iJRASET
International Journal For Research in Applied Science and Engineering Technology

INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 10 **Issue:** V **Month of publication:** May 2022

DOI: https://doi.org/10.22214/ijraset.2022.42685

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 7.538
Volume 10 Issue V May 2022- Available at www.ijraset.com

A Review on Method Development, Validation, Optimization and Applications of HPLC

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Abstract: ***High-Performance Liquid Chromatography (HPLC) is a technique used to separate components from the mixture. The technology consists of two phases, the stationary phase, and the mobile phase. Component separation is based on differences in partition coefficients in two phases.***
The present review article focuses on the principle, types, instrumentation, process validation, application, advantages, disadvantages of HPLC. HPLC methods development and validation play important roles in the discovery, development, manufacture of pharmaceutical drugs, and various other studies related to humans and animals. An analytical procedure is developed to test a defining characteristic of the drug substance or drug product against established acceptance criteria for that characteristic. Validation of HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like Accuracy, precision, specificity, linearity, range, the limit of detection, the limit of quantification, robustness, and system suitability testing.
Keywords: HPLC, Types, Method validation, Method development, Instrumentation, and Applications.

I. INTRODUCTION

High-Performance Liquid Chromatography (HPLC) involves separations in which the components to be separated are distributed between two immiscible phases.

One of these phases is the mobile phase, and the other is a stationary phase[1,2]. High-Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It can separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High-performance liquid chromatography (HPLC) is the most accurate analytical method widely used for the quantitative as well as qualitative analysis of drug products[3]. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through the column with the help of gravity but in the HPLC technique, the solvent will be forced under high pressure up to 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities[4-9]. n HPLC, pumps will be used to pass pressurized liquid solvent including the sample mixture which is allowed to enter into a column filled with a solid adsorbent material. The interaction of each sample component will be varied and this causes the difference in flow rates of each component and finally leads to the separation of components of the column.

Chromatography can be depicted as a mass exchange process including adsorption. HPLC depends on pumps to pass a pressurized fluid and an example blend through a section loaded with adsorbent, prompting the partition of the specimen segments. The dynamic segment of the section, the adsorbent, is regularly a granular material made of solid particles (e.g. silica, polymers, etc.) 2 µm to 50 µm in size.

The segments of the example mixture/blend are isolated from each other because of their distinctive degrees of connection with the retentive particles. The pressurized fluid is commonly a blend of solvents (e.g. water, acetonitrile/methanol) and is known as the 'mobile phase'. Its organization and temperature play an important part in the partition procedure by affecting the connections occurring between sample segments and adsorbent[10-17].

HPLC is recognized from traditional ("low weight") liquid chromatography because operational pressures are fundamentally higher (50 bar to 350 bar), while normal liquid chromatography regularly depends on the power of gravity to pass the portable stage through the segment. Because of the small sample amount isolated in scientific HPLC, column section measurements are 2.1 mm to 4.6 mm distance across, and 30 mm to 250 mm length. Additionally, HPLC segments are made with smaller sorbent particles (2 µm to 50 µm in normal molecule size). This gives HPLC high determining or resolving power (the capacity to recognize components) while isolating mixtures, which makes it a prominent chromatographic method.

A. *Types of HPLC*

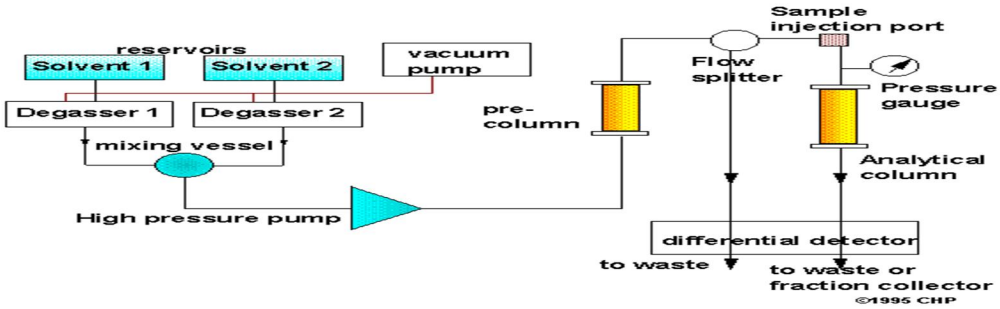
Depending on the substrate used i.e. stationary phase used, the HPLC is divided into the following types

