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ESTIMATION OF ANTIDIABETIC DRUGS BY HIGH PERFORMANCE LIQUID CHROMATOGHRAPHY METHOD

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ABSTRACT

The determination of Metformin was carried out in pure and tablet forms. The drug was found almost stable to neutral and photolytic condition. Hence, the proposed method is suitable for application in quality-control laboratories for quantitative analysis of drugs individually. This research project deals with the studies on the "Development and validation of liquid chromatographic methods for the quantification of anti-diabetic drugs in formulations and biological samples. The need for drug analysis, chromatographic methodology (method development and optimization) and experimental results of drug quantification in dosage forms, biological matrix and validation of the HPLC method was used for single drug of antidiabetic class; Metformin as a development of the assay method. The mobile phase consisting of water and methanol in the ratio of 50: 50 at wavelength 239 nm, column C18 (phenomenex) and the flow rate 1 mL $min^{-1}$and retention time 2.6 mins. A simple, rapid, accurate and stability indicating HPLC method was developed which is economic, sensitive and time saving than other chromatographic procedures. It is user-friendly and importance tool for analysis of combined dosage form. The developed method was successfully applied to the determination of metformin in pharmaceutical formulations.

KEYWORD: HPLC, Antidiabetic Drugs, Metformin etc.

INTRODUCTION

High performance liquid chromatography (HPLC) is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying or purifying the individual components of the mixture. Before the

invention of HPLC, chemists had column chromatography at their disposal, and column chromatography was time consuming.[1] HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational Pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column.[2] Also HPLC columns are made with smaller adsorbent particles (2–50 μm in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique.[5] It relies on pumps to pass pressurized liquid solvent containing the sample mixture through a column. filled with a solid adsorbent material.[3] HPLC is distinguished from traditional. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter, and 30–250 mm length. Also HPLC columns are made with smaller adsorbent particles (2–50 μm in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique. Chemists, wanting to speed the separation process up, first experimented with the introduction of a vacuum source or a high pressure source. However, they found with the increased negative or positive pressure, the column length would have to be increase linearly in order to acquire a valid separation that could be used for analytical data with a high confidence level. Chemists realized that with the development of pressurized systems, reducing the particle size would increase the efficiency.[4] Today, HPLC has many uses including medical (e.g. detecting vitamin D levels, urine sample, biological sample of synthetic substance) and the solvent reservoir to the pump, where it is becomes highly pressurized. The prepared sample is then injected into the line, where it travels with the solvent into the HPLC column. The detector detects when these molecules detach from the silica and reports the data in the form of a chromatogram.[5] The mixture moves through the column at varying velocities and interact with the sorbent, also known as the stationary phase. The velocity of each component in the mixture depends on its chemical nature, the nature of the column and the composition of the mobile phase. The time at which a specificanalyte emerges from the column is termed as its retention time. The retention time is measured under specific conditions and considered as the identifying characteristic of a given analyte. The composition of the mobile phase is either maintained as a constant or as varied during the chromatographic analysis. The composition of the mobile phase is either maintained as a constant or as varied during the chromatographic analysis.[6] The constant approach is effective for the separation of the sample components that are not very dissimilar in their

affinity for the stationary phase. In the varied approach, the composition of the mobile phase differs from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times where high eluting strength produces fast elution. The composition of the mobile phase is chosen based on the intensity of interactions between several sample components and the stationary phase.[7] The HPLC partitioning process is quite similar to the liquid-liquid extraction process except that the former is a continuous process unlike the latter which is a step-wise process. It is recommended that trial partitioning processes be performed to determine the exact HPLC method that would provide adequate separation.[8]

METHOD'S OF HPLC

High-performance liquid chromatography or high-pressure liquid chromatography (HPLC) is a chromatographic method that is used to separate a mixture of compounds in analytical chemistry and biochemistry so as to identify, quantify or purify the individual components of the mixture.[9] Analytical method development is considered as a critical process in pharmaceutical availability of the different type of column, operating parameters, mobile phase composition, diluent and pH value make it critical to develop an analytical methods. A good analytical methods should be simple, used column, mobile phase and buffer should be common. It can be done easily step by step.

Following are the common methods for development steps

1. Selection of HPLC analytical methods
2. Selection of chromatographic condition
3. Parameter optimization

During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C18column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point. A column is of course, the starting and central piece of a chromatograph. A appropriately selected column can produce a good chromatographic separation which provides an accurate and reliable analysis. An improperly used column can often generate confusion, inadequate, and poor separations which can lead to results that are invalid or complex to interpret. The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Choosing the best column for application requires consideration of stationary phase chemistry, retention capacity, particle size, and

