

Acceptance criteria for Assay by HPLC:

- RSD for area response Methylprednisolone hydrogen succinate from the reference solution should be not more than 2.0

Result:

Sr. No.	Acceptance criteria	Results
1	RSD for area response Methylprednisolone hydrogen succinate from the reference solution should be not more than 2.0	0.1%

Data interpretation:

From the above results, it is concluded that the system is suitable for Analytical Method Verification.

4.2 SYSTEM PRECISION:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to six injections of standard. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of series of measurements.

Acceptance criteria:

- The RSD for the retention time of peak from six replicate injections of Reference solution should be NMT 2.0%
- The RSD for peak area of replicate injections of Reference solution should not be more than 2.0%

Results:

Inj. No.	Methylprednisolone hydrogen succinate	
	Retention time (min)	**Area response**
1	23.493	3384785.146
2	23.473	3385391.722
3	23.487	3384809.416
4	23.480	3388404.546
5	23.493	3388824.617
6	23.487	3389929.282
Mean	**23.486**	**3387024.122**
% RSD	**0.0%**	**0.1%**

Data interpretation:

From the above results, it is concluded that retention time and area responses were consistent as evidenced by relative standard deviation. Hence, it is concluded that the system precision parameters meet the requirement of verification.

4.3 METHOD PRECISION:

In method precision, a homogeneous sample of a single batch should be analyzed six times. This indicates whether a method is giving consistent results of a single batch.

Acceptance criteria for Assay by HPLC:

- The results should be within specification limit.
- % RSD of 6 results shall be not more than 2.0%.

Specification Limit:

Specification limit	Not less than 97.5% and not more than 102.0% on dried substance.

Results:

Set No.	% Assay on Dried basis
1	99.7 %
2	99.8 %
3	98.6 %
4	100.5 %
5	99.5 %
6	99.1 %
Mean	**99.5 %**
% RSD	**0.7 %**

Data interpretation:

From the above results, it is concluded that the method precision parameters meet the requirement of specification and method verification protocol.

4.4 SUMMARY OF RESULTS:

<table>
<tr><th colspan="2">Parameter</th><th>Acceptance criteria</th><th colspan="2">Results</th></tr>
<tr><td colspan="2">System suitability</td><td>RSD for the area response of Methylprednisolone hydrogen succinate from the reference solution should be not more than 2.0</td><td colspan="2">0.1%</td></tr>
<tr><td rowspan="9">Precision</td><td rowspan="2">System precision</td><td>The RSD for the retention time of peak from six replicate injections of Reference solution should be NMT 2.0%</td><td colspan="2">0.0%</td></tr>
<tr><td>The RSD for the peak area of 6 replicate injections of Reference solution should be NMT 2.0%</td><td colspan="2">0.1%</td></tr>
<tr><td rowspan="9">Method precision</td><td>Results should meet the specification limit.</td><td colspan="2">Results were within the specification limit</td></tr>
<tr><td rowspan="8">% RSD of 6 results shall be not more than 2.0%</td><td>Set. No.</td><td></td></tr>
<tr><td>1</td><td>99.7</td></tr>
<tr><td>2</td><td>99.8</td></tr>
<tr><td>3</td><td>98.6</td></tr>
<tr><td>4</td><td>100.5</td></tr>
<tr><td>5</td><td>99.5</td></tr>
<tr><td>6</td><td>99.1</td></tr>
<tr><td>Mean</td><td>99.5</td></tr>
<tr><td>%RSD</td><td>0.7%</td></tr>
</table>

CONCLUSION

The quality control (QC) laboratory plays a critical role in pharmaceutical production, for both in-process and finished product testing. Been at the QC department of Mylan laboratories provided me with a lot of worthwhile experiences.

High performance or pressure liquid chromatography is a very important analytical tool in the pharmaceutical industry. This project provides an insight into the principles, instrumentation, operations and applications of the instrument.

Furthermore, the test assay by HPLC been updated in the current BP/Ph. Eur, 8.6 monograph was verified and the method was found to be suitable and precise.

METHOD DESCRIPTION

Verification of Assay by HPLC method for Methylprednisolone hydrogen succinate drug substance was carried out as per BP/Ph.Eur., 8.6 monograph.

***Method verification procedure*:**

The following parameters were considered to verify the Assay by HPLC test method for Methylprednisolone hydrogen succinate drug substances at QC.