1) *Normal Phase HPLC:* In this method, the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is hexane, chloroform, and diethyl ether. The polar samples are retained on column [58]
2) *Reverse Phase HPLC:* It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is nonpolar or hydrophobic. The more is the non-polar nature the more it will be retained
3) *Size-exclusion HPLC:* The column will be incorporated with precisely controlled substrate molecules. Based on the difference in molecular sizes, the separation of constituents will occur.
4) *Ion-exchange HPLC:* The stationary phase is having ionically charged surface opposite to the sample charge. The mobile phase used is an aqueous buffer that will control pH and ionic strength[18-27].

II. INSTRUMENTATION

The HPLC instrumentation involves a pump, injector, column, detector, integrator, and display system. In the column, the separation occurs. The parts include:

1) *Solvent Reservoir:* The contents of the mobile phase are present in a glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of a sample, the polar and non-polar solvents will be varied.
2) *Pump:* The pump suctions the mobile phase from the solvent reservoir and forces it to column and then passes to a detector. 40000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate, and composition of the mobile phase



Instrumentation of HPLC

3) *Sample Injector:* The injector can be a solitary infusion or a computerized infusion framework. An injector for an HPLC framework should give an infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).
4) *Columns:* The heart of the system is the column. To achieve high efficiency of separation, the column material (micro-particles, 5-10 µm size) is packed in such a way that the highest numbers of theoretical plates are possible. Silica (SiO2, H2O) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si-O-Si) in a rigid three-dimensional structure containing interconnecting pores. Thus a wide range of commercial products is available with surface areas ranging from 100 to 800 m2/g. and particle sizes from 3 to 50 µm
5) *Detector:* Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase. These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase before the detection. Solute property detectors that do not require the removal of the mobile phase before detection include spectrophotometric (UV or UV-Vis) detector, fluorescence detectors, polarographic, electro-chemical, and radio-activity detectors, whilst the moving wire flame ionization detector and electron capture detector both require removal of the mobile phase before detection. UV-Vis and fluorescent detectors are suitable for gradient elution because many solvents used in HPLC do not absorb to any significant extent
6) *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.[28-29].

III. METHOD DEVELOPMENT ON HPLC

A step involved in the method development of HPLC is as follows:

1) Understanding the Physicochemical Properties of a drug molecule.
2) Selection of chromatographic conditions.
3) Developing the approach of analysis.
4) Sample preparations
5) Method optimization
6) Method validation

A. *Understanding the Physicochemical Properties of Drug Molecules*

The Physicochemical Properties of a drug molecule play an important role in method development. For Method development, one has to study the physical properties like solubility, polarity, pKa, and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. The solubility of molecules can be explained based on the polarity of molecules. Polar, e.g. water, and nonpolar, e.g. benzene, solvents do not mix. In general, like dissolves like i.e., materials with similar polarity are soluble in each other. The selection of mobile phase or diluents is based on the solubility of an analyte. The analyte must be soluble in diluents and must not react with any of its components. pH and pKa play an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion. $pH = - \log10[H3O+]$

Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times.[30,31]

B. *Selection Of Chromatographic Conditions*

1) *Selection of Column:* 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, columns must be stable and reproducible.[32,33]
2) *Buffer Selection:* Choice of a buffer is governed by the pH that is desired. The typical pH range for reversed-phase on silica-based packing is pH 2 to 8. The buffer must have a pKa close to the desired pH since the buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value >2 units of the desired mobile phase pH.[34]
3) *Buffer Concentration:* Generally, a buffer concentration of 10-50 mm is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds.[35]
4) *Isocratic and Gradient Separations:* In this mode of separation includes constant eluent composition, which means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant. The peak capacity is low and the longer the component is retained on the column the wider is the resultant peak. Gradient mode of separation includes significantly increases the separation power of a system mainly due to an increase of the apparent efficiency (decrease of the peak width). Peak width varies depending on the rate of the eluent composition variation. In deciding whether a gradient or isocratic would be required an initial gradient run is performed and the ratio between the total gradient time and the difference in the gradient time between the first and last component is calculated. The calculated ratio is 0.25 gradient would be adequate. [36]
5) *Internal Diameter:* The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded into a column. [37]
6) *Particle Size:* Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles. These silica particles come in many sizes with 5 µm beads being the most commonly used. The smaller particles usually provide more surface area and better separations but the pressure required for the optimum linear velocity increases by the inverse of the particle diameter squared. Larger particles are used in preparative HPLC where column diameters are in the range of 5 cm to >30 cm and for non-HPLC applications such as solid-phase extraction. [38,39]