column dimensions.[10] The three main components of an HPLC column are the hardware, the matrix, and the stationary phase. There are several types of matrices for support of the stationary phase, including silica, polymers, alumina, and zirconium. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One short coming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography. Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds. Commonly used reverse phase columns and their uses are listed below.[7] Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) and peptides with hydrophobic residues, and other large molecules. C3–C5columns generally retain non-polar solutes more poorly when compared to C8 or C18 phase.[8] These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8, MOS) phases have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids. Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible.[8] The separationselectivity election of Chromatographic mode: chromatographic modes based on the analyte's molecular weight and polarity. All case studies will focus on reversedphase chromatography (RPC), the most common mode for small organic molecules. Ignitable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents. Optimization of Mobile phase: Buffer Selection: Different buffers such as potassium phosphate, sodium phosphate and acetate were evaluated for system suitability parameters and overall chromatographic performance.[9] Effect of pH.:- If analytes are ionisable, the proper mobilephase pH must be chosen based on the analyte pKa so the target analyte is in one predominate ionization state, ionized or neutral. Effect of organic modifier: - Selection of the organic modifier type is relatively simple in reverse phase HPLC. The usual

choice is between acetonitrile and methanol (rarely THF).[10] Gradient elution is usually employed with complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. Selection of detector and wavelength: After the chromatographic separation, the analyte of interest is detected by using suitable detectors. Some commercial detectors used in LC are: ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis.[11]

INSTRUMENTION OF HPLC

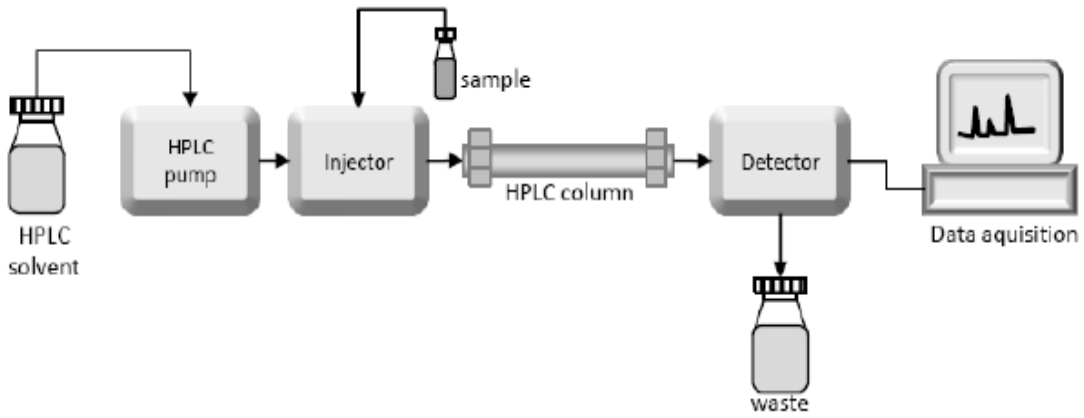


Figure 1: Block diagram of HPLC.

Block Diagram of an HPLC

Solvent mixture travels through capillary tubes, from the solvent reservoir to the pump, where it is becomes highly pressurized. The pump is also used to control the flow rate of the mobile phase substance, which is typically measured in mL/minute. The prepared sample is then injected intothe line, where it travels with the solvent into the HPLC column. There are many different columns you can choose from, depending on the sample you want analyzed. As the solvent moves through the column, molecules from your sample will stick to the silica in the column and detach at different times, making them distinguishable from one another. The detector detects when these molecules detach from the silica and reports the data in the form of a chromatogram. Various types of detectors can be used such a UV-VIS, fluorescence, or an evaporative-light scattering detector (ELSD). Once the solvent has traveled through the column it goes into a waste container, or can be collected.[13] The parameters of the HPLC, like any instrument, are important and are dependent on your sample. The solvent mixture, containing a strong solvent and a weak solvent, will depend on

whether your sample is polar in nature or not. Common solvents include water, methanol and acetonitrile. Two different solvent methods can be use, isocratic or gradient. With isocratic, the solvent mixture stays the same, 50:50 for example. With a gradient, the solvent will start with a 100:0 ratio of weak solvent: strong solvent and increase in increments over time to the final.[13,14]

The basic HPLC system consists of the following parts: 1. Solvent or mobile phase reservoir 2. High pressure pump 3. Injector 4.Column5. Detector 6. Data recording and interpretation unit.

1. Solvent or Mobile phase reservoir: The solvents or buffers mixture of solvents and buffers in the form of homogenous mixture are stored in solvent reservoir and are allowed to enter the mixing chamber through mechanical pump via flowing tube reservoirs mainly made up of glass bottled with properly covered.

2. High pressure pump: Different types of pumps are used in high pressure liquid chromatography which can be classified as direct gas pressure pump, pneumatic intensifier pump, reciprocating pump and syringe pumps. only two types of pumps are employed in HPLC equipments, one is mechanical controlled which delivers mobile phase at a constant flow rate and another one is pneumatic controlled which provides and works at constant pressure. In mechanical pumps, the most frequently used is the reciprocating piston type in which a motor or mechanical driven which drives sapphire plunger into small liquid end chamber to force out the mobile phase. In the meantime the check valves control the flow of the solvent into and out of the liquid end which prevents back flow of liquid. Because of the continuous flow by means of pulses the plunger moves constantly in and out, during these movements there is a possibility of pressure fluctuation which may cause upset of 17 baseline during analysis. In case of pneumatic pumps may be either the gas displacement type which uses direct pressure from a highly compressed gas to force solvent out of a tube or the pneumatic amplifier type which compress gas at a lower pressure impinges on the large end of a piston to force the smaller end to deliver the liquid. The amplification of pressure is proportional to the ratio of the areas of the two ends of the piston.