- System Suitability
- Precision
  - System Precision
  - Method Precision

SYSTEM SUITABILITY

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set.

Injected the blank (1injection), the Reference solution (5 Injection), and checked for system suitability parameters.

PRECISION

System precision; The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to six injections of standard. The precision of analytical method is usually expressed as the standard

deviation or relative standard deviation (Coefficient of variation) of series of measurements.

Injected Blank (1injection), Reference solution (6 injections) into the chromatograph. Recorded the chromatographs Calculated relative standard deviation for retention time and area response of 6 determinations.

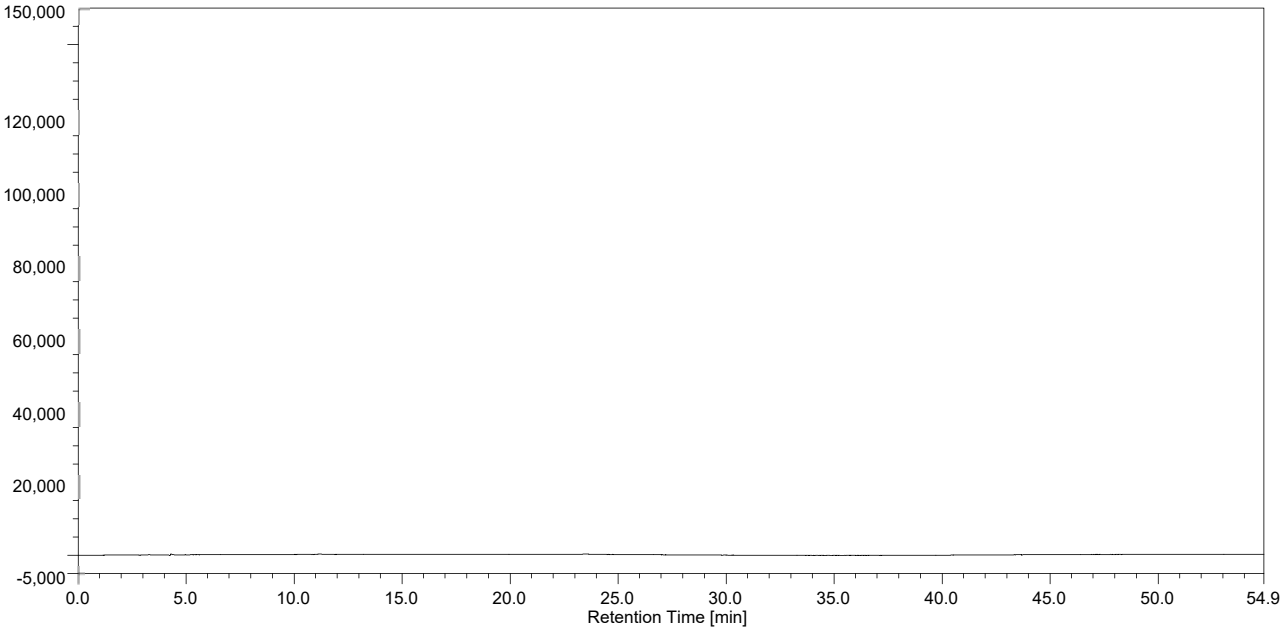
method precision; In method precision, a homogeneous sample of a single batch should be analyzed six times. This indicates whether a method is giving consistent results of a single batch.

Analyzed the sample six times of a single batch as per analytical procedure, Calculated the % impurity.