7) *Pore Size:* Pore size of column defines the ability of the analyte molecules to penetrate inside the particle and interact with its inner surface [40]

8) *Selection of Mobile Phase:* The mobile phase affects resolution, selectivity, and efficiency. Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. Acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205, and 212nm respectively. These solvents are miscible with water. A mixture of acetonitrile and water is the best initial choice for the mobile phase during method development. [41]

Mode	Solvent type used	Type of compound used
Reversed Phase	Water/Buffer, ACN, Methanol	Neutral or non-ionized compounds which can be dissolved in water/ organic mixtures.
Ion-pair	Water/Buffer, ACN, Methanol	Ionic or Ionizable compounds
Normal Phase	Organic solvents	Mixtures of isomers and compounds not soluble in Organic/ Water mixtures.
Ion exchange	Water/Buffer	Inorganic ions, proteins, nucleic acids, organic acids.
Size exclusion	Water, Tetrahydrofuran, chloroform	High molecular weight compounds.

7) *Selection of Detectors:* Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analyses, potential interference, the limit of detection required, availability, and/or cost of detector. The UV-visible detector is a versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Photodiode Array (PDA). The Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive index chromatographic and spectral sensitivity, stability, and reproducibility make this detector the ideal solution for the analysis of components with limited or no UV absorption. Multi-wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds. [42,43]

Detector	Type of compound can be detected
UV-Visible &Photodiode array	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system.
Conductivity detector	Charged compounds, such as inorganic ions and organic acid.
Electrochemical detector	For easily oxidized compounds like quinines or amines
Refractive Index detector & Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors, eg.polymers, saccharides.

C. Developing the Approach for Analysis

While developing the analytical method on RP-HPLC the first step which is followed is the selections of various chromatographic parameters like a selection of mobile phase, selection of column, selection of flow rate of mobile phase, selection of pH of the mobile phase. All of these parameters are selected based on trials and followed by considering the system suitability parameters. Typical parameters of system suitability are e.g. retention time should be more than 5 min, the theoretical plates should be more than 2000, the tailing factor should be less than 2, resolution between 2 peaks should be more than 5, % R.S.D. of the area of analyte peaks in standard chromatograms should not be more than 2.0 %.like other. Detection wavelength is usually an isosbestic point in the case of simultaneous estimation of 2 components. After this, the linearity of the drug is studied to know the range of concentrations up to which the drug follows the linear pattern. Analysis of the laboratory mixture is also carried out to know the practicability of the developed method for simultaneous estimation. After that analysis of marketed formulation is carried out by diluting the marketed formulation up to concentration range of linearity. [44-49]

D. Sample Preparation

Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column. The aim of sample preparation is a sample aliquot that, Is relatively free of interferences, Will not damage the column, and Is compatible with the intended HPLC method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column.[50]

E. Method Optimization

Identify the "weaknesses" of the method and optimize the method through experimental design. Understand the method performance with different conditions, different instrument setups, and different samples. [51]

F. Method Validation

Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for specific intended use are fulfilled. A process of evaluating method performance and demonstrating that it meets a particular requirement. In essence, it knows what your method is capable of delivering, particularly at low concentrations.

1) Scope of Process Validation

Validation is one of the wide and most difficult areas because it engaged in all levels of product manufacturing. The scope of validation in different fields follows Analytical, Instrument Calibration, Process Utility services, Raw materials, Packaging materials, Equipment, Facilities, Manufacturing operations, Product Design, Cleaning, Operators.

a) Importance of Validation: Assurance of quality, Time-bound, Process optimization, Reduction in rejections, Increased output, Avoidance of capital expenditures, Fewer complaints about process-related failures, Reduced testing in-process and in finished goods, More rapid and reliable start-up of new types equipment, Easier scale-up for development work.

b) Significance of Process Validation: Process validation is defined as "establishing documented evidence which provides a high degree of assurance that a specific system, related equipment, and process consistently meet the approved specifications and produce products meeting predetermined quality attributes.