3. Injector: Injector is mainly used to inject the sample components either in the form ofliquid or gaseous form into the column compartment which allows mixing up with mobile phase and gets detected. There are two types of injectors, one is manual injector using

Injection valves and which is called Rheodyne injector and another one is fully automated electronically controlled device. The manual injector operates by loading sample loop by means of glass or plastic syringes while the mobile phase is pumped into the column. In case of automated injector, devices control automatically number of injections, injection volumes and time gap between injections which ensures the reproducibility of injections. In auto injector the volume of injections can be modified at any time depends upon requirements.

4. **Columns:** The column is the heart of a High pressure liquid chromatography system and its importance needs to elaborate. HPLC columns are either polymers or silica based. Silica based packings are the most popular and often used systems. The chemical natures ofthese packing vary with the stationary phase; their physical characteristics are similar.[12a] The average particle diameter of the packing's is between 3 to 10 urn with a narrow size distribution[13], These are produced by grinding and sizing of the irregularly shaped, bulk manufactured silica; the HPLC columns packings should be based on spherical silica 18 particles having good mechanical strength and a narrow size distribution. The bulk particles are usually precipitated from silica solutions in the presence offoraldehyde and urea at a pH of 2.[14a] The columns of smaller particles permit faster separation than columns of larger particle size. Higher strength particles provide columns that exhibit lower back pressures long lifetime.[15] Small molecules are usually chromatographed on particles having 5-12 nm pore size while large molecules are usually separated with particles having a pore size of 12 to 30 nm, e.g., proteins are usually separated on 30 nm pore size particles.[16] The silica surface contains various kinds of SiOH (silanol) groups and those particles heated at high temperatures, e.g., 800°C, are devoid of such groups and hence cannot be used for columns. Individual silanols exist in three general types. The SiOH groups on a fully hydroxylated surface occur mainly in the hydrogen- bonded form.[17] The second type contains hydroxylated and free silanol and the third type contains very few silanol sites. Free silanols are undesirable for the separation of the basic molecules. The addition of appropriate alkali can eliminate problems of secondary interactions between the basic compounds and acidic silanols (e.g., triethylamine). The secondary reaction between the acidic solutes can also be prevented by the addition of small amount of carboxylic acid into the mobile phase.[18] A more detailed discussion of silanol related problems and their correction is given in reference.[19] Most bonded phase silica packings are made surface reacted.

Organosilanes. Various alkyl and alkyl substituted silica are made with this reaction. A few packings use a polymerized surface layer that results from the reaction of di or tri functional silanes with silica particles. The stability of the bonded phase is important in HPLC reproducibility. Long chain alkyl bonded phase packings (e.g Cg or Cjg) generally are more stable than monomeric phases (e.g, diols). End capping is often used to more completely bond (silanize) packings and consists of a subsequent reaction with trimethylchlorosilane or hexamethyldisilanize, to increase the cover support and to 19 minimize unwanted reaction with free silanols.[20,21] Reversed phase separations can be made using polymer (polystyrene) particles, which are spherical and porous. Column specifications should specify particle size, length, internal diameter etc. Normal columns are 3-25 cm in length and internal diameter of most analytical columns is 0.4-0.5 cm. Micro-bore column (0.1-0.2 cm i.d) are used for interfacing with detectors such as mass spectrometer.[22] The column plate number N is the single most important characteristic of a column and defines the ability of the column to produce sharp, narrow peaks and to achieve good resolution for band pairs with small a values. N depends on the particle size and is usually expressed per meter length of the column. The values of N for a 25 cm column should be approximately 10000. The shape of the peaks produced by the column is equally important. The peak symmetry should be between 0.9 to 1.1. Retention time reproducibility or consistency in RRT is the criterion for a good column. Knox and Parcher[23] report that the most efficient chromatography can be expected ifthe injected solution is introduced centrally at the top or inlet of the column.

Types of column use in HPLC

1. Normal Phase Columns for Students in HPLC Training

The normal phase column uses polarity to separate the compounds of the sample. In normal phase chromatography, the stationary phase is polar and the mobile phase is nonpolar. Once the sample reaches the column, its least polar compounds will separate first and its most polar compounds will separate last. because each compound is extracted at different times.

2. Reverse Phase Columns for Students in HPLC Courses

The reverse phase column is very similar to normal phase, but the polarities are reversed. In this type of column, the stationary phase is nonpolar and the mobile phase is polar. The reverse phase column is the most commonly used method and offers a range of testing options for students in HPLC courses. Due to its great range of testing options, students can use the reverse phase column to test a wide variety of samples.