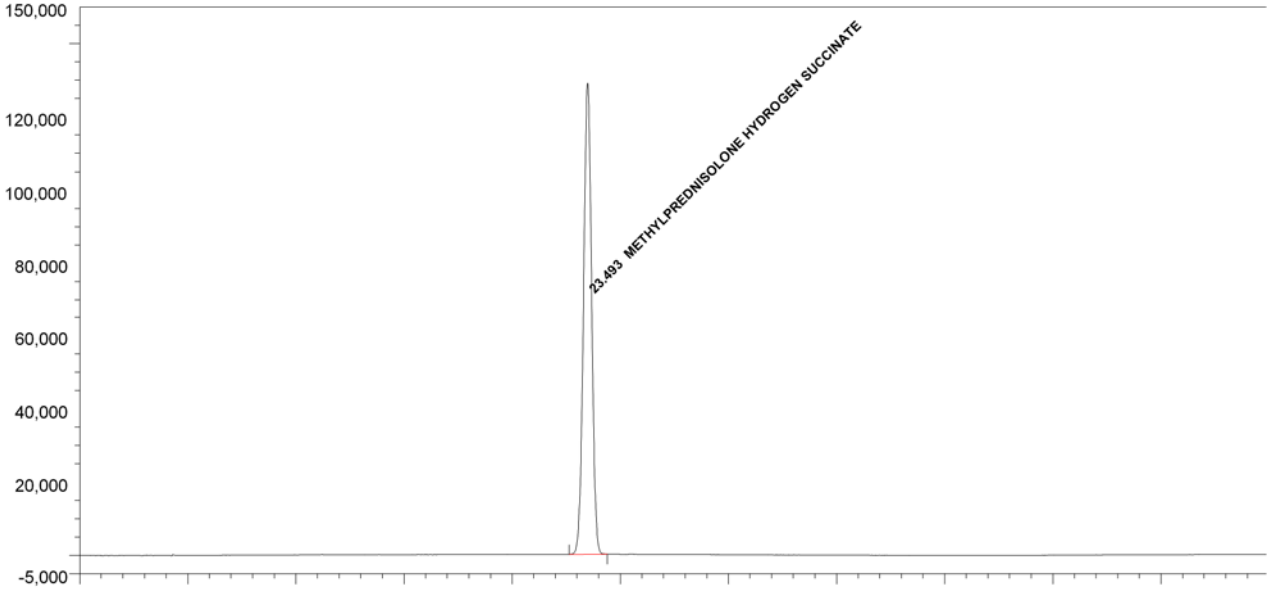
CHROMATOGRAMS

In all chromatograms provided below, Y axis corresponds to the absorbance and X axis corresponds to the retention time in minutes.

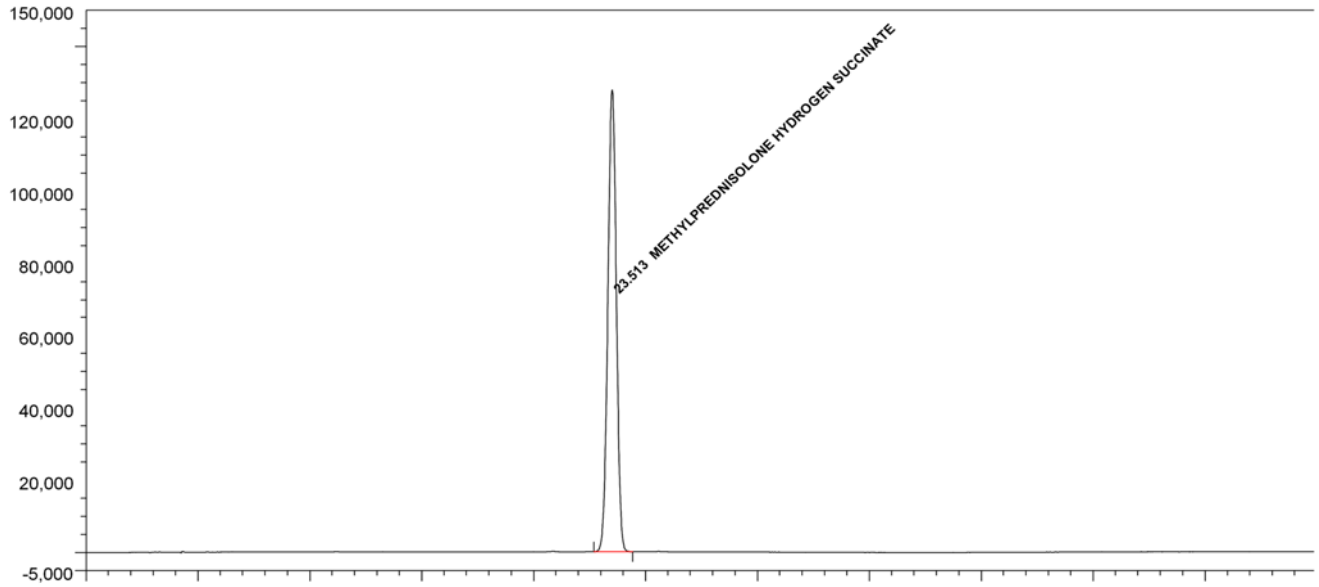
Blank:



Standard:



Sample



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