Process validation is a basic factor for drug product safety and quality and thus a fundamental component of the quality assurance system used by pharmaceutical manufacturers. The basic principle of Quality Assurance is that a drug should be produced that is fit for its intended use. Effective Process Validation contributes significantly to assure the drug quality

2) Types of Process Validation

a) Prospective Validation: Prospective validation is defined as the establishment of documented evidence that a system does what it purports to do based on a pre-planned protocol. The objective of prospective validation is to prove or demonstrate that the process will work under a validation master plan or protocol prepared for pilot product trials. It is not limited to

- List of analytical methods, as appropriate.
- Proposed in-process controls with acceptance criteria
- Additional testing to be carried out, with acceptance criteria and analytical validation, as appropriate.
- Sampling plan.
- Methods for recording and evaluating results. Functions and responsibilities.

b) Retrospective Validation: Validation of such processes should be based on historical data. The steps involved require the preparation of a specific protocol and the reporting of the results of the data review, leading to a conclusion and a recommendation. The basis for retrospective validation is “Valid in-process specifications for such characteristics shall be consistent with drug product final specifications and shall be derived from previous acceptable process average and process variability estimates where possible and determined by the application of suitable statistical procedures where appropriate.”

Some of the essential elements are;

- Batch size/strength/manufacturer/year/period.
- Master manufacturing/packaging documents.
- Current specifications for active materials/finished products.
- List of process deviations, corrective actions, and manufacturing changes.

c) *Concurrent Validation:* In-process monitoring of critical processing steps and end-product testing of current production can provide documented evidence to show that the manufacturing process is in a state of control. It is similar to the perspective, except the operating firm will sell the product during the qualification runs, to the public as its market price.

d) *Revalidation:* It is the repetition of a validation process or a part of it. Some of the changes in revalidation are Changes in the source of active raw material manufacturer. Changes in packaging material Changes in the process Changes in the plant/facility.

3) *Various Approaches in Process Validation*

a) *Process Design:* The goal of this stage is to design a process suitable for routine commercial manufacturing that can consistently deliver a product that meets its quality attributes.

b) *Process Qualification:* This stage has two elements:

- Design of the facility and qualification of the equipment and utilities.
- Process performance qualification (PPQ).

c) *Continued Process Verification:* The goal of the third validation stage is continual assurance that the process remains in a state of control (the validated state) during commercial manufacture.

Three stages of lifecycle approaches are

- Product/process design and development
- Qualification of the commercial manufacturing equipment
- Maintenance of the process in a state of control during production.

d) *Validation protocols:* It is a written form that explains how the validation should conduct. Which is documented and used for the testing of various products, processes, etc?

4) *Protocol* ***as follow***s;

a) General information.

b) Objective.

c) Background/revalidation.

d) Summary of development and technology transfer (form R & D or another site activity to justify in-process testing and controls Any previous validations.

e) List of equipment and their qualification status.

f) Facilities qualification.

g) Process flow chart.

h) Manufacturing procedure narrative.[52-66]

IV. APPLICATIONS OF HPLC

HPLC has several applications in the fields of pharmacy, forensic, environment, and clinical. It also helps in the separation and purification of a compound.

1) Pharmaceutical Applications: The pharmaceutical applications include controlling drug stability, dissolution studies, and quality control.

2) *Environmental Applications:* Structure elucidation and Monitoring of unknown pollutants and detecting components of drinking water.

3) *Forensic Applications:* Analysis of textile dyes, quantification of drugs and steroids in biological samples.

4) *Food and Flavour Applications:* Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives.

5) *Clinical Applications:* Detecting endogenous neuropeptides, analysis of biological samples like blood and urine.

V. CONCLUSION

This review is based on HPLC which is an assertive analytical technique with sophisticated technologies that have been extensively practiced for decades. It is having several advantages. With the use of HPLC, one can produce extremely pure compounds. It can be used in both laboratory and clinical science.

With the use of HPLC the accuracy, precision, and specificity can be increased. The practice of HPLC is bygone limited to analyzers but is now widely performed by students, chemists, biologists, production workers, and other novices in academia, research, and quality control laboratories.