3. Ion Exchange Columns for Students in HPLC Training

The ion exchange column uses either cationic or anionic charged ions. Cationic have a net positive charge of ions and anionic have a net negative charge. This will affect the way the sample responds and the lab technician will likely choose which charge to use based on the sample they are testing. When the mobile phase and sample enter the column, the sample will begin to respond to the charged ions.

5. Size Exclusion Columns for Students in HPLC Courses

Unlike the other columns, size exclusion columns do not rely on the interaction of mobile phase, stationary phase, and sample to separate the sample. The sample is separated when it's filtered through a material that has different sized pores. The pores are made up of mesopores and micropores. Mesopores have a diameter between two and 50 nanometres, while the micropores have a diameter less than two nanometres. Based on each compounds' ability to pass through the varying sized pores, they will separate at different rates. Larger compounds will pass more quickly, because they will avoid the holes, while smaller compounds that pass through the pores will take longer. Size exclusion columns are typically used to test proteins and carbohydrates.

DETECTORS

The most commonly used detectors in LC are concentration sensitive. The detector output signal is a function of the concentrations of the analytes passing through the detector cell. In order to use the information for quantitation, the detector must respond linearly to changes in concentration over a wide concentration range, which is called the linear dynamic range of the detector.[23] Criteria for the evaluation of the quality or the suitability of the detector are as follows: the magnitude of the linear dynamic range, the noise level, the sensitivity, and the selectivity. The sensitivity is determined by the specific characteristics of the analytes and by the extent to which these differ from the characteristics of the sample matrix. The most important parameters are noise, drift, detection limit (sensitivity), selectivity, stability, and compatibility with various elution modes

UV-VISIBLE DETECTORS

The UV-visible absorbance detector is the most common HPLC detector in use today since many compounds of interest absorb in the UV (or visible) region (from 190–600 nm). Sample concentration, output as absorbance, is determined by the fraction of light transmitted through the detector cell by Beer's Law.

A ¼ logðI0=IÞ ¼ Ebc

Where, A is absorbance, I0 is the incident light intensity, I is the intensity of the transmitted light, e is the molar extinction coefficient of the sample, b is the path length of the cell in cm, and c is the molar sample concentration.[24] UV absorbance occurs as a result of the transition of electrons from p! p, n ! p, or n !r molecular orbitals; most aromatic compounds absorb strongly at or below 260 nm, compounds with one or more double bonds (e.g., carbonyls, olefins) at 215 nm, and aliphatic compounds 205 nm.

There are three different types of UV detectors.

FLUORESCENCE DETECTORS

Fluorescence detectors (FL) measure the optical emission of light by solute molecules after they have been excited at a higher energy wavelength and can be very sensitive for compounds that have native fluorescence or that can be made to fluoresce through derivatization. Schematically, they resemble Figure 4, except that the grating is replaced by a filter or monochromator at a right angle to the incident light to simplify the optics and reduce background noise. The light source is usually a broad spectrum deuterium or xenon flash lamp. The excitation wavelength (often close to the UV kmax) is selected by a filter or monochromator between the lamp and the flow cell, always at a higher energy (lower wavelength) than the emission wavelength. To compare the two vectors A and B, angle H is calculated after the spectra are reduced point by point to vectors. If vectors A and B are totally different, H ¼ 90. If A and B totally identical detectors can be as much as 100 times more sensitive than a UV detector, making them particularly useful for trace analyses, or in sample limited or low concentration sample situations.

ELECTROCHEMICAL DETECTORS

For compounds that can be oxidized or reduced the electrochemical (EC) detector is one of the most sensitive and selective HPLC detectors available.[25,26] EC detectors require the use of electrically conductive HPLC mobile phases (buffers suffice) and, when properly used and maintained, are the standard bearer when it comes to response levels for the HPLC analysis of compounds such as catecholamines and neurotransmitters. EC detectors for HPLC usually contain three separate electrodes; a working, a counter (auxiliary), and a reference electrode. Common electrode materials are carbon, gold, silver, or platinum. A fixed potential difference is applied between the working electrode and the reference electrode to drive an

electrochemical reaction at the working electrode's surface. Current produced from the electrochemical reaction as compounds are oxidized or reduced at the working electrode is balanced by a current flowing in the opposite direction at the counter electrode. The EC detector response output is the amplified current resulting from the electrochemical reaction at the working electrode. Amperometric EC detectors use disk type thin-layer electrodes operated at a constant potential. They exhibit low conversion efficiencies due to diffusion limitations and are only capable of measuring 5–10% of an electrochemically reactive analytes; however, in spite of the low conversion efficiencies, they are still quite sensitive. Amperometric EC detectors can be run in a pulsed mode (referred to as pulsed amperometric detection, or PAD) where a cycled potential is used to clean and restore the electrode surfaces several times a second, prior to measuring the signal output. PAD's are the detector of choice when sensitive carbohydrate measurements are required. Coulametric EC detectors overcome the limited conversion efficiencie

RADIOACTIVITY DETECTORS

Radioactivity detectors (sometimes referred to as radiometric or radio-flow detectors) are used to measure radioactive analytes as they elute from the HPLC column. Most radioactivity detectors are based on liquid scintillation technology to detect phosphors caused by the radioactive nuclides such as low-energy b-emitters. the most common. A liquid scintillator can be added post column (called homogeneous operation) or the flow cell can be packed with beads of a permanent solid state scintillator (heterogeneous operation). The radioactivity detector can be very sensitive and is extremely useful for the detection of radiolabeled compounds in toxicological, metabolism, or degradation studies. Large flow cell volumes are typically used in radioactivity detectors to increase analyte residence time, which increases the number of radioactive decays that can be detected. Peak tailing and broadening caused by the larger cell volume can be minimized by using larger volume columns assuming that sufficient sample is available for larger mass injections to compensate f Analysis of galactosamine impurities in heparin according to the USP monograph. Separation A is from a standard solution; B from a digested spiked heparin sample. Peak 1 is galactosamine (1% level w=w), peak 2 is glucosamine. Additional method.[25,26]

CONDUCTIVITY DETECTION

The conductivity detector is a bulk property detector that measures the conductivity of the mobile phase. Conductivity detectors are the detector of choice for ion chromatography or ion

exchange separations when the analyte does not have a UV chromophore. In a conductivity detector, the resistance (or strictly the impedance) between two electrodes in the flow cell is measured. For many applications, particularly ion chromatography, where conductive buffers are required in the mobile phase, a suppressor column is used post-analytical column (before the detector) in order toreduce the background conductance of the mobile phase.

CHEMILUMINESCENT NITROGEN DETECTOR

The chemiluminescent nitrogen detector is an element specific detector where the column effluent is nebulized with oxygen and a carrier gas of argon or helium and pyrolyzed at 1050C.[27,28] Nitrogen containing compounds (except N2) are oxidized to nitric oxide, which is then mixed with ozone to form nitrogen dioxide in the excited state. The nitrogen dioxide decays to the ground state with the release of a photon, which is detected by a photometer. The resultant signal is directly proportional to the amount of nitrogen in the original analyte. Because of this relationship

CHIRAL DETECTORS

Many compounds, particularly drugs, exist in enantiomeric forms that can possess significantly different pharmacological properties, and chromatographic separation of enantiomers can be complimented by the use of detectors capable of responding to the different chiral forms. Chiral detectors in flow cell form essentially mimic their bench top counterparts; polarimeters (PL), optical rotary dispersion (ORD), and circular dichrosm detectors (CD).

REFRACTIVE INDEX DETECTION

The refractive index (RI) detector is a universal bulk property detector, and is the original, oldest LC detector. RI detectors measure the difference in optical refractive index between mobile phase and the sample; no chromophore on the solute molecule is required. For this reason, RI detection has been used very successfully for the analysis of sugars, triglycerides, and organic acids. The most common RI detector design is the deflection refractometer where the light from a tungsten source lamp is directed through a pair of wedge-shaped flow cells, (reference and sample). The reference cell contains trapped or static mobile phase and the column effluent is sent through the sample cell. As the light passes through the two detector cells it is refracted differently, measured by a pair of photodiodes that convert the signal to a measurable output voltage. Modern RI detectors use thermostatted flow cells due to the susceptibility of RI measurements to temperature fluctuations.[29]

LIGHT SCATTERING DETECTORS

Recent improvements in the ability to efficiently nebulize an HPLC column effluent has lead to increased utility of light scattering detectors. The most popular detector of this type is the evaporative light scattering detector (ELSD). The ELSD works on the principle of evaporation (nebulization) of the mobile phase followed by measurement of the light scattered by the resulting particles. The column effluent is nebulized in a stream of nitrogen or air carrier gas in a heated drift tube and any nonvolatile particles are left suspended in the gas stream. Light scattered by the particles is detected by a photocell mounted at an angle to the incident light beam. Carrier gas flow rate and drift tube temperature must be adjusted for whatever mobile phase is used. Detector response is related to the absolute quantity of analyte present, and while decreased sensitivity will be obtained for volatile analytes, unlike the UV detector, no chromophores are required and it has orders of magnitude more response than the RI detector.[30,31,32] ELSD also has the advantage over RI detection in that the response is independent of the solvent, so it can be used with gradients, and is not sensitive to temperature or flow rate fluctuations. Mobile phases of course must be volatile, similar to those used for MS detection. Linearity can be limited in some applications, but is certainly quantitative over a wide enough range if properly calibrated. Recent applications of the ELSD have also been extended to UHPLC; an example separation comparing low wavelength UV to ELSD (and another example of the advantages of orthogonal detection) for the separation of some antibiotics.

CORONA DISCHARGE DETECTION

Corona charged aerosol detection (CAD), sometimes referred to as corona discharge detection(CDD) is a unique technology gaining in popularity in which the HPLC column eluent is first nebulized with a nitrogen (or air)carrier gas to form droplets that are then dried to remove mobile phase, producing analyte particles.[33,34] The primary stream of analyte particles is met by a secondary stream of nitrogen (or air) that is positively charged as a result of having passed a high-voltage, platinum corona wire. The charge transfers diffusionally to the opposing stream of analyte particles and is further transferred to a collector where it is measured by a highly sensitive electrometer, generating a signal in direct proportion to the quantity of analytepresent.