The review focuses on the types, instrumentation, process validation, applications of HPLC. The pump delivers the mobile phase from a reservoir and onto a column packing material that typically consists of 3-5 µm silica particles. Sample solutions are injected using pressure and leak-resistant injector onto the mobile phase just before the column. It follows isocratic or gradient elution techniques and the substance eluted from the column is detected using one or more detectors.

It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. It has wide applications in different fields in terms of quantitative and qualitative estimation of active molecules.

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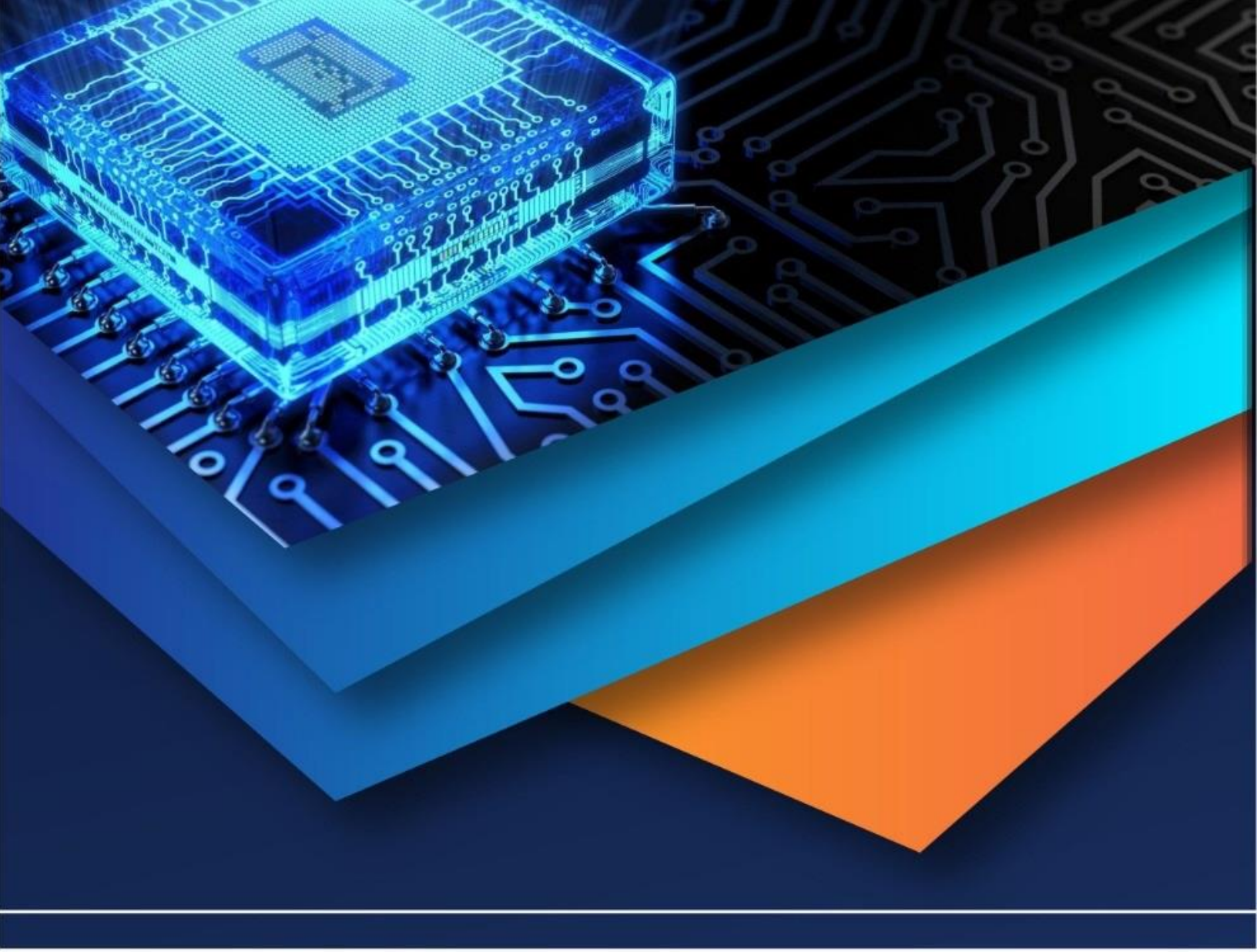

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