A simplified schematic of how the CAD works is illustrated. Because the entire process involves particles and direct measurement of charge, CAD is highly sensitive, provides a

consistent response, and has a broad dynamic range, which offers some real advantages, particularly when analyzing compounds lacking UV chromophores.[35,36] Often compared to other universal- type HPLC detectors, like RI and ELSD, CAD has been shown to be much easier to use, and unlike RI, can accommodate gradients. In addition, CAD response is not dependent upon the chemical characteristics of the compoundsof interest, but on the initial mass concentration of analyte.

TYPE OF HPLC

1. Reversed-phase chromatograph
2. Normal-phase and adsorption chromatography
3. Ion exchange chromatography
4. Size exclusion chromatography
5. Bio-affinity chromatography

Ion-exchange chromatography

The stationary bed 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 size exclusion chromatography the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. Mainly for historical reasons, this technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a "gel". Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography.

Normal phase chromatography

The stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Reversed-phase chromatography

The inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

ADVANTAGES OF HPLC[37]

1. Speed, Efficiency and Accuracy

Compared to other chromatographic techniques, such as TLC, HPLC is extremely quick and efficient. It uses a pump, rather than gravity, to force a liquid solvent through a solid adsorbent material, with different chemical components separating out as they move at different speeds. The process can be completed in roughly 10 to 30 minutes, and it delivers high resolution. It is accurate and highly reproducible. Because it is largely automated, basic HPLC runs can be performed with minimal training.

2. Cost and Complexity

Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics. Techniques such as solid phase extraction and capillary electrophoresis can be cheaper and even quicker, especially for analysis under good manufacturing practice. Although it is relatively easy to use existing HPLC methods, it can be complex to troubleshoot problems or to develop new methods. This is largely because of the array of different modules, columns and mobile phases.

3. Sensitivity and Resolution

In general, HPLC is versatile and extremely precise when it comes to identifying and quantifying chemical components. With many steps involved, the precision of HPLC is largely down to the process being automated and therefore highly reproducible. HPLC does have low sensitivity for certain compounds, and some cannot be detected as they are irreversibly adsorbed. Volatile substances are better separated by gas chromatography.

DISADVANTAGE OF HPLC

1. Coelution

Because of the speed of HPLC and it reliance on different polarities of compounds, two compounds with similar structure and polarities can exit the chromatography apparatus at the same time or nearly the same time. This is known as coelution. Coelution makes determining exactly which portion of the mixture eluted at what point difficult.

2. Adsorbed Compounds

HPLC typically uses a glass column filled with beads made of different materials. The mixtures being forced through the column have chemicals that bind with different strengths to the beads. The strength of the binding, which depends on the Somecompounds bind so strongly that they are essentially never released from the beads in the column and are never measured in the solution exiting the column..

3. Cost

Typical laboratory separation techniques involve developing an assay, or method of separation, and then implementing that assay to separate individual compounds from a solution. However this usually results in multiple solutions that also need to undergo procedures, leading to an exponential increase in complexity. Although HPLC can often simplify and speed up this process, the cost of developing an HPLC apparatus can become tremendous.

4. Complexity

HPLC is not only used to separate simple compounds, it is also used to isolate specific proteins out of a cellular mixture. In this case the beads in the column are usually coated with an antibody specific to the protein you need to collect. The proteins bind the the antibodies and the remaining solution is passed through the column, then the proteins are released using another solution and collected. This requires a highly skilled technician to monitor the column at all times and make sure that the process is running exactly as planned.

APPLICATIONS OF HPLC

In the pharmaceutical industry, the qualitative type of HPLC analysis is widely used. In the research and development wing, both qualitative and quantitative methods are employed.

1. In quality control, it is used to check if the manufactured products comply with the specified standards. These specific standards are fixed by the pharmacopeias and other drug regulating bodies. The guidelines mentioned in the pharmacopeia will give an idea of how the peak of the drug in the formulation should look, when run with specified HPLC mobile phases are used. If the peaks do not correspond to those shown in pharmacopeia, the batch cannot be passed for quality check.[38]

2. In R & D, it is used to identify the specific molecule or component in the 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 some time is tested in bioavailability studies. Then the sample released is taken and injected into the 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 drug formulations like injections, solutions, dissolved form of solid dosage forms are injected into HPLC injector to record the peaks of the individual constituents.

3. Also, any new molecule under development or in a 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, bioavailability, etc. of the formulation or chemical moieties under development.

4. In plant constituents, there are many molecules with similarity in chemistry like isoflavones, glycosides, saponins, etc. but the different activity or nutritional value. These compound can't be precisely determined by other methods. Hence they are determined by HPLC analysis through separation into individual components and thereby identification.

5. This method of chromatography finds vast use in.

1. Clinical diagnosis of diseases, disorders.
2. In scientific research for discovery.
3. In pharmaceutical labs for analysis.
4. In the food industry for quality control.
5. For standards control by government.
6. For separation of similar molecules.[38]

VALIDATION OF HPLC

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.

The validation scope, boundaries and responsibilities for each process or groups of similar processes or similar equipment's must be documented and approved in a validation plan.

These documents, terms and references for the protocol authors are for use in setting the scope of their protocols.[39] It must be based on a Validation Risk Assessment (VRA) to ensure that the scope of validation being authorised is appropriate for the complexity and importance of the equipment or process under validation. Within the references given in the VP the protocol authors must ensure that all aspects of the process or equipment under qualification; that may affect the efficacy, quality and or records of the product are properly qualified. Qualification includes the following steps.

Selectivity and Specificity Selectivity of the analytical method is defined as the degree to which a method can quantify the analyte in the presence of inerferents.[40] The other components which may be present include impurities, degradants, matrix, etc. The term specificity and selectivity is often used interchangeably. 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 that provides responses for a number of chemical entities that may or may not be distinguished from each other. The International Union of Pure and Applied Chemistry (IUPAC) have expressed the view that "Specificity is the ultimate of Selectivity'. The IUPAC discourages use of the term specificity and instead encourages the use of the term selectivity.[41]

Precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability is the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision. It is assessed by making six sample determinations at 100% concentration or by preparing three samples at three concentrations in triplicates covering the specified range for the procedure. It involves repeated determination of same sample. Intermediate precision expresses within laboratories variation: different days, different analyst, different equipments, etc. It is the term synonymous with the term 'ruggedness', defined by USP. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. To study intermediate precision, use of an experimental design is encouraged.

Range: Range of an analytical method is the interval between the upper and lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range is normally derived from the linearity studies and depends on the intended application of the procedure. The following minimum specified ranges should be considered precision is generally studied by multiple preparations of sample and standard solution.

Accuracy: The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value found. Practically no measurement process is ideal, therefore, the true or actual value cannot be exactly known in any particular measurement. The accepted true value for accuracy assessment can be assessed by analyzing a sample with known concentration. The accuracy studies are usually carried out by determining the recovery of the spiked sample of analyte into the matrix of the sample (a placebo) or by comparing the result to the results of a certified reference material of known purity. If the placebo of the sample is not available, the technique of standard addition is used. In case of methods for quantitation of impurities, the sample with known amount of impurities is assessed. Accuracy should be assessed using minimum of nine determinations over a minimum of three concentration levels covering the specified range (for e.g., three concentrations/ three replicates each of the total analytical procedure.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is partially evaluated during method development stages. The aim of the robustness study is to identify the critical operating parameters for the successful implementation of the method. These parameters should be adequately controlled and a precautionary statement included in the method documentation. In case of an HPLC method, robustness study involves method parameters like pH, flow rate, column temperature and mobile phase composition which are varied within a reasonable range. The system suitability parameters obtained for each condition are studied to check the parameter which significantly affects the method.

System suitability System: suitability testin (SST) is an integral part of many analytical procedures. The tests are based on the concept that the equipment, analytical operations and

samples are the integral part of the system that can be evaluated as such. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis.

DIABETIES MELLITUS (DM)

Diabetes mellitus commonly referred to as diabetes, is a group of metabolic disorders in which there are high blood sugar levels over a prolonged period. Symptoms of high blood sugar include frequent urination, increased thirst, and increased hunger. If left untreated, diabetes can cause many complications. Acute complications can include diabetic ketoacidosis, hyperosmolar hyperglycemic state, or death. Serious long-term complications include cardiovascular disease, stroke, chronic kidney disease, foot ulcers, and damage to the eyes. Diabetes is due to either the pancreas not producing enough insulin, or the cells of the body not responding properly to the insulin produced. There are three main types of diabetes mellitus:

Type 1 DIABETIES MELLITUS

It results from the pancreas' failure to produce enough insulin due to loss of beta cells. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes".

Type 2 DIABETIES MELLITUS

It begins with insulin resistance, a condition in which cells fail to respond to insulin properly. As the disease progresses, a lack of insulin may also develop. This form was previously referred to as "nonInsulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". The most common cause is a combination of excessive body weight and insufficient exercise.

Gestational diabetes is the third main form, and occurs when pregnant women without a previous history of diabetes develop high blood sugar levels.

CLASSIFICATION OF ANTIDIABETIC DRUGS BELONG CLASS TWO DRUG

Class	Mechanism of action	Side effects	Contraindications
Biguanide (metformin)	• Enhances the effect of insulin	• Lactic acidosis • Weight loss • Gastrointestinal complaints are common (e.g. diarrhea, abdominal cramps) • Reduced vitamin B12absorption	• Chronic kidney disease • Liver failure • Metformin must be paused before administration of iodinated contrast medium and major surgery.
Sulfonylureas (e.g., glyburide, glimepiride)	• Increase insulin secretion from pancreaticβ-cells	• Risk of hypoglycemia • Weight gain • Hematological changes: agranulocytosis, hemolysis	• Severe cardiovascular comorbidity • Obesity • Sulfonamide allergy • (particularly long-acting substances)
Meglitinides (nateglinide, repaglinide)	• Increase insulin secretion from pancreaticβ-cells	• Risk of hypoglycemia • Weight gain	• Severe renal or liver failure
DPP-4 inhibitors (saxagliptin, sitagliptin)	• Inhibit GLP-1 degradation → promotes glucose-dependent insulin secretion	• Gastrointestinal complaints • Pancreatitis • Headache, dizziness • Arthralgia	• Liver failure • Moderate to severe renal failure
GLP-1 agonists (incretin mimetic drugs: exenatide, liraglutide, albiglutide)	• Direct stimulation of the • GLP-1 receptor	• Nausea • Increased risk of pancreatitisand possibly pancreatic cancer	• Preexisting, symptomatic gastrointestinal motility disorders
SGLT-2 inhibitors(canagliflozin, dapagliflozin, empagliflozin)	• Increased glucosuria through the inhibition of SGLT-2 in the kidney	• Genital yeast infections and urinary tract infections • Polyuria and dehydration • Diabetic ketoacidosis	• Chronic kidney disease • Recurrent urinary tract infections
Alpha-glucosidase inhibitors(acarbose)	• Reduce intestinal glucose absorption	• Gastrointestinal complaints (flatulence, diarrhea, feeling of satiety)	• Any preexisting intestinal conditions (e.g., inflammatory bowel disease) • Severe renal failure

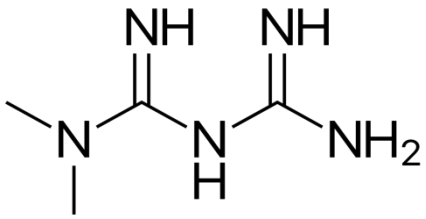
Thiazolidinediones(pioglitazone)	• Reduce insulin resistance through the stimulation of PPARs (peroxisomeproliferator-activated receptors) • Increase transcription of adipokines	• Weight gain • Edema • Cardiac failure • Increased risk of bone fractures (osteoporosis)	• Congestive heart failure • Liver failure
Amylin analogs (pramlintide)	• Reduce glucagon release • Reduce gastric emptying • Increase satiety	• Risk of hypoglycemia • Nausea	• Gastroparesis

HPLC OF ANTIDIABETIC DRUG

METFORMIN

Metformin and its derivatives have been widely used for a long time for the treatment of type 2 diabetes mellitus. It lowers both basal and postprandial (i.e. after eating a meal) plasma glucose and is not chemically or pharmacologically related to any other classes of oral antihyperglycemic agents.[43]

A biguanide hypoglycemic agent used in the treatment of non-insulin-dependent diabetes mellitus not responding to dietary modification. Metformin improves glycemic control by improving insulin sensitivity and decreasing intestinal absorption of glucose.[44] Metformin is a first line agent for the treatment of type 2 diabetes that can be used alone or incombination with sulfonylureas, thiazolidinediones or other hypoglycemic agents. Metformin has not been linked to serum enzyme elevations during therapy and is an exceeding rare cause of idiosyncratic clinically apparent acute liver injury.[44]



METFORMIN[43]

MOLECULAR FORMULA:- $C_4H_{11}N_5$

MOLECULARWEIGHT :- 129.167 g/mol

IUPAC NAME :- 3-(diaminomethylidene)-1,1-dimethyl

MELTING POINT :- 223-226 °C

SOULUBILITY:- Freely soluble in water; slightly soluble in alcohol.

Practically insoluble in ether, chloroform, acetone, methylene chloride.[44]

CONTENT	RANGE
1.Method	High Performance liquid chromatoghraphy (HPLC)
2.U V Detector(wavelength)	239nm
3.Flow rate	1 mL min−1
4.Column	Column C18(phenomenex)
5.Mobile phase	Methanol :water(50:50)
6.Retention time	2.6min

DISCUSSION AND RESULT

To the best of our knowledge, there has been no HPLC method developed to determine metformin concentration utilizing volume sizes as small as 0.1 mL, using a liquid–liquid extraction procedure with a column specifically designed for reverse phase chromatography (e.g. C18 column).

The retention of metformin was examined on C18 with mobile phases that consisted of a mixture of phosphate solutions and acetonitrile. Metformin retention was very close to the column dead volume and no separation could be achieved from endogenous components using such columns and mobile phase composition.

For the present method workup, the extractability of metformin from plasma was tested in recovery experiments methanol : water (50:50% v/v) mixture.

CONCLUSION

The innovative method displayed high calibers of sensitivity and selectivity for monitoring concentrations of Metformin based on a small sample volume. It was simple in terms of sample preparation and involved liquid-liquid extraction, which was rapid and accessible by the virtue of using a single C18 column. The method was reproducible and can be use for estimation of Metformin.

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